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Formation and Possible Functions of α-Putrescinylthymine in Bacteriophage φW-14 DNA: Analysis of Bacteriophage Mutants with Decreased Levels of α-Putrescinylthymine in Their DNAs

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The DNA synthesized in the nonpermissive host by the noncomplementing mutants *am*36 and *am*42 of bacteriophage φW-14 contains about half the wild-type level of α-putrescinylthymine (putThy) and a correspondingly greater level of thymine. The mechanisms whereby thymine nucleotides are excluded from replicating DNA are functional in both mutants because neither of them incorporates exogenous thymidine into DNA. It is proposed that (i) in wild-type φW-14, the conversion of hydroxymethyluracil to putThy at the polynucleotide level is sequence specific, but that to thymine is nonspecific; and (ii) in the mutants, the sequence-specific recognition is impaired so that more thymine and less putThy are formed. The thymine-rich DNA can be packaged into phage particles. In the case of *am*42, the phage particles are morphologically indistinguishable from and have essentially the same polypeptide composition as wild-type particles. However, the DNA molecules they contain are about 11% shorter than those in wild-type phage. *am*42rev4, a revertant of *am*42, contains DNA with about 70% of the normal level of putThy; these molecules are about 3% shorter than wild-type DNA. The properties of *am*42 and *am*42rev4 are consistent with the suggestion that putThy facilitates the very tight packing of φW-14 DNA (Scraba et al., Virology **124**:152–160, 1983). It also appears that the putThy content of φW-14 DNA can be reduced by no more than 30% without adversely affecting the production of viable progeny; for example, the burst size of *am*42rev4 is about 25% of that of the wild type.

In the DNA of the *Pseudomonas acidovorans* bacteriophage φW-14, 50% of the thymine is replaced with α-putrescinylthymine (putThy) (4). Both of these bases are formed from hydroxymethyluracil (hmUra) at the polynucleotide level (7, 11). The presence of this unusual base markedly affects the properties of φW-14 DNA (4) and of the virion (13). The ratio of thymine to putThy does not vary in the wild-type phage, suggesting that the putThy content of the DNA is carefully regulated. Since the distribution of putThy within φW-14 DNA is ordered (14), its content could be regulated by the sequence-specific conversion of hmUra to thymine and putThy. One approach to determining the factor(s) that regulates the putThy content is to isolate and characterize phage mutants defective in the formation of thymine or putThy or both.

The preliminary characterization of several such amber mutants of φW-14 was reported recently. Two of them, *am*36 and *am*42, do not complement each other, and in the nonpermissive host they synthesize DNA in which the ratio of thymine to putThy is increased significantly over that of the wild type (10).

This paper presents a more detailed analysis of *am*36 and *am*42. The results show that, like the wild type, these mutants do not incorporate exogenous thymidine into their DNAs. The increased thymine content is a consequence of a defect in the conversion of hmUra to thymine and putThy, rather than in the exclusion of thymine nucleotides from the DNA (7, 11). Furthermore, their physical properties also support the conclusion that putThy facilitates the packing of the DNA into the φW-14 head (13).

MATERIALS AND METHODS

The following materials and methods were described previously: bacterial and phage strains (3, 5, 10), culture media (4, 6), growth and infection of bacteria (6, 7), measurement of DNA synthesis (7), measurement of tritium release from [5-^{3}H]uracil (8), purification of phage (6), extraction and purification of intracellular DNA (7), purification of phage DNA (6),

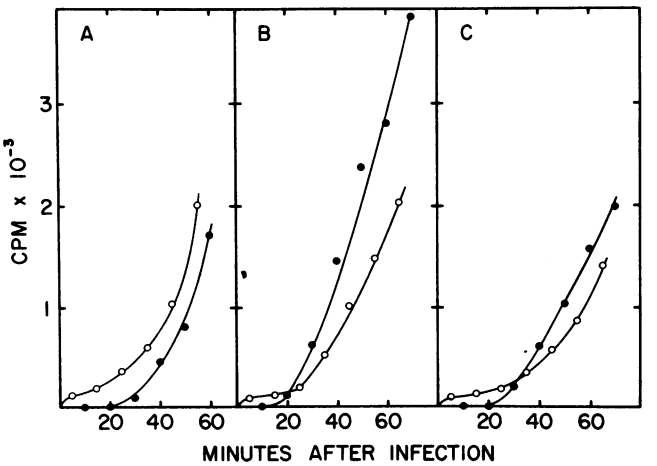

FIG. 1. DNA synthesis and tritium release from [5-^{3}H]uracil by infected cells of strain 3L. [5-^{3}H]uracil (specific activity, 0.1 μCi μg^{-1}, 1 μCi ml^{-1}) was added to an exponentially growing culture of strain 3L at a cell density of 3 × 10^8 ml^{-1}. At intervals, samples were removed for the determination of alkali-resistant, acid-insoluble radioactivity (○) and of the amount of tritium released from the uracil (●). Panels: A, wild-type φW-14; B, *am*36; C, *am*42.

hydrolysis of DNA and thin-layer chromatography of bases (7), analysis of monopyrimidine tracts (14), isopycnic density gradient centrifugation of DNA (6), thymidine incorporation (7), electron microscopy (13), and labeling and electrophoretic analysis of polypeptides (13). Spontaneous revertants of *am*42 were selected by picking single plaques grown on the permissive host into 10-ml cultures of the same host, incubating the cultures to lysis, plating the lysates on the nonpermissive host, and then selecting a single plaque from each lysate.

RESULTS

DNA synthesis in infected cells. The precursor [5-^{3}H]uracil can be used to measure both the synthesis of DNA and the synthesis of 5-substituted pyrimidines derived from uracil in phage-infected cells of strain 3L, a nonpermissive thymidine auxotroph of *P. acidovorans* strain 29 (3, 8).

In strain 3L cells infected with wild-type

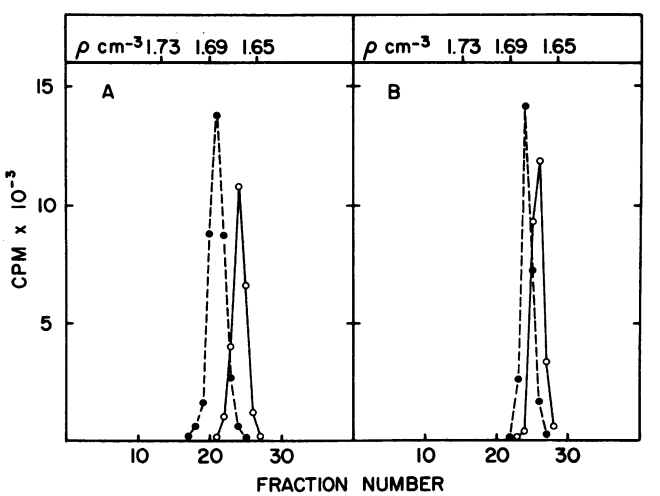

FIG. 2. Isopycnic density gradient centrifugation of DNAs. Mutant DNAs were labeled with [6-^{3}H]uracil (●), and wild-type DNA was labeled with $^{32}PO_4$ (○). Panels: A, *am*42 intracellular DNA from infected nonpermissive cells; B, *am*42rev4 DNA from phage particles. Wild-type DNA has a buoyant density of 1.666 g cm^{-3}.

phage, tritium release and the incorporation of uracil into phage DNA started together 20 min after infection and continued at the same rate (Fig. 1). In cells infected with either of the mutants, incorporation lagged behind release and continued at a slower rate (Fig. 1).

The DNA synthesized by cells infected with *am*36 and *am*42 was of uniform buoyant density in both cases and denser than wild-type DNA (Fig. 2). The mutant DNAs were of similar base composition because they were not clearly separable in CsCl gradients (data not shown). The DNAs synthesized by the mutants in the permissive host were of the same buoyant density as wild-type DNA.

Composition of mutant DNAs. The DNA synthesized by *am*36 and *am*42 in the nonpermissive host contained more thymine and less putThy than did wild-type DNA (Table 1). The only pyrimidine deoxynucleosides present in the hydrofluoric acid hydrolysates were deoxycytidine, thymidine, and α-putrescinyldeoxythymidine. Enzymatic digestion (8) released only the nucleotides of thymine, cytosine, and putThy (data not shown), showing that the DNAs did not contain any unusual, acid-labile intermediate(s) in the sequences of reactions converting hmUra to thymine and putThy (8). The putThy/thymine ratios in the monopyrimidine tracts of the DNAs, 0.40 for *am*42 and 0.47 for *am*36, were similar to the overall ratios of putThy/thymine. The putThy/thymine ratio in the monopyrimidine tracts of wild-type DNA is 2.4; the overall ratio is 1.0 (13).

Origin of the thymine in mutant DNAs. Thymidine incorporation into DNA by strain 3L stops after infection with wild-type φW-14 because the phage encodes a dTTPase (7) and an inhibitor of the host thymidylate synthase (11). Thymidine incorporation was also stopped by infection with either mutant (Fig. 3). Therefore, the distorted putThy/thymine ratios in the mutant DNAs resulted from a defect in the sequences of reactions converting hmUra to thymine and putThy at the polynucleotide level (7, 8, 11).

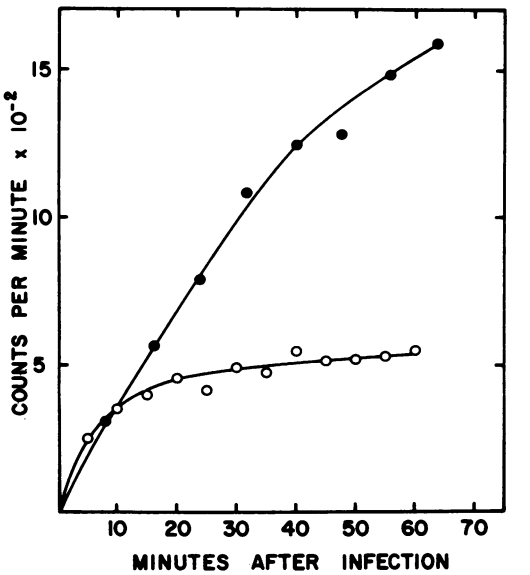

FIG. 3. Effect of phage infection on thymidine incorporation. [*methyl*-^{3}H]thymidine (specific activity, 0.1 μCi μg^{-1}, 250 μg ml^{-1}) was added to an exponential-phase culture of strain 3L at a cell density of 3×10^8 ml^{-1}. The culture was divided immediately, and one half was infected with *am*42 phage. Samples were removed at intervals for the determination of acid-insoluble radioactivity in the uninfected (●) and infected (○) culture. *am*36 behaved similarly.

Packaging of mutant DNAs. For both mutants, at least 50% of the alkali-resistant, trichloroacetic acid-insoluble radioactive material synthe-

TABLE 1. DNA base compositions[a]

DNA	Host	putdThd cpm[b]	Thymidine cpm[b]	Cytidine cpm[b]	putdThd/ thymidine ratio	(putdThd + thymidine)/cytidine ratio
*am*42 intracellular	Sup$^+$	6,904	7,219	14,007	0.96	1.00
*am*42 particles	Sup^0	2,780	9,289	12,062	0.30	1.00
*am*42 intracellular[c]	Sup^0	15,707	61,377	79,725	0.26	0.97
*am*42 particles[c]	Sup^0	1,935	6,522	9,974	0.30	0.85
*am*42 intracellular	Sup^0	9,854	26,139	31,099	0.38	1.16
*am*36 intracellular	Sup^0	6,207	13,471	16,231	0.46	0.82
*am*36 intracellular	Sup^0	2,333	7,602	11,809	0.31	0.84
*am*42rev4 particles	Sup^0	8,548	13,888	19,392	0.62	1.16
*am*42rev4 particles	Sup^0	5,575	10,757	19,939	0.52	0.82

[a] DNA was labeled, extracted, and hydrolyzed, and the pyrimidine deoxynucleosides were separated by two-dimensional thin-layer chromatography on cellulose sheets (13).

[b] Counts per minute in the area cut from the chromatogram. The total recovery of applied radioactivity was 90%.

[c] Samples from the same infected culture.

sized from [6-^{3}H]uracil in the nonpermissive host was DNase resistant. This suggested that some of the DNA was being packaged.

Since *am*36 and *am*42 appeared to have identical phenotypes, the following experiments were performed with *am*42 only.

Lysates of nonpermissive cells infected with *am*42 in the presence of [6-^{3}H]uracil, or with wild-type phage in the presence of $^{32}P_i$, were purified by differential centrifugation by the protocol used for phage purification. The resulting preparations were mixed and banded in a CsCl gradient (Fig. 4). The *am*42 preparation contained material of buoyant density greater than that of wild-type phage. The material at the top and bottom of the gradient was assumed to be fragments of DNA associated with ruptured virions and DNA released from ruptured virions, respectively. ϕW-14 ruptures during prolonged centrifugation in CsCl gradients (Warren, unpublished observations).

Nature of the packaged DNA. Lysates of nonpermissive cells infected with *am*42 were purified by differential centrifugation and banding on a discontinuous CsCl gradient (6). The method of purification was chosen because of the instability of ϕW-14 particles during prolonged centrifugation in CsCl (Fig. 4). The "phage" band was removed from the discontinuous gradient and dialyzed against TN buffer (0.01 M Tris-hydrochloride, 0.15 M NaCl, pH 7.5). The particles obtained were indistinguishable from wild-type ϕW-14 by electron microscopy (data not shown).

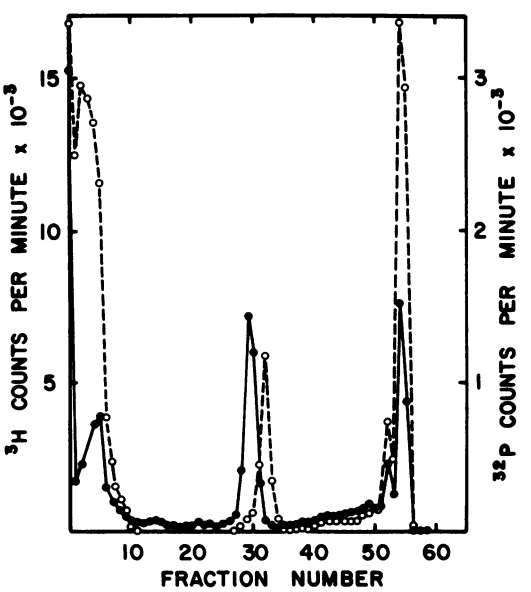

FIG. 4. Isopycnic density gradient centrifugation of phage particles. Symbols: (●) *am*42 particles prepared from nonpermissive cells infected in the presence of [6-^{3}H]uracil; (○) wild-type particles labeled with $^{32}PO_4$.

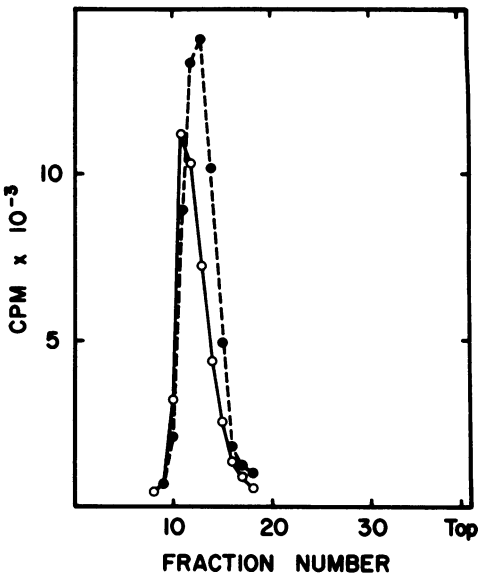

FIG. 5. Sucrose gradient sedimentation of phage DNAs. Wild-type DNA was labeled with $^{32}PO_4$ (○), and *am*42 DNA was labeled with [6-^{3}H]uracil (●) in the nonpermissive host.

The packaged DNA had the same base composition as the DNA extracted from cells 50 min after infection (Table 1). Therefore, DNA with more putThy than the average in the replicating pool was not packaged preferentially.

Electron microscopy showed earlier that the DNA in these *am*42 particles was some 11% shorter than wild-type DNA (13). This was confirmed by rate zonal centrifugation in sucrose gradients (Fig. 5); the ratio of *am*42 to wild-type DNA molecular weights was calculated to be 0.89 by using the formula derived by Burgi and Hershey (1). This value is in exact agreement with that obtained by measurements of relative lengths of molecules by electron microscopy (Table 2). The correspondence of molecular weights obtained by the two methods suggests that the same configuration (presumably B) is maintained in the wild-type and *am*42 DNAs; that is, *am*42 DNA contains fewer base pairs than wild-type DNA rather than the same number of base pairs compressed into a shorter length.

Revertants. The properties of the mutants suggested that any alteration in the putThy/thymine ratio might be lethal. However, in three out of ten spontaneous revertants of *am*42, the putThy/thymine ratio was less than that of the wild type. *am*42rev4 gave the lowest putThy/

TABLE 2. Comparison of φW-14 DNA molecules

Bacteriophage source	putdThd/ thymidine ratio	DNA length (μm)	No. of base pairs ($\times 10^3$)[a]	Average base pair mol wt[b]	DNA mol wt ($\times 10^6$)	Relative mol wt
φW-14 wild type	1.0	59.6	173	672	116	1.00
*am*42	0.3	53.1	154	666	103	0.89
*am*42rev4	0.6	57.7	167	669	112	0.97

[a] Values obtained by comparison with T4D DNA whose length was determined to be 62.1 μm under identical conditions (13) and which contains 180×10^3 base pairs (2).

[b] Values are calculated for the sodium salt with the assumption that each putrescinyl group displaces Na^+.

thymine ratio (Table 1); the buoyant density of its DNA was between those of the wild type and *am*42 (Fig. 2). Its particles had the same morphology as those of wild-type φW-14 (data not shown). The DNA molecules released from the particles by osmotic shock were slightly shorter than those from the wild type, but longer than those from *am*42 (Fig. 6, Table 2). The burst sizes of *am*42rev4 on the permissive and nonpermissive hosts were 110 and 130, respectively. The corresponding values for the wild type were 370 and 480. The ratio of the burst size on the nonpermissive host to that on the permissive host was 1.3 for the wild type and 1.2 for *am*42rev4. The buoyant density of *am*42rev4 DNA was the same from the permissive and the nonpermissive host (data not shown).

Phage structural polypeptides. There were no significant differences in the polypeptide compositions of virions of the wild type, *am*42 grown in the nonpermissive host, and *am*42rev4 (Fig. 7). Densitometer scans (data not shown) of the radioautographs suggested two small differences in the *am*42 profile.

DISCUSSION

The mechanisms excluding thymine from φW-14 DNA apparently continue to function in nonpermissive cells infected with *am*36 and *am*42 because exogenous thymidine is not incorporated into their DNAs. Therefore, the mutants are defective in the postreplicational modification of hmUra. Since the distribution of putThy

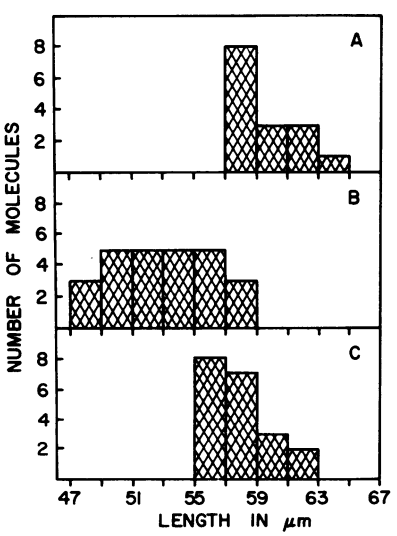

FIG. 6. Histograms of the lengths of DNA molecules released from phage particles by osmotic shock. Panels: A, wild type (mean length of DNA, 59.6 ± 1.0 μm); B, *am*42 (mean length of DNA, 53.3 ± 3.1 μm); C, *am*42rev4 (mean length of DNA, 57.5 ± 1.9 μm). The data for (A) and (B) are taken from Scraba et al. (13).

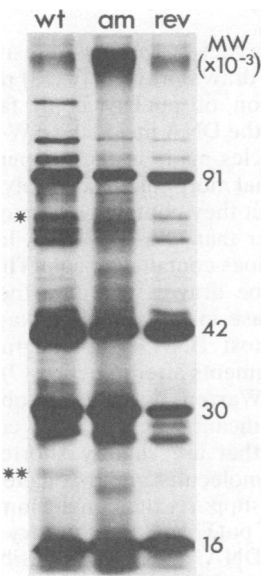

FIG. 7. Polypeptide compositions of phage particles. Fluorograms of 10% sodium dodecyl sulfate-polyacrylamide gels containing ^{3}H-amino acid-labeled proteins. Preparation and electrophoresis were as described previously (13). The major structural proteins of φW-14 have molecular weights of 91,000, 42,000, 30,000, and 16,000 (13). Abbreviations: wt, wild type; am, *am*42; rev, *am*42rev4.

is ordered (14), the modifying enzymes must, at some stage, recognize nucleotide sequence. The putThy/thymine ratios in the mutant DNAs indicate that the mutants lack the normal specificity for the conversion of hmUra to putThy. If it is assumed that the conversion of hmUra to 5-[(hydroxymethyl)-*O*-pyrophosphoryl]uracil (8) is the sequence-specific reaction, that 5-[(hydroxymethyl)-*O*-pyrophosphoryl]uracil is not a precursor of thymine, and that the formation of thymine is not sequence specific, the following working hypothesis can be proposed. Modification is coupled tightly to replication; the first modifying reaction is the sequence-specific conversion of some hmUra residues to 5-[(hydroxymethyl)-*O*-pyrophosphoryl]uracil, which is then converted to putThy; any hmUra residues that are not pyrophosphorylated are subsequently converted nonspecifically to thymine. Within the context of this hypothesis, the mutants *am*36 and *am*42 could be partially defective in the pyrophosphorylating enzyme, which would result in more thymine and less putThy being formed in the DNA.

DNA synthesis from [5-^{3}H]uracil by nonpermissive cells infected with *am*37 stops prematurely, whereas tritium release continues normally (8). It seems likely, therefore, that it is the accumulation of 5-[(hydroxymethyl)-*O*-pyrophosphoryl]uracil in *am*37 DNA, rather than the absence of putThy, that leads to the cessation of replication.

The properties of *am*42 are consistent with the conclusion drawn previously (13) that an important function of putThy is to facilitate tight packing of the DNA inside the ϕW-14 head. The *am*42 particles made in the nonpermissive host are of normal morphology and polypeptide composition, but they contain DNA molecules some 11% shorter than those of the wild type. Since this DNA does contain some putThy, no conclusions can be drawn regarding the role of the modified base in the actual packaging process. Although host DNA is not degraded to acid-soluble fragments after infection (7), none of it is packaged (Warren, unpublished observations).

It is significant that *am*42rev4 contains DNA molecules that are slightly shorter than wild-type DNA molecules and that have less putThy. This again supports the conclusion (13) that the presence of putThy allows the very tight packing of ϕW-14 DNA. Since the distribution of the lengths of the DNA molecules was broader for *am*42 than for the wild type and *am*42rev4 (Fig. 6), it is possible that putThy, in addition to influencing packing density, also has sequence-specific effects on the packaging process. *am*42 reverts at a frequency of $\sim 10^{-4}$, indicating that it does not contain multiple suppressible mutations. The *am*42rev4 phenotype is not suppressible because it produces DNA of the same buoyant density in both the permissive and nonpermissive hosts. The burst size of *am*42rev4 is not significantly different in the permissive and nonpermissive hosts. It seems unlikely, therefore, that the partial reversion of the *am*42 phenotype in *am*42rev4 is the consequence of mutation at a second site. Overall, the properties of *am*42 and *am*42rev4 suggest that the gene product defined by *am*42 is crucial for the production of ϕW-14 DNA containing the normal amount of putThy. The similarity in morphology, head volume, and polypeptide composition of wild-type, *am*42, and *am*42rev4 virions show also that it is the putThy content of the DNA that is the major factor in determining DNA packing density in this virus.

The decreased putThy content of *am*42rev4 DNA is surprising because the content of the wild-type DNA has never been observed to vary. However, the burst size of *am*42rev4 is only 25% of that of the wild type. This strain resembles those mutants of ϕe in which up to 20% of the hmUra can be replaced by thymine without affecting phage viability (9, 12). It appears that a decrease in the putThy content of ϕW-14 DNA of up to at least 30% is not lethal.

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