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DNA Synthesis and Gene Expression in *Bacillus subtilis* Infected with Wild-Type and Hypermodification-Defective Bacteriophage SP10

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A hypermodified base (Y-Thy) replaces 20% of the thymine (Thy) in mature DNA of *Bacillus subtilis* phage SP10. Two noncomplementing hypermodification-defective (*hmd*) mutants are described. At 30°C, *hmd* phage carried out a normal program, but at temperatures of ≥37°C, the infection process was nonproductive. When cells were infected at 37°C with *hmd* phage, DNA synthesis started at its usual time (12 min), proceeded at about half the normal rate for 6 to 8 min, and then stopped or declined manyfold. All, or nearly all, of the DNA made under *hmd* conditions consisted of fully hypermodified parental DNA strands H-bonded to unhypermodified nascent strands. The reduced levels of DNA synthesis observed under *hmd* conditions were accompanied by weak expression of late genes. A sucrose gradient analysis of SP10 *hmd*$^+$ replicating DNA intermediates was made. Two intermediates, called VF and F, were identified. VF consisted of condensed DNA complexed to protein; VF also contained negatively supercoiled domains covalently joined to relaxed regions. F was composed of linear concatenates from which mature DNA was cleaved. None of those intermediates was evident in cells infected at 37°C with *hmd* phage. Shiftup experiments were performed wherein cells infected with *hmd* phage at 30°C were shifted to 37°C at a time when replication was well under way. DNA synthesis stopped or declined manyfold 10 min after shiftup. The *hmd* DNA made after shiftup was conserved as a form sedimentationally equivalent to the F intermediate, but little mature DNA was evident. It is proposed that Y-Thy is required for replication and DNA maturation because certain key proteins involved with these processes interact preferentially with hypermodified DNA.

Bacillus subtilis phage SP10 belongs to a group of viruses in which thymine (Thy) in mature DNA is partially replaced by a hypermodified base (10, 37, 39). The hypermodified base in SP10 DNA (designated Y-Thy) has not been completely characterized with respect to chemical structure (37). Other members of this group include *B. subtilis* phage SP15 and *Pseudomonas acidovorans* phage ØW-14, in which Thy is replaced by 5-(4′,5′-dihydroxypentyl)uracil (DHP-uracil) and α-putrescinylthymine, respectively (4, 21). In the case of SP15, DHP-uracil and Thy are assembled as free deoxynucleoside triphosphates that are subsequently inserted into the DNA polymer (36). In the case of SP10 and ØW-14, however, neither Thy nor the respective hypermodified base is assembled as a free mononucleotide but, rather, each originates by post-replicational modification of a common precursor base that is probably 5-hydroxymethyl (HM) uracil (10, 27, 31, 37, 39). SP10 and ØW-14 are the only known biological systems in which Thy in DNA does not arise via the deoxythymidylate synthetase reaction (37).

Little is known about the biological relevance of hypermodified bases in DNA. Recent studies suggested that DHP-uracil plays no major role in differential transcription of SP15 DNA (16) but that normal levels of the hypermodified base are required for proper DNA maturation, phage assembly, or both (6, 36). These earlier studies were confounded by the fact that no situation could be defined in which SP15 DNA devoid of DHP-uracil was made (6, 36).

This paper concerns the isolation and characterization of hypermodification-defective (*hmd*) mutants of phage SP10. The results of these experiments suggest that Y-Thy is indispensable to SP10 development. Specifically, Y-Thy

seems to be required for proper replication of the phage genome as well as cleavage of concatenates to unit-size DNA.

MATERIALS AND METHODS

Phage and bacteria. SP10 *hmd*1 and SP10 *hmd*2 are heat-sensitive mutants defective in hypermodification (see below and reference 39). SP10 *hmd*$^+$ is the same clear-plaque variant used in all previous studies (29, 30). *B. subtilis* W23 was the host in all experiments.

Mutagenesis. Cells infected with SP10 *hmd*$^+$ at 30°C were mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine as described by Kahan (20).

Screening for *hmd* mutants. Survivors of mutagenesis were plated at 30°C. Plaques were picked with sterile toothpicks and scored for their ability to plate at 42°C. Lysates of heat-sensitive mutants were prepared at 30°C and used to infect cells at 42°C in the presence of 6-(*p*-hydroxyphenylazo)uracil, an inhibitor of bacterial DNA polymerase III (5, 32) that has no remarkable effect on SP10 development (29, 30). The cells were labeled with [6-^{3}H]uracil (10 μCi/ml) for 15 min. Cellular DNA was isolated (6) and acid hydrolyzed to a mixture of purine bases and pyrimidine deoxynucleosides (36). The digests were chromatographed in one direction on unmodified cellulose thin layers (10), using deoxythymidine (dThd) and Y-dThd as optical markers. Spots corresponding to the optical markers were eluted (10, 29), and the amount of label present was determined. In those cases where no label was recovered in the Y-dThd spot, a sample of [^{3}H]DNA was enzymatically degraded to mononucleotides (36, 39) that were fractionated by two-dimensional chromatography on unmodified cellulose thin layers (7). Of the 184 single-plaque isolates tested to date, only 2 generated DNA at 42°C which was totally devoid of Y-dThd. The bases replacing Y-Thy in SP10 *hmd*1 and SP10 *hmd*2 DNAs, under nonpermissive conditions, are 5-(hydroxymethyl-*O*-pyrophosphoryl)uracil and HM-uracil, respectively (39).

Sucrose gradient centrifugational analysis of replicating DNA. Cultures were infected as described previously (29, 30). The labeled precursor, typically $^{32}P_i$ (10 μCi/ml), was introduced 12 min after infection. At the desired times, 5-ml samples were removed and mixed with 20 ml of ice-cold medium that contained 10 mM KCN. The cells were harvested by centrifugation and suspended into 2 ml of 10 mM Tris-hydrochloride (pH 7.9), 10 mM disodium EDTA (pH 7.9), 1 mM KCN, and 1 mg of egg white lysozyme per ml. The cells were incubated at 37°C for 20 min, and 0.1 ml of 11% (wt/vol) sodium lauroyl sarcosine was added to complete lysis.

A 100-μl portion of the extract was mixed with 100 μl of a solution containing marker [^{14}C]DNA. Each marker was present at 0.5 μg. The entire mixture was layered onto a 5.8-ml linear sucrose gradient (5 to 25% sucrose in 10 mM Tris-hydrochloride, pH 7.9–1 mM disodium EDTA, pH 7.9–1 M NaCl–0.01% sarcosine). All gradients were spun in an SW50.1 rotor at 15°C. The exact conditions of centrifugation are given in the appropriate figure captions. Fractions (100 μl) were collected from the top with an ISCO density gradient fractionator, using Fluorinert FC-48 as the chase solution. The fractions were individually precipitated (6), and the amount of label in DNA was determined.

Isolation of RNA. Unlabeled RNA was isolated from 2-liter cultures by the method of Bolle et al. (2) as modified by Legault-Demare and Chambliss (22). [^{3}H]RNA was isolated from 50-ml cultures labeled with [5-^{3}H]uracil (5 μCi/ml).

Isolation of SP10 DNA strands. Strands of SP10 DNA were separated in CsCl on the basis of their differential binding of polyuridylate-polyguanidylate (16). The CsCl-purified strands were self-annealed and passed through hydroxyapatite columns to separate single strands from residual duplexes (19).

Other methods. DNA-RNA hybridization and hybridization-competition were carried out as described by Gillespie and Spiegelman (14). Enzyme assays and definition of activity units are given elsewhere (29, 39). Serum blocking power was determined by the method of DeMars (8). Protein concentration was measured by the method of Lowry et al. (26). The incorporation of radioactive precursors into DNA was measured as described by Lembach and Buchanan (23).

Materials. Radioactive materials were purchased from New England Nuclear Corp. (Boston, Mass.), except [6-^{3}H]HM-deoxyuridine, which was synthesized by the method of Alegria (1). Nitrosoguanidine was purchased from Aldrich Chemical Co. (Milwaukee, Wis.). Enzymes were obtained from Boehringer-Mannheim (Indianapolis, Ind.). Sucrose and Fluorinert FC-48 were purchased from Schwarz/Mann (Orangeburg, N.Y.) and Instrumentation Specialties Co. (Lincoln, Neb.), respectively. All nonradioactive materials were purchased from Sigma Chemical Co. (St. Louis, Mo.).

RESULTS

Thermolability of *hmd* phage. Wild-type (*hmd*$^+$) phage produced high bursts at all temperatures but, by this criterion, the infection process was most efficient between 30 and 37°C (Table 1). At temperatures of ≤ 30°C, mutant (*hmd*) phage gave bursts that were within 60% of normal, but at higher temperatures the burst size declined sharply (Table 1). For this paper, *hmd*$^+$ and *hmd* phage were compared at 37°C since this is the temperature at which most biochemical studies with SP10 were performed (10, 29, 30, 39, 40).

TABLE 1. Burst size of SP10 *hmd*$^+$ and SP10 *hmd* phages at various temperatures[a]

Phage	Burst size				
	20°C	30°C	33°C	37°C	42°C
SP10 *hmd*$^+$	31	87	89	83	63
SP10 *hmd*1	20	54	12	$\leq 1 \times 10^{-4}$	$\leq 1 \times 10^{-4}$
SP10 *hmd*2	21	58	12	$\leq 1 \times 10^{-4}$	$\leq 1 \times 10^{-4}$

[a] Cells were grown at 37°C to a density of ca. 2×10^8/ml. The cells were harvested by centrifugation and resuspended into 0.95 volume of fresh medium equilibrated to the indicated temperature. Ten minutes later, the cells were infected by adding 0.05 volume of medium that contained the phage (input multiplicity of infection = 0.5 PFU/cell). The remainder of the procedure was identical to the one used by Bott and Strauss (3). All platings were done at 30°C.

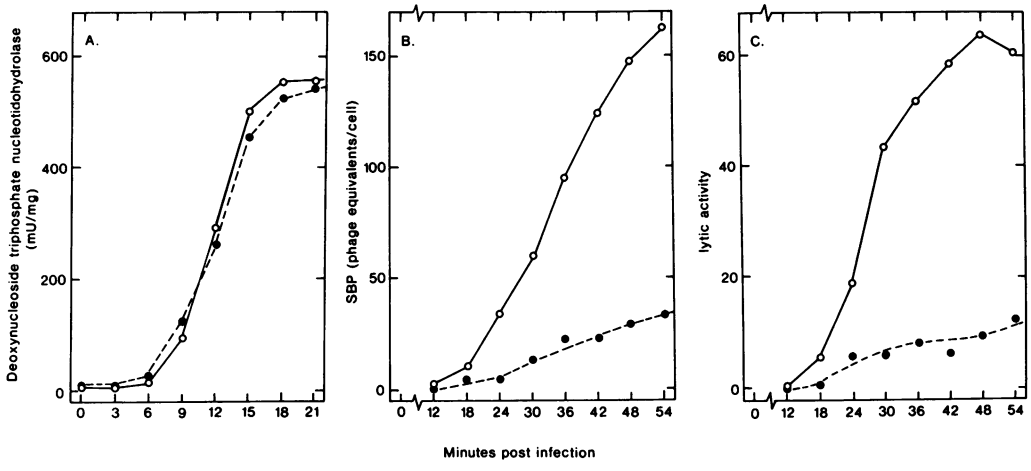

FIG. 1. Synthesis of SP10-coded proteins under *hmd*$^+$ and *hmd* conditions. Cultures (300 ml) were infected with SP10 *hmd*$^+$ or SP10 *hmd*1 at 37°C. At the times specified in the graph, 25-ml samples were removed. Extracts were prepared and assayed for deoxynucleoside triphosphatase (A), serum blocking power (B), and lytic enzymes (C). Symbols: (○) SP10 *hmd*$^+$; (●) SP10 *hmd*1.

Complementation of *hmd* mutations. When cells were doubly infected with *hmd*1 and *hmd*2, the burst size was no greater than that obtained when cells were infected with only one of the mutants (data not shown). These data suggest that *hmd*1 and *hmd*2 do not complement, which, in turn, suggests that both mutations are in the same gene.

Synthesis of phage-coded proteins. The conditionally lethal nature of *hmd* mutations suggested that some important aspects of SP10 development were aberrant in the absence of hypermodification. To see if the defect involved early or late functions, synthesis of SP10 proteins was compared under *hmd*$^+$ and *hmd* conditions. In this and all other experiments, only data obtained with *hmd*1 phage will be presented unless stated otherwise.

A typical early enzyme, deoxythymidine triphosphate nucleotidohydrolase (29), was synthesized normally under *hmd* conditions (Fig. 1A), whereas two late functions, structural proteins responsible for serum blocking power (Fig. 1B) and lytic enzymes (Fig. 1C), were synthesized poorly. Therefore, under the conditions defined, the absence of hypermodification limited late gene expression.

Synthesis of phage-coded RNA. Since hypermodification is an event at the DNA polymer level (39), it seemed reasonable to assume that the low levels of late protein synthesis observed under *hmd* conditions were due to poor transcription of late genes. To confirm this, DNA-RNA hybridization competition experiments were done (29). For these experiments, parallel cultures of *B. subtilis* W23 were infected under *hmd*$^+$ conditions. One culture was labeled with [5-^{3}H]uracil from 4 to 12 min postinfection (early [^{3}H]RNA), and the other was labeled from 12 to 20 min postinfection (late [^{3}H]RNA) (29). Unlabeled competitor RNAs were isolated 12 and 20 min postinfection by *hmd*$^+$ and *hmd* phage.

Unlabeled 12-min RNAs isolated from cells infected under *hmd*$^+$ and *hmd* conditions were equivalent with respect to their ability to compete against early [^{3}H]RNA (Fig. 2A). By the same token, *hmd*$^+$ and *hmd* 12-min [^{3}H]RNAs each competed against sequences that accounted for about 60% of the label in late [^{3}H]RNA (Fig. 2A). These data indicate that 12-min RNAs present in *hmd*$^+$- and *hmd*-infected cells were equivalent. This conclusion is supported by the observation that unlabeled *hmd*$^+$ and *hmd* 12-min RNAs competed equally well against *hmd* 4- to 12-min [^{3}H]RNAs (data not shown).

On the other hand, *hmd*$^+$ and *hmd* 20-min RNAs displayed marked differences (Fig. 2B). Specifically, sequences amounting to 30% of the label in late [^{3}H]RNA were either absent from 20-min *hmd* RNA or present in low concentrations. This difference is better illustrated by a "mixed competitor" experiment (2) in which late [^{3}H]RNA was incubated simultaneously with a saturating concentration of *hmd*$^+$ 12-min RNA and various concentrations of *hmd*$^+$ or *hmd* 20-min RNA (Fig. 2C). Overall, these data are consistent with the interpretation that genes transcribed uniquely at times later than 12 min of a normal SP10 program were weakly expressed under *hmd* conditions.

Kinetics of DNA synthesis. In many phage systems, expression of late genes is, in some measure, dependent on DNA synthesis (15, 18). Although the screening procedure showed that some DNA synthesis took place under *hmd* conditions, the possibility still existed that the

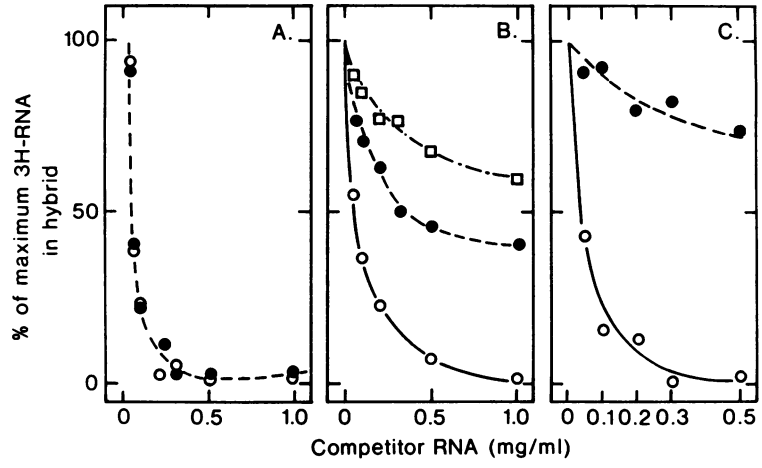

FIG. 2. DNA-RNA hybridization competition between early and late SP10 RNAs made under *hmd*$^+$ and *hmd* conditions. Early [^{3}H]RNA was labeled from 4 until 12 min postinfection by *hmd*$^+$ phage; late [^{3}H]RNA was labeled 12 to 20 min postinfection by *hmd*$^+$ phage (29). Unlabeled RNAs were isolated 12 or 20 min postinfection by *hmd*$^+$ or *hmd*1 phage. Hybridization mixtures contained 1 μg of [^{3}H]RNA and filters that contained 20 μg of heat-denatured phage DNA. (A) Hybridization competition of early [^{3}H]RNA. The specific activity of [^{3}H]RNA was ca. 32,000 cpm/μg and, in the absence of unlabeled competition RNA, ca. 11% of the input was retained. Unlabeled RNA was extracted 12 min postinfection by *hmd*$^+$ (○) or *hmd*1 (●) phage. (B) Hybridization competition of late [^{3}H]RNA. The specific activity of [^{3}H]RNA was 23,500 cpm/μg and, in the absence of competitor, 33% of the input was retained by the filter. The unlabeled competitors were (□) *hmd*$^+$ 12-min RNA, (○) *hmd*$^+$ 20-min RNA, and (●) *hmd*1 20-min RNA. (C) Mixed competition of late [^{3}H]RNA. Late [^{3}H]RNA was simultaneously competed by 1 mg of *hmd*$^+$ 12-min RNA and the indicated amounts of either (○) unlabeled *hmd*$^+$ 20-min RNA or (●) unlabeled *hmd*1 20-min RNA.

apparently weak expression of SP10 late genes observed under *hmd* conditions could reflect some upset in the mechanism of DNA synthesis.

Kinetics of DNA synthesis was monitored by the incorporation of [6-^{3}H]uracil into acid-precipitable, alkali-labile material (23). By this criterion, replication of the viral genome, under *hmd*$^+$ conditions, was first evident 9 to 12 min postinfection and continued for the duration of the latent period (Fig. 3). During an *hmd* program, DNA synthesis started at the normal time, proceeded at about half the normal rate until 18 to 21 min, and then stopped or declined many-fold (Fig. 3). The limited capacity for DNA synthesis under *hmd* conditions could not be attributed to a dearth of substrates inasmuch as dATP, dGTP, dCTP, and HM-dUTP pools actually expanded during an *hmd* program (data not shown). It is worth noting that depletion of dTTP pools occurred normally under *hmd* conditions (data not shown), which agrees with our observations that many early functions were expressed normally under these mutant conditions (Fig. 1A and 2A).

Intermediates in SP10 replication. In the case of SP01, several key intermediates in the replication cycle have been identified by sucrose gradient analysis (24, 25). Initially, parental DNA is converted to a form that sediments through neutral sucrose at ca. 300S; this is the so-called VF ("very fast") form. Approximately 10 min later, the VF form disappears and is replaced by

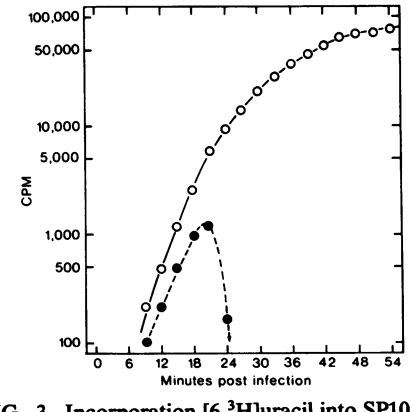

FIG. 3. Incorporation [6-^{3}H]uracil into SP10 *hmd*$^+$ and SP10 *hmd*1 DNA. Cultures (12 ml) were infected at 37°C (29). Various times postinfection, 500-μl samples were transferred to a fresh tube that contained 20 μl of [6-^{3}H]uracil (200 μCi/ml). The samples were labeled for 3 min and frozen in an acetone-solid CO_2 bath. At the end of the experiment, the samples were thawed, and the amount of label in DNA was determined (25). Symbols: (○) SP10 *hmd*$^+$; (●) SP10 *hmd*1.

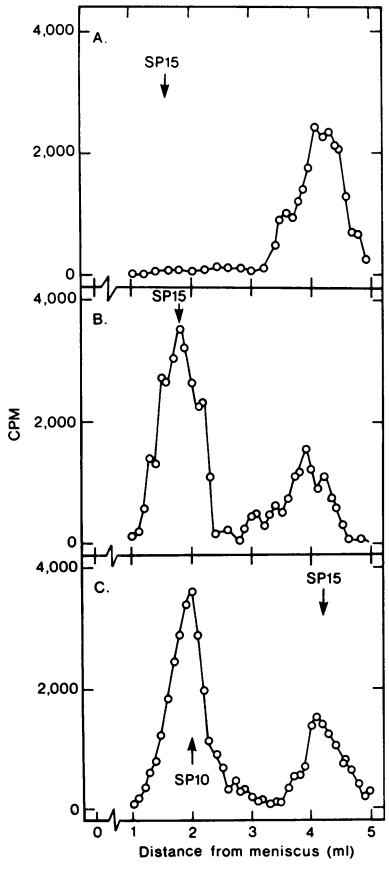

FIG. 4. Sample neutral sucrose gradient profiles of intermediates in SP10 *hmd*$^+$ DNA replication. A culture was infected with SP10 *hmd*$^+$ at 37°C. $^{32}P_i$ (10 μCi/ml) was added at 12 min. At the times given below, [^{32}P]DNA was extracted as described in the text. (A) [^{32}P]DNA extracted 18 min postinfection that was sedimented at 11,000 rpm for 4.5 h. (B) [^{32}P]DNA extracted 24 min after infection that was sedimented at 11,000 rpm for 4.5 h. (C) [^{32}P]DNA extracted 33 min after infection that was sedimented at 11,000 rpm for 15 h. Arrows mark the position of SP10 or SP15 virion [^{14}C]DNA (ca. 2,000 cpm) used as marker.

the more slowly sedimenting F form composed of linear concatenates that initially contain up to 20 phage equivalents of DNA. Mature DNA (54S) appears roughly 5 min after the F form, and as the amount of mature DNA increases, the average size of the F form diminishes.

A similar sequence of events seems adequate to explain the replicative cycle of SP10 *hmd*$^+$. Figure 4 depicts sample gradient profiles. Figure 5 presents a detailed summary of the amount of label in VF, F, and M (mature) forms of SP10 *hmd*$^+$ DNA. For these particular experiments, cells were labeled with $^{32}P_i$ beginning at the 12th min postinfection. At various times, extracts were prepared and sedimented through sucrose at a reduced speed to demonstrate the VF and F forms or at a higher speed to resolve the F and M forms. The internal markers were SP10 *hmd*$^+$ virion [^{14}C]DNA (80 megadaltons; 50S) and SP15 virion [^{14}C]DNA (250 megadaltons; about 100S).

Until 18 min, all [^{3}H]DNA sedimented through sucrose at a rate 2.5 to 3 times faster than did the SP15 [^{14}C]DNA marker; this evidently represented the VF form of SP10 *hmd*$^+$ replicating DNA (see below). Unlike the situation with SP01 (24), the VF intermediate of SP10 replication usually remained evident until late in the program. Between 18 and 21 min, a form sedimenting in the vicinity of SP15 [^{14}C]DNA appeared in SP10 *hmd*$^+$-infected cells which presumably corresponded to the F intermediate. The F intermediate reached its maximal level at 24 min and was maintained at approximately that level for the duration of the infection process. At 24 min, the F intermediate had a nominal sedimentation coefficient of 105 to 120S which corresponded to linear duplexes of mass 350 to 500 megadaltons (9). Experiments with alkaline sucrose gradients (not shown) suggested, by comparison with other systems (11, 25),

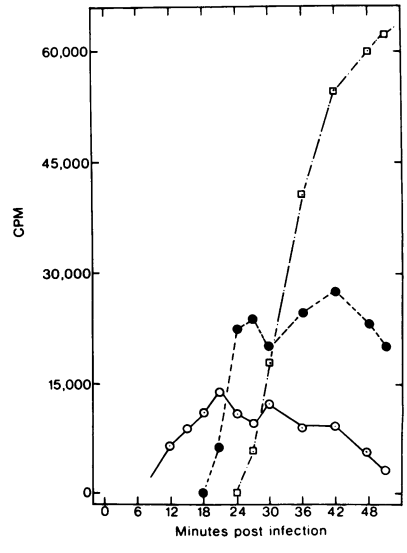

FIG. 5. Abundance of replicative intermediates various times postinfection by SP10 *hmd*$^+$. Sucrose gradient analyses were carried out as described in the text. Duplicate 100-μl samples of extracted [^{32}P]DNA were centrifuged at either 11,000 rpm for 4.5 h (VF and F forms) or 11,000 rpm for 15 h (F and M forms). The total amount of radioactivity recovered in a particular form is recorded. Since the F form is defined in both regimens used, the reported amount of label in this intermediate is the average of two values. Symbols: (○) VF; (●) F; (□) M.

that the F intermediate was a linear multigenomic structure containing, at 24 min, between four and six SP10 phage DNA equivalents. The M form of SP10 *hmd*$^+$ DNA appeared between 24 and 27 min postinfection and, thereafter, increased in level until it accounted for 60 to 70% of the [^{3}H]DNA present at 51 min (i.e., 4 min before lysis).

A pulse-chase experiment was performed to determine the precursor relationship between VF, F, and M forms of SP10 DNA. Label, as [6-^{3}H]HM-deoxyuridine, was introduced at 12 min, and incorporation was terminated at 17.5 min by the addition of dThd (25). Initially, all radioactivity was recovered in the VF intermediate (data not shown). Between 18 and 24 min, label appeared in the F intermediate at approximately the same rate at which it was lost from the VF intermediate. Label did not appear in the M form until 24 to 27 min postinfection. These data are consistent with the progression VF → F → M.

Under *hmd* conditions, replication of the SP10 genome was limited to an interval of time when VF was the only observable intermediate in normally infected cells (Fig. 3 and 4). This implies that, under *hmd* conditions, formation of the VF intermediate should not take place. Indeed, all [^{3}H]DNA made between 12 and 18 min of an *hmd* program sedimented through sucrose at 60S, i.e., 20% faster than mature DNA (data not shown). The fate of this intermediate has not been studied.

Characterization of the VF and F intermediates. The VF form was stable in sarcosine, which indicates that binding to the cell membrane plays little, if any, role in maintaining the structure of this intermediate (25). Similarly, treatment of VF with RNase A had no obvious effect on the sedimentation properties of this intermediate (Fig. 6A). On the other hand, treatment with pronase converted VF to a form that sedimented at a rate characteristic of the F intermediate (Fig. 6B); material produced by pronase treatment of VF will be called F*. These data indicate that VF represented a condensed intermediate caused by complexing of DNA to protein.

Novobiocin, an inhibitor of DNA gyrase (13), immediately arrested SP10 DNA synthesis at all times postinfection (data not shown). Evidently, negatively supercoiled (underwound) regions must be maintained for continuous replication (13). Sedimentation coefficient of negatively supercoiled DNA varies with ethidium bromide concentration in a characteristic fashion (35). Low dye concentrations unwind the supercoils, which results in a reduction in sedimentation coefficient; higher dye concentrations introduce positive supercoils with a corresponding increase in sedimentation coefficient. Forms of DNA that cannot be negatively supercoiled (i.e., linear duplexes and open circles) also show characteristic changes in sedimentation coefficient; in this case, the sedimentation coefficient decreases approximately linearly with increasing dye concentration.

To show that our system would give reliable results, we compared the effect of ethidium bromide concentration on the *Escherichia coli* nucleoid (41) in its native, negatively supercoiled form and its DNase I-relaxed form (Fig. 7A). For the native form, the characteristic biphasic curve, indicative of negatively supercoiled DNA, was readily obtained, whereas the sedimentation coefficient of the relaxed form decreased linearly across the range of dye concentrations tested.

The F and F* forms of SP10 *hmd*$^+$ DNA responded to increasing dye concentrations as relaxed forms (Fig. 7B). VF, on the other hand, responded in a peculiar fashion (Fig. 7B). For dye concentrations of ≤2 μg/ml, the sedimentation coefficient of VF decreased linearly in a manner similar to that obtained for F and F*. Higher dye concentrations, however, produced

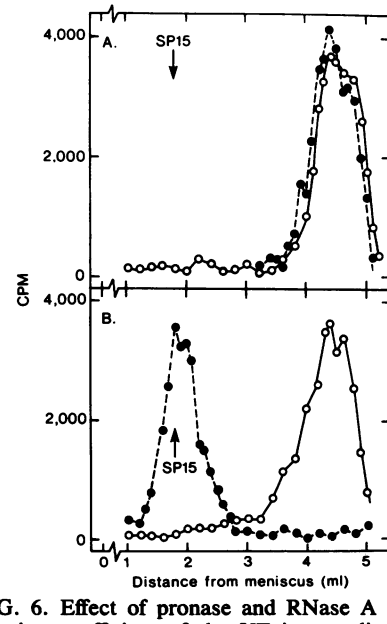

FIG. 6. Effect of pronase and RNase A on sedimentation coefficient of the VF intermediate. Cells infected at 37°C with SP10 *hmd*$^+$ received $^{32}P_i$ (10 μCi/ml) at 12 min. DNA was extracted at 18 min. A 100-μl portion of the extract was treated with either RNase A (10 μg/ml) or pronase (10 μg/ml) at 37°C for 1 h and then centrifuged at 11,000 rpm for 4.5 h. (A) RNase A; (B) pronase. Symbols: (○) incubated without enzyme; (●) incubated with enzyme. Arrows locate the position of marker [^{14}C]DNA.

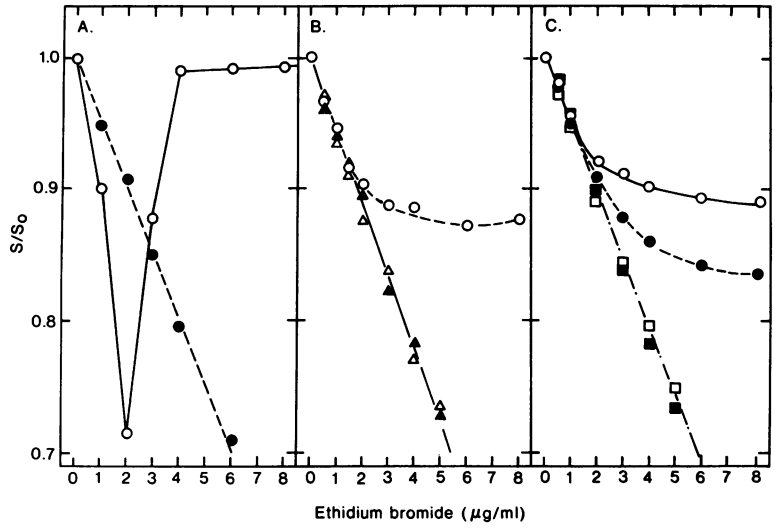

FIG. 7. Effect of ethidium bromide on sedimentation behavior of replicative intermediates. Extracts of $^{32}P_i$-labeled cells received the indicated concentration of dye and were sedimented in gradients containing an identical concentration of dye. (A) *E. coli* nucleoid (41) in its native, negatively supercoiled form (○) or its DNase I-relaxed form (●). (B) The F (▲), F* (△), and VF (○) forms of SP10 *hmd*$^+$ replicating DNA. F* is generated by pronase treatment of VF (see text). (C) DNase I-relaxed VF. Extracts were first treated with increasing concentrations of DNase I in an ice-water bath for 10 min under conditions that limit nucleolytic damage to single-strand breaks (41). Symbols: (○) no DNase I; (●) 0.5 μg of DNase I/ml; (■) 1 μg of DNase I/ml; (□) 2 μg of DNase I/ml.

no further reduction in the sedimentation coefficient of VF. That is to say, at concentrations of dye where positive supercoiling of restrained DNA would be expected, VF underwent no further reduction in sedimentation coefficient.

Such data could be interpreted to mean that VF DNA contains underwound and restrained regions that are covalently joined to relaxed regions. High concentrations of dye introduced positive supercoiling into the restrained regions which compensated for the continued unwinding of the relaxed regions. If so, introduction of sufficient single-strand breaks into VF DNA should relax the restrained regions, thereby resulting in a structure whose sedimentation coefficient declines linearly at dye concentrations of >2 μg/ml. Controlled incubation of VF with DNase I did, indeed, generate a form that responded to ethidium bromide concentration as a fully relaxed structure (Fig. 7C). The amount of DNase I treatment required to give a fully relaxed form of VF DNA did not affect the initial sedimentation coefficient (data not presented). Therefore, if we are correct in assuming that VF DNA is a condensed, protein-complexed intermediate, then supercoiling makes only a minor contribution to the rapidly sedimenting nature of this DNA. Similar conclusions have been reached for the condensed form of T4 DNA (17).

Characterization of DNA made under *hmd* conditions. The small amount of DNA made under *hmd* conditions seems to be in a form that does not occur in *hmd*$^+$ infections (unpublished data described above). Inasmuch as *hmd*1 and *hmd*2 DNAs replace Y-Thy with different bases (39), it followed that DNA made under *hmd* conditions would have a buoyant density in CsCl different from that of *hmd*$^+$ DNA and that this density differential could be used as a probe to explore the relationship between parental and newly replicated strands generated under *hmd* conditions.

For these experiments, cells were infected at 37°C with *hmd* phage that contained [^{32}P]DNA. At 12 min, [6-^{3}H]uracil was added. DNA was extracted 20 min postinfection and banded in CsCl gradients. In the case of *hmd*1, about 75% of parental [^{32}P]DNA and all of the nascent [^{3}H]DNA were denser than the SP10 *hmd*$^+$ marker [^{14}C]DNA (Fig. 8A). In the case of *hmd*2, 65% of the parental [^{32}P]DNA and all of the nascent [^{3}H]DNA were recovered in a band that was less dense than *hmd*$^+$ marker DNA (Fig. 8B). These data are consistent with the observation that the unique base in *hmd*1 DNA is more acidic than Y-Thy, whereas the one in *hmd*2 DNA is neutral (39).

When newly replicated *hmd* DNA was denatured and rebanded in neutral CsCl, the parental [^{32}P]DNA was recovered at the same density as

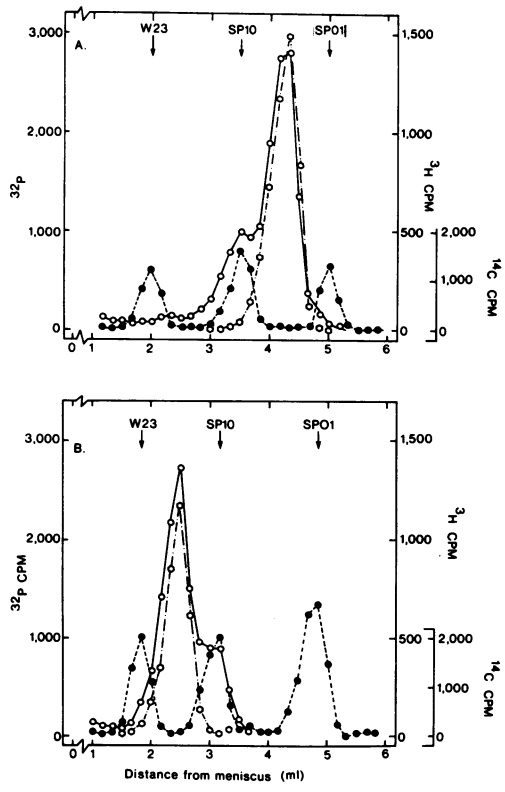

FIG. 8. Isopycnic centrifugation of DNA extracted from cells infected with SP10 *hmd*1 or SP10 *hmd*2. Bacteria were infected at 37°C with *hmd* phage that contained [^{32}P]DNA. At 12 min, [6-^{3}H]uracil (10 μCi/ml) was added. DNA was extracted 18 min postinfection and banded in neutral CsCl density gradients (6). (A) SP10 *hmd*1. (B) SP10 *hmd*2. Symbols: (○) parental [^{32}P]DNA; (○) newly replicated [^{3}H]DNA; (●) marker [^{14}C]DNAs run in a parallel tube.

denatured *hmd*$^{+}$ DNA, whereas the nascent [^{3}H]DNA was recovered in a separate band (Fig. 9). [^{3}H]DNA annealed to both H and L strands of SP10 *hmd*$^{+}$ DNA (Table 2), suggesting that newly replicated *hmd* DNA contained both complementary strands. Thus, it appears that replication under *hmd* conditions generated hybrid DNA molecules composed of a fully hypermodified parental strand H-bonded to a nascent unhypermodified strand. In other words, fully unhypermodified duplexes could not be generated in vivo.

In ethidium bromide, material made under *hmd* conditions behaved as if it were fully relaxed, and treatment with pronase had no effect on its sedimentation coefficient (data not shown). Therefore, it is not immediately obvious why material made under *hmd* consistently sedimented more rapidly than mature DNA. Many possibilities exist, but none has been tested.

Shiftup experiments. Strictly speaking, the results shown above indicate that hypermodification is required for the first round of DNA synthesis. However, is hypermodification continuously required for DNA synthesis? To obtain some idea on this point, shiftup experiments were performed. Cells were infected with *hmd* phage at 30°C. Various times later, a sample was removed and heat shocked at 50°C for 2 min to rapidly destroy the thermolabile function. After the heat shock, the sample was placed at 37°C and $^{32}P_i$ was added. Control experiments demonstrated that heat shock had no effect on *hmd*$^{+}$ infections (data not shown).

Only the results of a typical experiment are shown here (Fig. 10). In this case, shiftup came 50 min postinfection at 30°C, which is roughly equivalent to 36 min postinfection at 37°C. When an *hmd*$^{+}$ culture was shifted up in the manner described, incorporation of $^{32}P_i$ continued for about 21 min, at which time the culture lysed. In the *hmd* culture, however, the apparent rate of

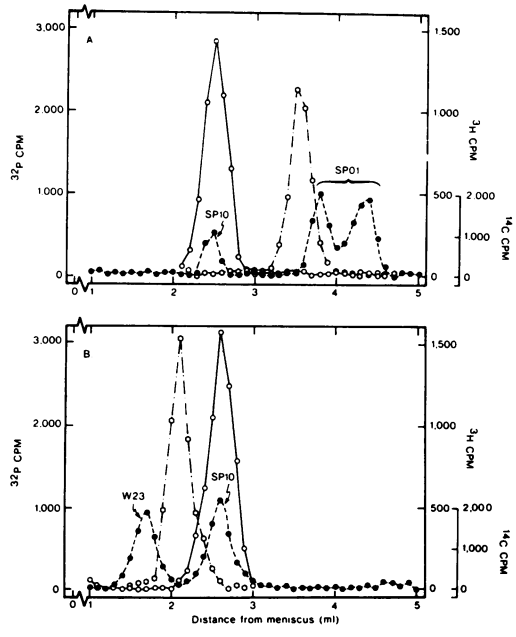

FIG. 9. Isopycnic centrifugation of denatured DNA extracted from cells infected with SP10 *hmd*1 and SP10 *hmd*2. This is the companion experiment to the one reported in Fig. 8. Material with a density different from *hmd*$^{+}$ native DNA was isolated from pooled gradient fractions and denatured by dialysis against distilled water followed by heating for 2 min in a boiling water bath (39). The denatured DNA was rebanded in neutral CsCl gradients. (A) SP10 *hmd*1; (B) SP10 *hmd*2. Symbols: (○) parental [^{32}P]DNA; (○) newly replicated [^{3}H]DNA; (●) denatured marker [^{14}C]DNAs run in a parallel tube.

TABLE 2. Annealing of DNA made in vivo to the L and H strands of SP10 *hmd*$^+$ DNA[a]

Labeled DNA	Unlabeled DNA	Input cpm[b]	S1 nuclease-resistant cpm	
			Mock hybridized[b]	Hybridized[b]
^{32}P-labeled parental	None	2,463	13	82
	L strand	2,876	71	686
	H strand	2,626	66	649
^{3}H-labeled nascent	None	1,251	18	47
	L strand	1,338	41	305
	H strand	1,119	27	381
^{32}P-labeled L strand[c]	None	2,476	41	93
	L strand	2,509	73	176
	H strand	2,818	81	1,572
^{32}P-labeled H strand[c]	None	2,011	45	87
	L strand	2,256	53	1,703
	H strand	2,256	58	201

[a] Cells were infected, at 37°C, with SP10 *hmd*1 that contained [^{32}P]DNA and labeled with [6-^{3}H]uracil (10 μCi/ml) from 12 until 18 min postinfection. DNA was extracted and banded in neutral CsCl density gradients (Fig. 8A). All material that banded more heavily than marker SP10 *hmd*$^+$ DNA was collected and denatured by dialysis against distilled water followed by heating in a boiling water bath for 2 min (36, 39). The denatured DNAs were rebanded in neutral CsCl; material corresponding to parental [^{32}P]DNA and nascent [^{3}H]DNA was reisolated (Fig. 9). Hybridization was carried out in 200 mM NaCl–10 mM Tris-hydrochloride (pH 7.9). The reaction mixtures contained ≤0.1 μg of labeled DNA and 25 μg of unlabeled DNA. The final volume was 200 μl. The reaction mixtures were incubated at 67°C for 4 h. This is not enough time to give maximal hybridization to unlabeled DNA, but self-annealing of the labeled DNA is negligible during this interval. The reaction mixtures were diluted twofold with 60 μM $ZnSO_4$ and 250 μg of denatured calf thymus DNA per ml. The S1 nuclease of *Aspergillus oryzae* (250 U) was added. The mixture was incubated at 50°C for 4 h, and the amount of precipitable label was determined.

[b] Three hybridization mixtures were constructed. One was precipitated immediately and used to determine the input label. The second was mock hybridized by incubation at 2°C for 4 h and was then incubated with S1 nuclease at 50°C. The third reaction mixture was hybridized at 67°C for 4 h and digested with S1 nuclease at 50°C.

[c] Prepared from SP10 *hmd*$^+$ [^{32}P]DNA.

DNA synthesis decreased rapidly and stopped within 10 to 12 min of the shiftup. The *hmd* culture lysed 30 min after the shiftup.

In a subsequent experiment, the shiftup was performed as described above. After shiftup, the cells were labeled with $^{32}P_i$ for 10 min. The DNA was extracted and centrifuged through neutral sucrose gradients. Approximately 50% of the *hmd*$^+$ [^{32}P]DNA was recovered as the M form, whereas the remainder sedimented as the F form (Fig. 11A). In the case of *hmd* [^{32}P]DNA, no more than 10% of the material sedimented as the M form (Fig. 11B). When denatured and banded in neutral CsCl gradients, the buoyant density of *hmd* [^{32}P]DNA made after the shiftup was between that of fully hypermodified strands and unhypermodified strands (Fig. 12), implying that, under these conditions, the unhypermodified DNA was covalently linked to hypermodified DNA made before shiftup. All in all, these data are consistent with the interpretation that concatenates containing unhypermodified DNA are inefficiently cleaved to the M form.

Shiftdown experiments. Shiftdown experiments were performed to see if restoration of the thermolabile *hmd* functions permitted completion of the infection process. For the sake of comparison, data are also shown for SP10 *rep*d, a heat-sensitive mutant defective in an as yet undefined aspect of replication.

In the case of *rep*d, shiftdown resulted in the reestablishment of DNA synthesis (Fig. 13) and production of viable phage (not shown); this mutant could be incubated at 37°C for up to 42 min without affecting the subsequent production of DNA and phage at 30°C in any major way. In the case of *hmd* mutants, normal amounts of DNA and progeny were generated at 30°C provided the shiftdown took place <12 min postinfection at the nonpermissive temperature; thereafter, the amount of DNA and progeny generated at 30°C decreased sharply (Fig. 13 and unpublished data). Under *hmd* conditions, a shiftdown later than 18 min postinfection yielded virtually no DNA synthesis or progeny production at 30°C (Fig. 13 and unpublished data).

DISCUSSION

Even though it is a minor component of SP10 DNA (37, 39), Y-Thy is clearly essential for intracellular development. At nonpermissive temperatures, SP10 *hmd*1 and SP10 *hmd*2 generate DNAs in which different intermediates accu-

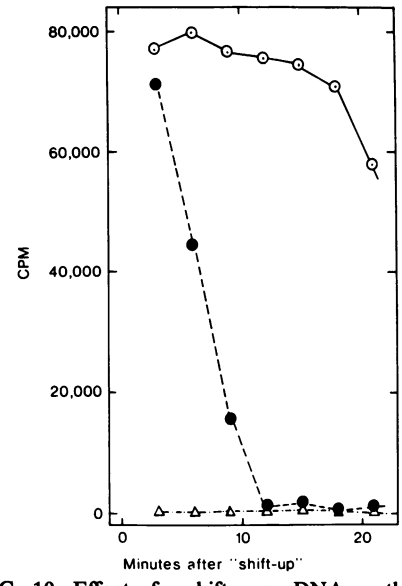

FIG. 10. Effect of a shiftup on DNA synthesis in cells infected with SP10 *hmd*$^+$ and SP10 *hmd*1. Bacteria were infected at 30°C, which is a permissive temperature for *hmd*1. At 50 min, the culture (5 ml) was transferred to a water bath (50°C) and swirled gently for 2 min. The culture was then placed at 37°C. Periodically, 500-μl portions were pulse-labeled with [6-^{3}H]uracil as described in Fig. 3. Symbols: (○) SP10 *hmd*$^+$; (●) SP10 *hmd*1; (△) SP10 *rep*d, a heat-sensitive mutant that is defective in DNA synthesis at 37°C (see text).

mulate in place of Y-Thy (39), yet both mutants show basically the same properties with respect to intracellular development (this paper and unpublished data). Therefore, intermediates in hypermodification cannot substitute for Y-Thy.

Under *hmd* conditions, replication of SP10 DNA stopped or declined manyfold after a few minutes (Fig. 3). The principal, if not only, product of SP10 DNA replication under *hmd* conditions consisted of duplexes composed of parental (hypermodified) strands H-bonded to nascent (*hmd*) strands (Fig. 8 and 9). DNA-DNA annealing experiments (not shown) indicate that *hmd* duplexes can be formed quite readily in vitro, so there is no reason to believe that any intrinsic properties of *hmd* strands per se prevent formation of such duplexes in vivo. Consequently, it seems reasonable to propose, as a working hypothesis, that the replicational machinery of SP10 cannot use an *hmd* strand as template, so replication stops at the point where these strands would have to serve as templates to sustain DNA synthesis.

Shiftdown experiments indicated that the thermolabile *hmd* function could not be rescued later than 18 min postinfection (Fig. 13). Although it could be that inactivation of the thermolabile *hmd* function becomes irreversible at 18 min, another explanation seems equally valid. The time at which *hmd* functions become unrescuable by shiftdown corresponds to the time at which DNA synthesis stops at the nonpermissive temperature (Fig. 3), i.e., the time at which *hmd*$^+$-*hmd* hybrid duplexes are completed (Fig. 8 and 9). Such hybrid duplexes are evidently inert as templates for DNA synthesis (Fig. 3 and 10). At 37°C, hypermodification of nascent SP10 *hmd*$^+$ DNA occurs no more than 8 to 10 s after polymerization (39). It could well be that hypermodification can occur only within a short time interval of DNA synthesis and that, beyond this interval, hypermodification cannot occur even if the *hmd* functions are restored. In this context, it is worth noting that *hmd*$^+$-*hmd* hybrid duplexes cannot be rescued by superinfection with *hmd*$^+$ phage even though superinfecting phage develops normally (H. Witmer and M. Franks, unpublished data).

Insofar as replication under *hmd*$^+$ conditions was concerned (Fig. 4 and 5), SP10 showed basically the same intermediates as SP01 (25).

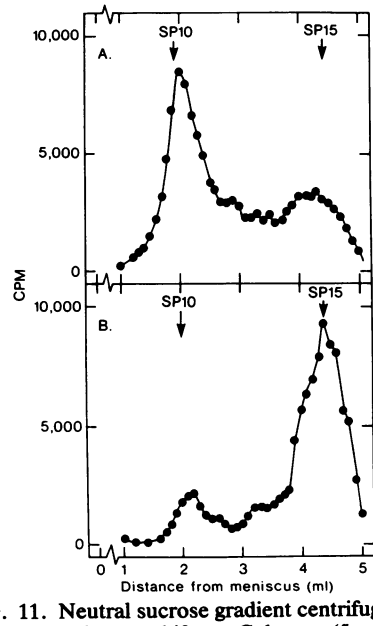

FIG. 11. Neutral sucrose gradient centrifugation of DNA made after a shiftup. Cultures (5 ml) were infected at 30°C with either SP10 *hmd*$^+$ or SP10 *hmd*1. At 50 min, the cultures were shifted up to 37°C as described in Fig. 12. $^{32}P_i$ (10 μCi/ml) was added 1 min after the culture was placed at 37°C. DNA was extracted 10 min later and sedimented through neutral sucrose gradients at 11,000 rpm for 15 h. Only the F and M forms of DNA (see text) are resolved by this procedure. (A) SP10 *hmd*$^+$; (B) SP10 *hmd*1. Arrows locate the position of marker [^{14}C]DNAs.

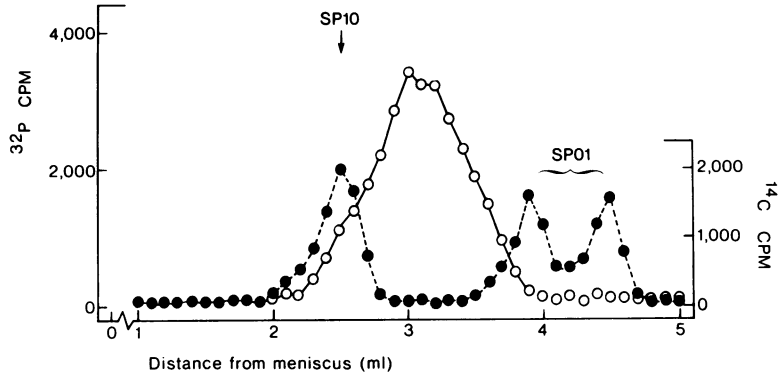

FIG. 12. Isopycnic centrifugation of denatured DNA made after a shiftup. Material that sedimented through sucrose like the F intermediate (Fig. 11) was reisolated, denatured, and banded in neutral CsCl density gradients (6, 39). Symbols: (○) [^{32}P]DNA extracted from SP10 *hmd*1-infected cells; (●) denatured marker [^{14}C]DNAs.

Therefore, at the present time, it does not seem necessary to assume that replication of hypermodified DNAs requires novel intermediates. However, one point of interest should be noted. Replication of the SP10 genome is novobiocin sensitive, yet underwinding of SP10 DNA is seemingly limited to relatively small domains in the VF intermediate (Fig. 7B). Maintenance of these underwound regions, which we assume to be essential for replication, requires continuous presence of proteins because F* DNA (Fig. 6B) behaves as if it were fully relaxed (Fig. 7B). When cells were infected under *hmd* conditions, SP10 DNA assumed nothing comparable to the VF form (Witmer and Franks, our unpublished data), so it is possible that some proteins required to generate and maintain the underwound regions require a fully hypermodified DNA. We are currently attempting to identify SP10-coded DNA-binding proteins to see if any bind preferentially to fully hypermodified DNAs. Considering the complex and reactive nature of the substituents in hypermodified bases (37), they would be ideally suited as target molecules for DNA-protein interactions.

DHP-uracil, the hypermodified base in SP15 DNA (4), is required for proper DNA maturation or phage assembly (6, 36). Evidence for this conclusion stems from the fact that, under conditions where only 5% of the replicating pool has a normal DHP-uracil content, over 50% of the DNA packaged into phage is normal with respect to base composition (6, 36). A similar conclusion seems to hold for Y-Thy in SP10 DNA inasmuch as little, if any, *hmd* DNA made after a shiftup matured (Fig. 11). Late protein synthesis is essential for DNA maturation, since chloramphenicol, added 12 min postinfection by *hmd*$^+$ phage, allows the VF and F intermediates to accumulate but no mature DNA is generated (data not given). At present, it is not known if SP10 mature DNA is cut from the concatenates before packaging, as is the case with SP01 (24), or, as in the case of coliphages ØX-174, T4, and λ (12, 33, 38), cleavage from the concatenate is the final step of packaging. Either way, one or more late proteins must interact with the F intermediate to generate mature DNA and, when the F intermediate contained unhypermodified regions (Fig. 12), this interaction was inefficient (Fig. 11).

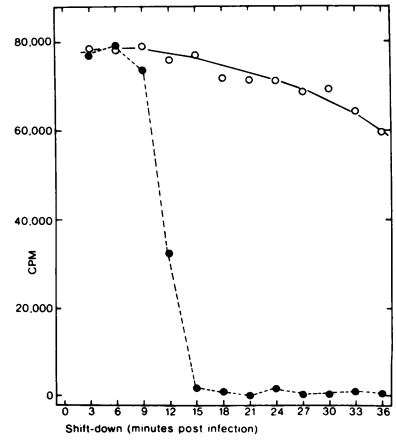

FIG. 13. Restoration of thermolabile functions: Shiftdown experiment. Parallel 12-ml cultures were infected at 37°C with either SP10 *hmd*1 or SP10 *rep*d (see text). Various times postinfection, 1-ml samples were transferred to a fresh tube and incubated, with gentle agitation, at 20°C for 2 min. The samples were placed at 30°C, and $^{32}P_i$ was added to a final concentration of 10 μCi/ml. The samples were incubated at 30°C until all the SP10 *rep*d-infected cultures lysed. The amount of label in DNA was determined (23). SP10 *hmd*1-infected cultures failed to lyse or lysed poorly if shiftdown came later than 18 min after infection at 37°C. Symbols: (○) SP10 *rep*d; (●) SP10 *hmd*1.

Thus far, it is clear that Y-Thy is required for replication as well as cleavage of mature DNA from concatenates. In turn, it is proposed that both defects arise through faulty interaction of certain key proteins with unhypermodified DNA. It is difficult to judge from the available data if Y-Thy plays a role in differential transcription of the SP10 genome. Although it is true that SP10 late genes were weakly expressed under *hmd* conditions (Fig. 2), this could just as easily be attributed to the low levels of DNA synthesis that occur under those conditions (Fig. 3), since late transcription of numerous phage genomes is, to some extent, coupled to replication (15, 20). The importance of unusual bases in differential gene expression varies somewhat from system to system. In the case of coliphage T4, late genes can be transcribed only from an HM-cytosine-containing template (42). Indeed, the *alc* gene product of phage T4 seems to modify cellular RNA polymerase such that HM-cytosine-containing DNA is a preferred template (34). On the other hand, Thy can substitute for, at least, 20% of the HM-uracil in φe DNA without affecting phage functions (28). Similarly, Thy can replace up to 70% of the DHP-uracil in SP15 DNA without affecting gene expression (6, 36). Thus, there is no a priori reason to believe that Y-Thy would play a direct role in transcription of the SP10 genome.

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LITERATURE CITED

1. **Alegria, A. H.** 1967. Hydroxymethylation of pyrimidine nucleotides with formaldehyde. Biochim. Biophys. Acta **149**:317–324.
2. **Bolle, A., R. H. Epstein, W. Salser, and E. P. Geiduschek.** 1968. Transcription during early bacteriophage T4 development: synthesis and relative stability of early and late RNA. J. Mol. Biol. **31**:325–348.
3. **Bott, K., and B. Strauss.** 1965. The carrier state of *Bacillus subtilis* infected with the transducing bacteriophage SP10. Virology **25**:212–225.
4. **Brandon, C., P. M. Gallop, J. Marmur, H. Hayashi, and K. Nakanishi.** 1972. Structure of a new pyrimidine from *Bacillus subtilis* phage SP-15. Nature (London) New Biol. **239**:70–71.
5. **Brown, N. C.** 1970. 6-(*p*-Hydroxyphenylazo)uracil: a selective inhibitor of host DNA replication in phage infected *Bacillus subtilis*. Proc. Natl. Acad. Sci. U.S.A. **67**:1454–1461.
6. **Casella, E., O. Markewych, M. Dosmar, and H. Witmer.** 1978. Production and expression of dTMP-enriched DNA of bacteriophage SP15. J. Virol. **28**:753–766.
7. **Dawid, I. B., D. D. Brown, and R. H. Reeder.** 1970. Composition and structure of chromosomal and amplified ribosomal DNA's of *Xenopus laevus*. J. Mol. Biol. **51**:241–260.
8. **DeMars, R. I.** 1955. The production of phage related material when bacteriophage development is interrupted by proflavine. Virology **1**:83–99.
9. **Donelli, G., E. Dore, C. Frontali, and J. Grandolfo.** 1975. Structure and physico-chemical properties of bacteriophage G III. A homogeneous DNA of molecular weight 5 × 10^8. J. Mol. Biol. **94**:555–565.
10. **Dosmar, M., and H. Witmer.** 1979. An atypical deoxynucleoside triphosphate in *Bacillus subtilis* infected with bacteriophage SP10c. Curr. Microbiol. **2**:361–364.
11. **Frankel, F. R.** 1968. Evidence for long DNA strands in the replicating pool after T4 infection. Proc. Natl. Acad. Sci. U.S.A. **59**:131–138.
12. **Frankel, F., M. Batcheler, and C. Clark.** 1971. The role of gene 49 in DNA replication and head morphogenesis in bacteriophage T4. J. Mol. Biol. **62**:439–463.
13. **Gellert, M., M. O'Dea, T. Itoh, and J. Tomizawa.** 1976. Novobiocin and coumermycin inhibit DNA supercoiling by DNA gyrase. Proc. Natl. Acad. Sci. U.S.A. **73**:4474–4478.
14. **Gillespie, D., and S. Spiegelman.** 1965. A quantitative assay for DNA-RNA hybrids with DNA immobilized on a membrane. J. Mol. Biol. **12**:829–842.
15. **Glassberg, J., R. A. Slomiany, and C. R. Stewart.** 1977. Selective screening procedure for the isolation of heat- and cold-sensitive, DNA replication-deficient mutants of bacteriophage SP01 and preliminary characterization of the mutants isolated. J. Virol. **21**:54–60.
16. **Guha, A., and W. Szybalski.** 1968. Fractionation of the complementary strands of coliphage T4 DNA based on the asymmetric distribution of the Poly U and Poly U, G binding sites. Virology **34**:608–616.
17. **Hamilton, S., and D. E. Pettijohn.** 1976. Properties of condensed bacteriophage T4 DNA isolated from *Escherichia coli* infected with bacteriophage T4. J. Virol. **19**:1012–1027.
18. **Hemphill, H. E., and H. R. Whiteley.** 1975. Bacteriophages of *Bacillus subtilis*. Bacteriol. Rev. **39**:257–315.
19. **Jayaraman, R., and E. B. Goldberg.** 1969. A genetic assay for mRNA's of phage T4. Proc. Natl. Acad. Sci. U.S.A. **64**:198–204.
20. **Kahan, E.** 1966. A genetic study of temperature-sensitive mutants of the *subtilis* phage SP82. Virology **30**:650–660.
21. **Kropinski, A. M. B., R. J. Bose, and R. A. J. Warren.** 1973. 5-(4-Aminobutylaminoethyl)uracil, an unusual pyrimidine from the deoxyribonucleic acid of bacteriophage ØW-14. Biochemistry **12**:151–157.
22. **Legault-Demare, L., and G. H. Chambliss.** 1974. Natural messenger ribonucleic acid-directed, cell-free protein synthesizing system of *Bacillus subtilis*. J. Bacteriol. **120**:1300–1307.
23. **Lembach, K., and J. M. Buchanan.** 1970. The relationship of protein synthesis to early transcriptive events in bacteriophage T4-infected *Escherichia coli* B. J. Biol. Chem. **245**:1575–1587.
24. **Levner, M. H.** 1972. Replication of viral DNA in SP01-infected *Bacillus subtilis*. II. DNA maturation during abortive infection. Virology **48**:417–429.
25. **Levner, M. H., and N. R. Cozzarelli.** 1972. Replication of viral DNA in SP01-infected *Bacillus subtilis*. I. Replicative intermediates. Virology **48**:402–416.
26. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. **193**:265–275.
27. **Maltman, K. L., J. Neuhard, H. A. Lewis, and R. A. J. Warren.** 1980. Synthesis of thymine and α-putrescinylthymine in bacteriophage ØW-14-infected *Pseudomonas acidovorans*. J. Virol. **34**:354–359.
28. **Marcus, M., and M. C. Newlon.** 1971. Control of DNA synthesis in *Bacillus subtilis* by phage φe. Virology **44**:83–93.
29. **Markewych, O., A. Boghosian, M. Dosmar, D. Ende, and H. Witmer.** 1977. SP-10 bacteriophage-specific nucleic acid and enzyme synthesis in *Bacillus subtilis* W23. J. Virol. **21**:84–95.
30. **Markewych, O., E. Casella, M. Dosmar, and H. Witmer.** 1979. Deoxythymidine nucleotide metabolism in *Bacillus subtilis* W23 infected with bacteriophage SP10c: preliminary evidence that dTMP in SP10c DNA is synthesized by

a novel, phage-specific mechanism. J. Virol. **29**:61–68.
31. **Neuhard, J., K. L. Maltman, and R. A. J. Warren.** 1980. Bacteriophage ØW-14-infected *Pseudomonas acidovorans* synthesizes hydroxymethyldeoxyuridine triphosphate. J. Virol. **34**:347–353.
32. **Neville, M. M., and N. C. Brown.** 1972. Inhibition of a discrete bacterial DNA polymerase by 6-(*p*-hydroxyphenylaso)-uracil and 6-(*p*-hydroxyphenylazo)-isocytosine. Nature (London) New Biol. **240**:80–82.
33. **Sinsheimer, R. L., R. Knippers, and T. Komano.** 1968. Stages in the replication of bacteriophage ØW174 DNA *in vivo*. Cold Spring Harbor Symp. Quant. Biol. **33**:443–448.
34. **Snyder, L., L. Gold, and E. Kutter.** 1976. A gene of bacteriophage T4 whose product prevents true late transcription on cytosine-containing T4 DNA. Proc. Natl. Acad. Sci. U.S.A. **73**:2098–2102.
35. **Vinograd, J., J. Lebowitz, R. Radloff, R. Watson, and P. Laipis.** 1965. The twisted circular form of polyoma viral DNA. Proc. Natl. Acad. Sci. U.S.A. **53**:1104–1111.
36. **Walker, M. S., and M. Mandel.** 1978. Biosynthesis of 5-(4',5'-dihydroxypentyl)uracil as a nucleoside triphosphate in bacteriophage SP15-infected *Bacillus subtilis*. J. Virol. **25**:500–509.
37. **Warren, R. A. J.** 1980. Modified bases in bacteriophage DNA's. Annu. Rev. Microbiol. **34**:137–158.
38. **Weissbach, A., P. Bartl, and L. Salzman.** 1968. The structure of replicative lambda DNA-electron microscope studies. Cold Spring Harbor Symp. Quant. Biol. **33**:525–532.
39. **Witmer, H.** 1981. Deoxythymidylate and the unusual nucleotide in mature SP10 phage DNA are synthesized by postreplicational modification of 5-hydroxymethyldeoxyuridulate. J. Virol. **39**:536–547.
40. **Witmer, H., and M. Dosmar.** 1978. Synthesis of 5-hydroxymethyldeoxyuridine triphosphate in extracts of SP10c-infected *Bacillus subtilis* W23. Curr. Microbiol. **1**:289–292.
41. **Worcel, A., and E. Burgi.** 1972. On the structure of the folded chromosome of *Escherichia coli*. J. Mol. Biol. **71**:127–147.
42. **Wu, R., and E. P. Geiduschek.** 1975. The role of replication proteins in the regulation of bacteriophage T4 transcription. I. Gene 45 and hydroxymethyl-C-containing DNA. J. Mol. Biol. **96**:513–538.