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Polymer-Level Synthesis of Oxopyrimidine Deoxynucleotides by *Bacillus subtilis* Phage SP10: Characterization of Modification-Defective Mutants

HEMAN WITMER* AND CHRISTOPHER WIATR

Department of Biological Sciences, University of Illinois at Chicago, Chicago, Illinois 60680

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Bacillus subtilis **phage SP10 DNA has two oxopyrimidines, thymidine 5′-monophosphate (dTMP) and its hypermodified analog (YdTMP). Published data suggest that both are synthesized by postreplicational modification of 5-hydroxymethyldeoxyuridylate (HOMedUMP) in nascent DNA by the following pathway: HOMedUMP→PPOMedUMP→dTMP (85%) or YdTMP (15%); PPOMedUMP is 5-(hydroxymethyl-*O*-pyrophosphoryl)deoxyuridylate, the pyrophosphoric acid ester of the C_5CH_2OH function of HOMedUMP. This paper describes aberrant DNAs synthesized at nonpermissive temperatures by a complementary series of heat-sensitive, modification-defective (*mod*) mutants. Collectively, these mutants encompass the major steps in the complete modification of nascent SP10 DNA. DNA produced by *mod*A phage retains HOMedUMP as its sole oxopyrimidine, implying that (i) this mutant is defective in the pyrophosphorylation step and (ii) formation of PPOMedUMP is required for any further modification. Furthermore, studies with double mutants indicated that *mod*A is epistatic for all other *mod* mutants, which supports the hypothesis that *mod*A controls the earliest step in the modification pathway. Since their DNAs contain no YdTMP, *mod*C and *mod*D are defective in hypermodification (i.e., PPOMedUMP→YdTMP). However, dTMP occupies the entire oxopyrimidine fraction of *mod*C DNA, whereas *mod*D DNA has a normal dTMP content, but the now-missing YdTMP is replaced by either PPOMedUMP or a byproduct of abortive hypermodification. It is proposed that the *mod*D mutants are defective in the catalytic aspects of hypermodification and that *mod*C are defective in some regulatory function that promotes hypermodification at the expense of reductive modification (i.e., PPOMedUMP→dTMP). Reductive modification is defective in *mod*B phage, as evidenced by the absence of dTMP. In contrast to the others, *mod*B DNA has a complex oxopyrimidine content: HOMedUMP, ca. 30%; PPOMedUMP, ca. 40%; and YdTMP, ca. 30%. The expanded level of YdTMP suggests that at certain sites, reductive modification and hypermodification are competing reactions. Interestingly, the PPOMedUMP content of *mod*B DNA seemingly reflects the maximum degree to which phage DNA can be pyrophosphorylated, since the loss of YdTMP from *mod*B*mod*C and *mod*B*mod*D DNAs results in a unilateral increase in HOMedUMP content.**

The oxopyrimidine fraction of mature SP10 DNA contains two components (19; M. Mandel, unpublished data cited in reference 17). The major component, accounting for 85% of total oxopyrimidine, is thymidine 5-monophosphate (dTMP), and the minor component is a hypermodified analog of dTMP (YdTMP). The hypermodification is a δ-substituted derivative of L-glutamic acid linked via the α-amino function to the C-5 methyl of what is nominally dTMP. Both oxopyrimidines in mature SP10 DNA are synthesized at the polymer level (20).

A mechanism for polymer-level synthesis of dTMP and YdTMP has been proposed (20). Newly replicated DNA contains 5-hydroxymethyldeoxyuridylate (HOMedUMP) that is pyrophosphorylated to 5-(hydroxymethyl-*O*-pyrophosphoryl)deoxyuridylate (PPOMedUMP). Subsequently, the reducing or hypermodifying enzyme makes a nucleophilic attack at C-6 of the pyrimidine ring, generating a reactive exocyclic methylene by displacement of PP_i. Thereafter, the electrophilic exocycle receives either hydride to produce dTMP or the α-amino group of the hypermodification to produce YdTMP. Two critical features implicit within this model are that (i) reductive modification and hypermodification share a common step (i.e., formation of PPOMedUMP) and (ii) the frequency of each modification is rigidly controlled.

Heat-sensitive hypermodification-defective (*hmd*) mutants were described recently (20, 21). At nonpermissive temperatures, *hmd*-1 synthesized small amounts of DNA that contained normal levels of dTMP and an amount of PPOMedUMP equivalent to the now-missing YdTMP. Although such results certainly implicated PPOMedUMP as an intermediate in hypermodification, a similar role in reductive modification could only be assumed.

To date, little is known about the role of YdTMP in phage development except that presence of the atypical base is required for efficient DNA replication and packaging (21; unpublished data). However, it now seems clear that YdTMP plays an important role in protecting phage DNA from restriction by *Bacillus subtilis* R and, perhaps, other hosts (19; unpublished data).

This paper describes three new classes of heat-sensitive modification-defective mutants of SP10. These new mutants are designated *mod*A, *mod*B, and *mod*C. Their properties are compared with those of the previously described *hmd* mutants, which, for the sake of consistency, have been redesignated *mod*D. Collectively, these mutant classes seem to encompass the major steps in the complete modification of newly replicated SP10 DNA. At nonpermissive temperatures, the mutants make novel DNAs that should prove useful in the isolation of DNA-modifying enzymes.

* Corresponding author.

MATERIALS AND METHODS

All protocols have been published elsewhere and are cited at the appropriate places in the text. The generally permissive temperature was 30°C, and the generally nonpermissive temperature was 42°C. Each mutant was backcrossed five times to wild type (wt).

RESULTS

DNA metabolism in cells infected with *mod* mutants. SP10 wt DNA synthesis began 6 min postinfection at 42°C and rapidly accelerated to a high rate that was maintained until cell lysis at 45 min (Table 1; unpublished data). Between 6 and 12 min, all *mod* mutants synthesized DNA at or near the wt value; however, based upon their ability to sustain DNA synthesis at later times, the mutants could be separated into two groups. Those designated *mod*B and *mod*D-1 were unable to sustain DNA synthesis at later times, whereas those designated *mod*A, *mod*C, and *mod*D-2 continued to make DNA until the onset of cell lysis (ca. 105 min), albeit at a rate that evidently never exceeded 25 to 30% of the wt level (Table 1; unpublished data). Mutant DNA synthesis between 6 and 12 min will be referred to as early replication, and mutant DNA synthesis beyond 12 min will be referred to as late replication.

Contrary to an earlier report (21), *mod*D-2 was able to carry out late replication (Table 1). The earlier work was done with a mutant backcrossed once to wt, but the phage evidently retained a heat-sensitive mutation in a closely linked replication-defective gene. This particular *repd* gene codes for a protein that is dispensible during the earliest round(s) of replication but indispensible for later rounds (not shown). The "DNA arrest" genes of coliphage T4 have a similar phenotype, to cite a familiar example (4).

Products of early replication. (i) Isopycnic centrifugation. Preliminary evidence obtained during the mutant search (20) showed that no mutant-specific DNA made at 42°C had a normal oxopyrimidine content, and as a result, each should have an atypical buoyant density relative to wt (20, 21). Accordingly, mutant phage containing [^{32}P]DNA were prepared at 30°C and used to infect cells at 42°C. Newly made DNA was labeled with [6-^{3}H]dUrd from 6 until 11.5 min postinfection. Identically treated controls were infected with wt. DNA was extracted (2) and banded in neutral CsCl gradients (16, 20, 21).

With *mod*A, a hybrid peak containing parental [^{32}P]DNA and nascent [^{3}H]DNA was observed at a position denser than wt marker (Fig. 1A), whereas *mod*B hybrid material proved even denser (Fig. 1C). In contrast, material extracted from *mod*C-infected cells was less dense relative to wt (Fig. 1E). Hybrid material was repurified (21), denatured by dialysis against distilled water (16), and rebanded in neutral CsCl. In all cases, parental [^{32}P]DNA now cobanded with denatured wt DNA, whereas nascent material now banded at positions characteristic of each mutant DNA (Fig. 1B, D, and F). These data indicate that the atypical densities displayed by the hybrid DNAs stemmed exclusively from the atypical properties of mutant-specific nascent DNAs synthesized at 42°C. All labeled material extracted from control cultures banded with wt marker DNA (not shown).

Thus, for all mutants tested, early replication produced hybrid duplexes composed of a normally modified parental strand hydrogen bonded to a nascent aberrant strand. Presumably, such duplexes constitute the product of the first round of replication. A similar observation was made for *mod*D-1 and *mod*D-2 (21).

(ii) Deoxynucleotide composition. For deoxynucleotide analysis, cells were infected at 42°C with either wt or *mod* phage; cultures were labeled with [6-^{3}H]dUrd from 6 until 11.5 min. DNA was extracted, denatured, and CsCl purified. The DNA was enzymatically hydrolyzed to monodeoxynucleotides (16, 20) that were fractionated by either one-dimensional (20) or two-dimensional (3) chromatography.

All DNAs had the same dCMP content (Table 2). Moreover, all DNAs had the same total oxopyrimidine content, as evidenced by their comparable ratios of oxopyrimidine ^{3}H-deoxynucleotides to [^{3}H]dCMP. Composition of the oxopyrimidine fraction varied between DNAs. Nascent *mod*A- and *mod*C-specific DNAs each contained a single oxopyrimidine, HOMedUMP and dTMP, respectively. In agreement with published results (20), nascent *mod*D-specific DNA contained a normal quantity of dTMP, but YdTMP was now replaced by an equivalent amount of either PPOMedUMP (*mod*D-1) or HOMedUMP (*mod*D-2). Nascent *mod*B-specific DNA was unusual in that (i) although dTMP was absent, three other oxopyrimidines, HOMedUMP, PPOMedUMP, and YdTMP, were present in measurable amounts and (ii) YdTMP now occurred at twice the wt level. This would seem to indicate that *mod*B mutants are defective in reductive modification; how this could lead to conservation of PPOMedUMP and HOMedUMP as well as expanded levels of YdTMP will be discussed later.

Products of late replication. At later times of a modification-defective program, certain mutants were able to sustain low but significant levels of DNA synthesis (Table 1). In neutral CsCl gradients, native *mod*A and *mod*C DNAs synthesized at late times banded in the vicinity of the SP10 and bacterial DNA markers, respectively, whereas *mod*D-2

TABLE 1. DNA synthesis in bacteria infected with wt and *mod* SP10 phage[a]

Phage	DNA synthesis at the following time (min) postinfection (10^3 cpm per pulse)[b]								
	6–12	12–18	18–24	24–30	36–42	48–54	60–66	78–84	96–102
Wt	15.1	39.4	86.4	129.8	141.1	–[c]	–	–	–
*mod*A	12.6	9.3	11.7	15.8	21.5	27.9	27.1	30.0	29.4
*mod*B	10.1	1.4	<0.2	–	–	–	–	–	–
*mod*C	14.9	11.8	11.9	21.4	29.8	30.6	36.4	44.2	44.3
*mod*D-1	9.2	1.1	<0.2	–	–	–	–	–	–
*mod*D-2	13.6	13.1	17.8	22.4	26.7	23.5	29.3	38.4	40.1

[a] Cultures (25 ml) were infected at 42°C (10, 11) in the presence of 6-(*p*-hydroxyphenylazo)uracil, an inhibitor of bacterial DNA polymerase III (1, 13) that has no remarkable effect on SP10 development (10, 11). At various times postinfection, 1-ml samples were transferred to fresh tubes that contained 50 μl of [6-^{3}H]dUrd (0.5 μCi/μl). The samples were labeled for 6 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 (7).

[b] Only representative times are shown. The actual experiment included all 6-min intervals between infection and the onset of lysis.

[c] –, Sample not taken.

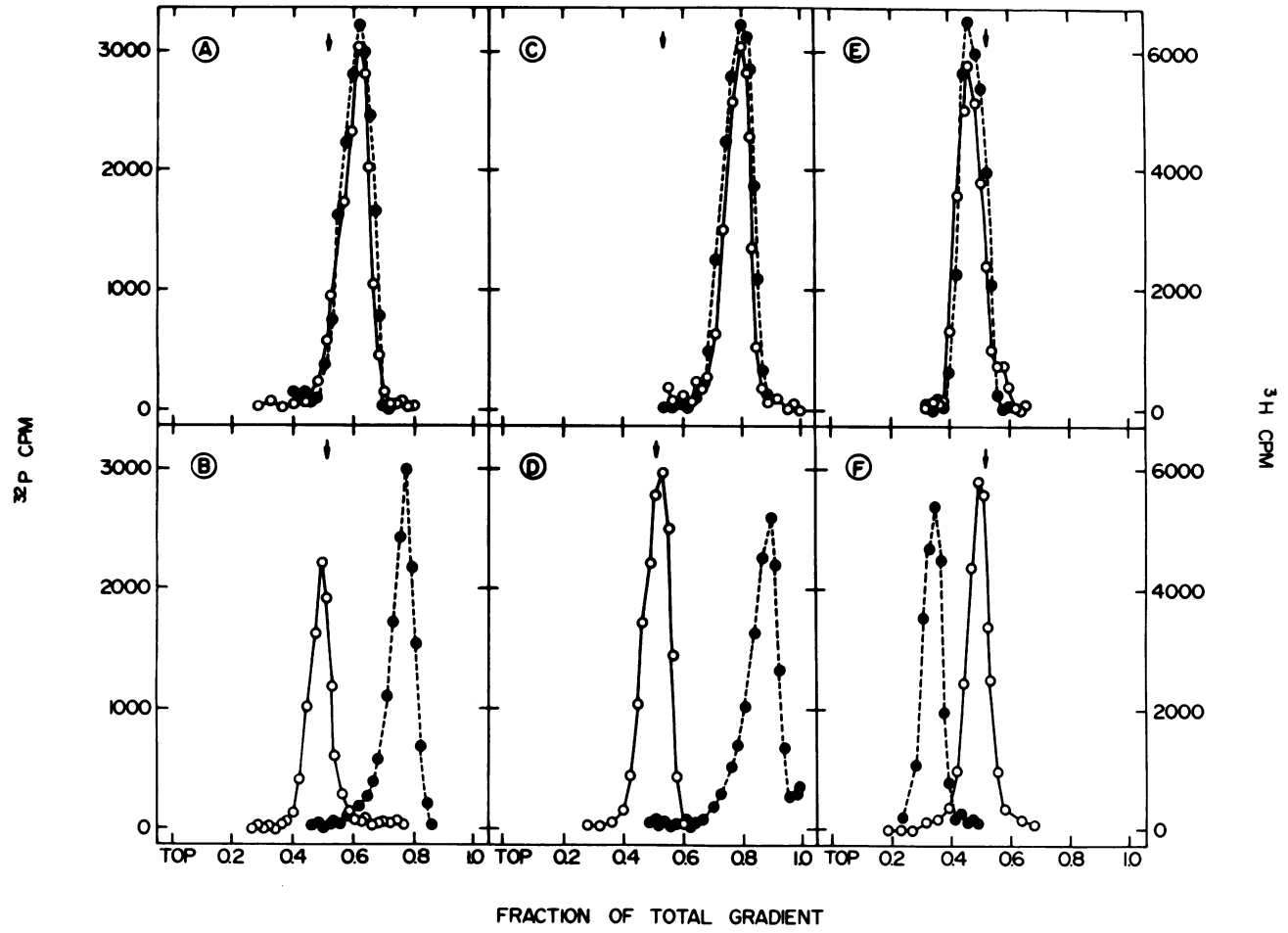

FIG. 1. Isopycnic centrifugation of mutant DNAs synthesized during early replication at 42°C. Cells were infected with phage containing [^{32}P]DNA. Newly replicated DNA was labeled from 6 until 11.5 min postinfection with [6-^{3}H]dUrd (10 μCi/ml). Samples of DNA were layered onto neutral CsCl solutions (1.720 g/ml) and centrifuged to equilibrium. All other protocols are in the text. Panels A, C, and E are native *mod*A, *mod*B, and *mod*C DNAs, respectively. Panels B, D, and F show denatured *mod*A, *mod*B, and *mod*C DNAs, respectively; in these cases, hybrid material from the native DNA gradients was extracted, denatured, and rebanded. Symbols: ○, parental [^{32}P]DNA; ●, nascent [^{3}H]DNA; and ↓, marker SP10 wt [^{14}C]DNAs run in a parallel gradient.

DNA banded at an intermediate position (Fig. 2A, C, and E). When denatured, mutant DNAs banded in the same positions relative to the aforementioned markers, but more importantly, denatured mutant-specific DNAs banded as single homogeneous peaks (Fig. 2B, D, and F). Chemical analyses revealed that HOMedUMP and dTMP occupied the entire oxopyrimidine fraction of *mod*A and *mod*C DNAs, respectively, whereas *mod*D-2 DNA contained dTMP and HOMedUMP in a molar ratio of ca. 5.5:1 (see below). Consequently, late replication represents the continued formation of homoduplexes composed of two aberrant strands. Homoduplexes first appeared 15 to 18 min after infection (not shown), which coincided well with the time at which *mod* DNA synthesis declined relative to wt (Table 1).

Characterization of double mutants. Mutants *mod*B and *mod*D-1 were unable to carry out late replication (Table 1). This could mean either that these two gene products are directly involved in replication at later times or that the

TABLE 2. Composition of mutant DNAs

DNA	% of recovered label[a]					[^{3}H]oxopyrimidine/[^{3}H]dCMP ratio
	HOMedUMP	PPOMedUMP	dTMP	YdTMP	dCMP	
Wt	–[b]	–	55.0	10.3	34.7	1.88
*mod*A	64.2	–	–	–	35.8	1.79
*mod*B	22.1	26.2	–	17.3	34.4	1.91
*mod*C	–	–	65.8	–	34.2	1.92
*mod*D-1	–	11.0	53.5	–	35.5	1.82
*mod*D-2	9.7	–	54.7	–	35.6	1.81

[a] Cells were infected at 42°C and labeled with [6-^{3}H]dUrd from 6 until 11.5 min. Chromatograms received 95,000 to 109,000 cpm of radioactivity, of which >96% was recovered. Purine nucleotides are ignored since they accounted for <200 cpm of the recovered label.

[b] –, spot containing <0.1% of the recovered label, ignored in the computations.

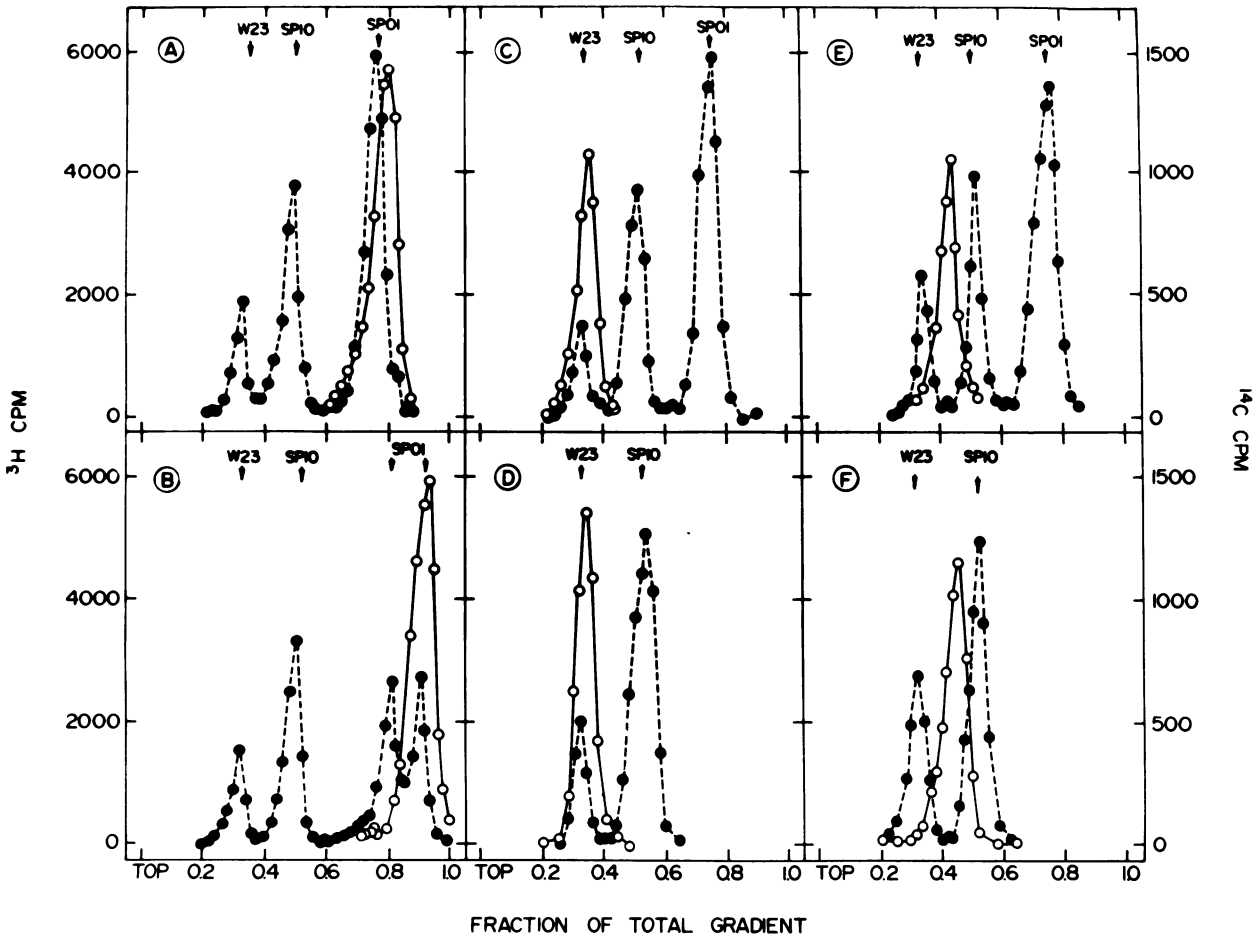

FIG. 2. Isopycnic centrifugation of mutant DNAs synthesized during late replication at 42°C. Cells were infected with unlabeled phage, and new DNA was labeled with [6-^{3}H]dUrd (10 μCi/ml) from 90 to 100 min postinfection. Panels A, C, and E are native *mod*A, *mod*C, and *mod*D-2 DNAs, respectively; panels B, D, and F are denatured *mod*A, *mod*C, and *mod*D-2 DNAs, respectively. Symbols: ○, [^{3}H]DNA; and ●, marker [^{14}C]DNAs run in the same tube.

DNAs made under nonpermissive conditions are unsuitable templates for continued replication. Since *mod*D-2 (an allele of *mod*D-1) was able to carry out late replication (Fig. 2C and F; Table 1), the latter possibility seemed more likely. The only common feature of *mod*B and *mod*D-1 DNAs synthesized at 42°C was the persistence of PPOMedUMP, implying that strands containing this highly charged nucleotide in quantity may not be suitable templates for the phage replicational machinery. As a result, replication terminates at the point where such strands would have to serve as templates to continue DNA synthesis. If so, conditions that remove PPOMedUMP should suppress the *mod*B and *mod*D-1 blockage on late replication. Accordingly, a complete set of double mutants was constructed. Each double mutant was tested for early and late replication as well as for the oxopyrimidine deoxynucleotide content of newly replicated DNA. For this analysis, DNA was synthesized in the presence of [*hydroxymethyl*-^{3}H]HOMedUrd, a precursor that labels only the oxopyrimidines in SP10 DNA (20).

Save for *mod*A *mod*B, all double mutants carrying *mod*B were defective in late replication (Table 3). The exceptional case synthesized DNA in a manner indistinguishable from that of *mod*A single mutants. On the other hand, *mod*A and *mod*C both suppressed the *mod*D-1 blockade on replication. Thus, suppression was indeed observed only under conditions wherein PPOMedUMP was not conserved in nascent DNA.

Several other points relating to the data in Table 3 seem noteworthy at this time. (i) *mod*A was epistatic to all other *mod* mutations, as evidenced by the fact that phage carrying *mod*A always conserved HOMedUMP as the sole oxopyrimidine. (ii) By the same reasoning, *mod*C was epistatic to *mod*D. (iii) *mod*B DNA contained HOMedUMP, PPOMedUMP, and YdTMP (Tables 2 and 3). As expected, *mod*B *mod*C and *mod*B *mod*D double mutants synthesized DNA that contained no YdTMP; interestingly, however, the loss of YdTMP was accompanied by a corresponding increase in HOMedUMP content and little, if any, change in the PPOMedUMP content.

DISCUSSION

Under nonpermissive conditions, the *mod*A mutation causes DNA to be conserved in what is tantamount to an unmodified state; i.e., HOMedUMP is the sole oxopyrimidine (Fig. 1A and B and 2A and B; Tables 2 and 3), implying that this mutation affects the earliest step in the modification process. Inferentially, mutations affecting the earliest step should be epistatic to mutations affecting later steps, and in fact, *mod*A is epistatic to all other *mod* mutations (Table 3). Pyrophosphorylation of HOMedUMP to PPOMedUMP is

the earliest known step in SP10 DNA modification (20). There is no compelling reason to believe that earlier steps exist, because increasing the reactivity of primary alcohols through the formation of their phosphoric or pyrophosphoric acid esters is a simple reaction found in many biochemical pathways (15). Using *mod*A-specific late DNA as template, we have recently succeeded in partially purifying the enzyme that converts HOMedUMP in DNA to PPOMedUMP with ATP (dATP) serving as the pyrophosphoryl donor (not shown). Interestingly, only the *mod*A-coded form of this enzyme is labile at 42°C (data not shown), thereby indicating that all pyrophosphoryl transfer to new SP10 DNA is carried out by a single enzyme.

The present data (Table 2) agree with earlier findings (20) that *mod*D-1 and *mod*D-2 substitute different nucleotides for YdTMP. As for *mod*D-1 DNA, PPOMedUMP occurs in place of YdTMP, implying that, at 42°C, the corresponding gene product becomes an absolute defective, incapable of any interaction with the precursor. On the other hand, replacement of YdTMP by HOMedUMP in *mod*D-2 DNA is more difficult to rationalize. It might be that, at 42°C, the *mod*D-2 gene product retains sufficient activity to make nucleophilic attack at C-6 of the pyrimidine ring, thereby forming the exocyclic methylene derivative, but is unable to transfer the hypermodification. Once formed, the exocycle is free to react with any available nucleophile. Normally, the enzyme would immediately present the α-amino group of the hypermodification, but in the absence of that transfer, it is conceivable that spontaneous hydrolysis of the enzyme · exocyclic methylene derivative complex would take place, creating free enzyme and HOMedUMP in DNA. Studies with model compounds suggest that such binary complexes could undergo nonenzymatic hydrolysis with a half-life of <1 s (14). Thus, under the unique conditions attendant to the *mod*D-2 mutation, it seems reasonable to assume that the occurrence of HOMedUMP reflects reformation of the ultimate precursor as a result of abortive hypermodification and not faulty pyrophosphorylation.

At 42°C, *mod*C and *mod*D phage synthesize DNA containing no YdTMP and, as such, behave as if they are both defective in some aspect of hypermodification. Notwithstanding, these mutations have fundamentally different effects on the oxopyrimidine content of DNA (Fig. 1E and F and 2C, D; Tables 2 and 3; reference 20). The oxopyrimidine fraction of *mod*D DNA still contains two components, i.e., a normal amount of dTMP along with an intermediate or byproduct of faulty hypermodification at a level equivalent to that of the absent YdTMP. Thus, *mod*D mutations do not affect the frequency of reductive modification. In contrast, dTMP occupies the entire oxopyrimidine fraction of *mod*C DNA, implying that reductive modification now occurs at positions normally hypermodified.

If complete modification of SP10 newly replicated DNA arises from the concerted activities of a regulated and unregulated process working on a common precursor, the seemingly paradoxical behavior of *mod*C and *mod*D mutants can be reconciled in terms of a simple, testable hypothesis. This hypothesis contains the assumption that reductive modification is an intrinsically unregulated process in that it can potentially occur at every oxopyrimidine site. Hypermodification, in contrast, is a regulated process that requires the cooperative and sequential action of two discrete functions. A regulatory function (*mod*C gene product) somehow commits a subset of all oxopyrimidine sites to hypermodification, and a catalytic function (*mod*D gene product) hypermodifies only the committed sites. Thus, under modification-defective conditions, *mod*D mutants make normal amounts of dTMP because the commitment aspect of hypermodification takes place, whereas *mod*C mutants, being defective in commitment, reductively modify each and every oxopyrimidine site. This hypothesis is also consistent with the fact that *mod*C mutations are epistatic to *mod*D mutations (Table 3).

In the above discussion, the somewhat ambiguous terms regulated and unregulated were used since it has yet to be determined that hypermodification of SP10 newly replicated DNA is a bona fide sequence-specific process. In this context, however, it is worth noting that dTMP but not YdTMP occurs at the central nucleotide position of 5′ . . . G-C-N-G-C . . . 3′ (R · *Fnu*4HI) sequences, whereas virtu-

TABLE 3. Analysis of DNA synthesized by double mutants at 42°C

Mutant	DNA synthesis (10^3 cpm)[a]		% of total oxopyrimidines[b]			
	Early	Late	HOMedUMP	PPOMedUMP	dTMP	YdTMP
wt	11.2	469.0	–[c]	–	82.4	17.6
*mod*A	9.4	374.6	100	–	–	–
*mod*B	6.8	0.8	31.1	40.3	–	28.6
*mod*C	10.8	412.1	–	–	100	–
*mod*D-1	6.1	1.1	–	16.2	83.8	–
*mod*D-2	7.7	361.5	15.1	–	84.9	–
*mod*A *mod*B	10.0	374.6	100	–	–	–
*mod*A *mod*C	9.1	339.1	100	–	–	–
*mod*A *mod*D-1	8.9	366.1	100	–	–	–
*mod*A *mod*D-2	10.1	391.0	100	–	–	
*mod*B *mod*C	7.0	0.7	59.3	40.7	–	–
*mod*B *mod*D-1	6.1	1.0	60.6	39.4	–	–
*mod*B *mod*D-2	7.2	1.3	50.2	41.8	–	–
*mod*C *mod*D-1	9.6	351.9	–	–	100	–
*mod*C *mod*D-2	9.1	391.0	–	–	100	–

[a] Cultures (25 ml) were infected as described in footnote *a* of Table 1 except that the labeled precursor was [*hydroxymethyl*-^{3}H]HOMedUrd. Sampling was started 6 min postinfection and continued until the onset of cell lysis. Cultures infected with mutant phage defective in late replication (see the text and below) failed to lyse; in these cases, sampling was terminated with the 18- to 24-min pulse since this typically was the last interval during which measurable incorporation was noted. "Early" denotes DNA synthesis between 6 and 12 min and is therefore the incorporation observed in the first sample. "Late" denotes DNA synthesis beyond min 12 and is reported here as the sum of incorporations obtained in all samples drawn at or after that time.

[b] Only results obtained with early DNA synthesis are presented here. In those cases where mutants carried out both early and late DNA synthesis, no significant differences in the nucleotide composition were ever observed. Chromatograms received 95,000 to 112,000 cpm, of which >97% was recovered.

[c] –, Nucleotide accounting for <0.1% of the recovered label, ignored in the computations.

ally all of the R · *Bsu*RI targets on SP10 DNA occur in the general sequence 5′ . . . G-G-C-C-YThy . . . 3′ (19). These observations suggest that at certain sites, reductive modification and hypermodification are mutually exclusive events. In other systems, hypermodification does appear to be a site-specific phenomenon. Coliphage Mu hypermodifies adenines in the general sequence, 5′ . . . (G,C)A(G,C)N(T,C) . . . 3′ (R. Kohman and D. Kamp, unpublished data cited in reference 5). *Pseudomonas acidovorans* phage ØW14 DNA contains equimolar amounts of dTMP and α-putrescinyl-dTMP (6), and the oxopyrimidines are distributed nonrandomly relative to each other (18). Like SP10, phage ØW14 synthesizes both oxopyrimidines at the polymer level from a common precursor (8, 9, 12).

DNA made at 42°C by *mod*B mutants contains no dTMP, and therefore, these phage seem defective in reductive modification (Fig. 1C and D; Tables 2 and 3). Absence of this process has remarkable effects on the oxopyrimidine content of DNA. (i) PPOMedUMP is conserved, which in turn supports the idea that this nucleotide is also an intermediate in reductive modification. (ii) It was noted above that, within the context of certain nucleotide sequences, reductive modification and hypermodification are mutually exclusive events. Notwithstanding, the fact remains that *mod*B-specific DNA has an overabundance of YdTMP, implying that, at certain other sites, the two processes compete to some degree. If SP10, like coliphage Mu, hypermodifies a family of related sequences, it is possible that the various included sequences are not hypermodified with unit efficiency. Restated in terms of the hypothesis outlined above, *mod*C-dependent commitment might be stringent at certain sites but relaxed, to various degrees, at others. (iii) Presence of HOMedUMP in *mod*B-specific DNA has interesting implications. Reformation of the ultimate precursor through abortive reductive modification seems an unlikely explanation in this case because it does not easily explain the continued retention of so much PPOMedUMP. Instead, a more plausible assumption would appear to be that, by virtue of the high negative charge density on PPOMedUMP, it is physically impossible to simultaneously pyrophosphorylate more than 40% of the HOMedUMP residues in DNA. Studies with double mutants (Table 3) showed that elimination of YdTMP from *mod*B DNA was accompanied by a corresponding increase in the HOMedUMP content with little apparent effect on the PPOMedUMP content; these latter data are consistent with the interpretation that SP10 DNA cannot be pyrophosphorylated beyond some critical point.

Overall, the results presented in this paper indicate that the complete modification of SP10 nascent DNA is a complex process involving the direct participation of at least four phage-coded functions. The novel DNAs synthesized by the mutant phage are proving useful in the identification and purification of modifying enzymes.

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