Deoxyribonucleoside Triphosphate Synthetase Complex*  

T4 Phage Gene 32 Protein as a Candidate Organizing Factor for the Deoxyribonucleoside Triphosphate Synthetase Complex*

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Linda J. Wheeler, Nancy B. Ray, Christian Ungermann, Stephen P. Hendricks, Mark A. Bernard, Eric S. Hanson, and Christopher K. Mathews*

From the Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon 97331-7305

After T4 bacteriophage infection of Escherichia coli, the enzymes of deoxyribonucleoside triphosphate biosynthesis form a multienzyme complex that we call T4 deoxyribonucleoside triphosphate (dNTP) synthetase. At least eight phage-coded enzymes and two enzymes of host origin are found in this 1.5-mDa complex. The complex may shuttle dNTPs to DNA replication sites, because replication draws from small pools, which are probably highly localized. Several specific protein-protein contacts within the complex are described in this paper. We have studied protein-protein interactions in the complex by immobilizing individual enzymes and identifying radiolabeled T4 proteins that are retained by columns of these respective affinity ligands. Elsewhere we have described interactions involving three T4 enzymes found in the complex. In this paper we describe similar analysis of five more proteins: dihydrofolate reductase, dCTPase-dUTPase, deoxyribonucleoside monophosphokinase, ribonucleotide reductase, and E. coli nucleoside diphosphokinase. All eight proteins analyzed to date retain single-strand DNA-binding protein (gp32), the product of T4 gene 32. At least one T4 protein, thymidylate synthase, binds directly to gp32, as shown by affinity chromatographic analysis of the two purified proteins. Among its several roles, gp32 stabilizes single-strand template DNA ahead of a replicating DNA polymerase. Our data suggest a model in which dNTP synthetase complexes, probably more than one per growing DNA chain, are drawn to replication forks via their affinity for gp32 and hence are localized so as to produce dNTPs at their sites of utilization, immediately ahead of growing DNA 3'-termini.

For some years our laboratory has investigated interactions among enzymes of deoxyribonucleoside triphosphate (dNTP) biosynthesis and mechanisms that coordinate dNTP synthesis with DNA replication (Mathews, 1993a, 1993b). Of particular interest is the question of how dNTP concentrations are maintained at saturating levels near replicative DNA polymerases, despite the relentless demand for precursors created by the extremely high rates of replicative DNA chain extension (over 500 s⁻¹ in prokaryotic cells). In studies of T4 phage-infected Escherichia coli, Greenberg's laboratory (Chiu et al., 1982) and ours (Allen et al., 1983; Moen et al., 1988) have described a multienzyme complex, called dNTP synthetase, which contains several phage-coded enzymes and at least two enzymes of host cell origin. In vitro, crude or purified preparations of this complex display kinetic facilitation of multi-step reaction pathways leading to dNTPs. In vivo, genetic evidence indicates that DNA replication draws from precursor pools that are very small in comparison with the total intracellular dNTP content (Ji and Mathews, 1993). Together, our data support a model in which the T4 dNTP synthetase complex is localized near replication sites and in which dNTPs destined for DNA replication are those produced by the complex in the immediate vicinity of replication forks.

Direct support for this model has been difficult to obtain, because as isolated in purified form, the dNTP synthetase complex does not contain DNA polymerase or other replication proteins (Moen et al., 1988). Accordingly, we have turned to other approaches, including protein affinity chromatography. In the T4 system, this approach was initially applied by Formosa et al. (1983) to analysis of interactions involving gp32, the single strand-specific DNA-binding protein encoded by gene 32. The protein was immobilized, and radiolabeled phage proteins bound to the chromatographic support were identified by two-dimensional gel electrophoresis. We have now applied the same approach to immobilized T4 dCMP hydroxymethylase (gp42), thymidylate synthase (gptd), and dCMP deaminase (gene cd). Each of these affinity ligands was found to retain several proteins of the dNTP synthetase complex and a few proteins of DNA replication and repair/recombination (Wheeler et al., 1992).  

In the present study, we have immobilized five more proteins: T4 ribonucleotide reductase, dCTPase-dUTPase, dihydrofolate reductase, deoxyribonucleoside monophosphokinase, and E. coli nucleoside diphosphokinase, with results similar to those seen in our earlier analyses. Unexpectedly, all eight proteins analyzed to date retain gp32 fairly strongly. Although some of the interactions may be indirect, we found that immobilized T4 thymidylate synthase tightly binds purified gp32, indicating a direct interaction between these two proteins. These observations suggest that dNTP synthetase complexes might be localized just ahead of growing DNA chains in the replication complex, drawn to these sites by their affinity for gp32 and functioning there to maintain local dNTP concentrations sufficient to sustain maximal replication rates.
MATERIALS AND METHODS

Recombinant E. coli Strains—All of the purified proteins in this study came from expression of recombinant genes inserted into plasmids and cloned in E. coli. Table I lists the source of each plasmid. Unless otherwise indicated, conditions for growth of the bacteria and induction of the recombinant genes were as described in the references cited.

Purification of Recombinant Proteins—All recombinant proteins were isolated from induced E. coli cultures of 1–3 liters. All purification schemes yielded proteins that were at least 95% homogeneous, as estimated by 45 and 60% acrylamide gel electrophoresis.

Deoxyribonucleoside Monophosphate Kinase (gpl)—The fractionation scheme was similar to that of Brush et al. (1990), with some differences. Cells were disrupted by sonication rather than French pressure cell treatment. Nucleic acids were precipitated with proline sulfate (0.2%) rather than streptomycin sulfate. In our hands, enzyme precipitated between 30 and 70% saturation with ammonium sulfate rather than 0–45%. Instead of Cibacron blue affinity chromatography, we used Mono-Q anion exchange chromatography in a Pharmacia FPLC apparatus with a 0–0.5 M NaCl gradient, in buffer A (50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 50 mM KCl, 1 mM dithiothreitol and 0.1 mM phenylmethylsulfonyl fluoride, 10% glycerol). Finally, our gel filtration step used a Superose-12 column and was also carried out in the FPLC apparatus also in buffer A.

dCTPase-dUTPase (gpl56)—The plasmid pLAM71* carries gene 56 downstream from a heat-inducible λ phage promoter. Induction was carried out by incubation at 42°C for 30 min. After collection of the cells by centrifugation, enzyme was isolated by a scheme based upon that of Warner and Barnes (1966) for the nonrecombinant gp56. Cells were disrupted by sonication, and nucleic acids were precipitated with streptomycin sulfate and then discarded. These and the subsequent DEAE-cellulose column chromatography were exactly as described by Warner and Barnes. The next step, gel exclusion chromatography, was carried out in the FPLC apparatus with a Superose-12 column. The buffer was 0.2 M potassium phosphate, pH 6.9, containing 1.0 M β-mercaptoethanol and 0.1 M NaCl. The final step, also carried out in the FPLC apparatus, involved hydrophobic interaction chromatography on a phenyl-Sepharose column. The gradient was from 5 to 0 M NaCl in 0.2 M potassium phosphate buffer, pH 7.0. Active fractions were pooled and concentrated by pressure dialysis. For further details, see Ungermann (1993).

E. coli Nucleoside Diphosphokinase (gndk)—E. coli DH5α cells transformed with plasmid pKT8P3 were incubated overnight in nutrient broth plus 100 μg/ml ampicillin. After centrifugation, bacteria were resuspended in buffer B (20 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 10 mM β-mercaptoethanol, 10% glycerol) and disrupted by sonication. Fractionation with ammonium sulfate followed with enzyme precipitating between 30 and 70% saturation with ammonium sulfate. The precipitated protein was added 0.3 volumes of 8% streptomycin sulfate, and the precipitated nucleic acid was discarded. After dialysis against buffer C (20 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, 50 mM KCl, 1 mM β-mercaptoethanol, 10% glycerol), the protein was applied to a 12-mL column of blue Sepharose (Pharmacia Biotech Inc.) pre-equilibrated in buffer C. N.D kinase was specifically eluted by 2 mM thymidine diphosphate in distilled water. After pressure dialysis, the concentrated material was loaded onto a Mono-Q FPLC column, and elution was carried out in a 0–0.5 M KCl gradient in buffer C. For complete details, see Ray (1992).

Dihydrofolate Reductase (gphfrd)—This procedure was based upon one originated in our laboratory (Purshet and Mathews, 1984). Bacteria were disrupted by sonication, and nucleic acids were precipitated from the extract with 0.3 volumes of 7% streptomycin sulfate. Enzyme was precipitated by the addition of ammonium sulfate to 45% of saturation. Hydrophobic interaction chromatography followed with a gradient of 1.7–0 M ammonium sulfate in 50 mM Tris-HCl buffer, pH 7.0, containing 10 mM β-mercaptoethanol. Active fractions were pooled, and then eluted with a 0–0.4 M NaCl gradient in 50 mM Tris-HCl, pH 7.0.

Ribonucleotide Reductase (gprndA and gprndB)—Induction of bacteria containing the plnrdAB plasmid was carried out by growing a 1-liter culture in LB broth plus 100 μg/ml ampicillin to an A₆₆₀ of about 0.7 and adding to that culture 400 μM isopropyl-thiogalactoside and 10 μM -mercaptoethanol. Bacteria were disrupted by sonic disruption, and nucleic acids were precipitated from the clear supernatant by adjustment to 1.0% streptomycin sulfate. Ammonium sulfate was added to 40% of saturation, and the precipitated protein was dissolved in buffer D and desalted into the same buffer. Chromatography was carried out in a Mono-Q anion-exchange column in the FPLC apparatus, using a gradient of 40–200 mM NaCl in buffer D. This column separated the R1R2 holoenzyme from its constituent unassociated R1 and R2 subunits. A typical 1.0-liter culture yielded about 1 mg of free R1 subunit, 2.5 mg of free R2 subunit, and 4.5 mg of R1R2 holoenzyme. For complete details, see Hanson (1994).

Thymidylate Synthase (gptd)—Recombinant T4 thymidylate synthase was prepared as described elsewhere.

Single-strand DNA-binding Protein (gpy56)—This procedure was a modification of a method described by Shamoo et al. (1986). Gene 32 expression was induced in E. coli AR120 carrying plasmid pY56 by overnight growth at 37°C in the presence of 0.12 mg/ml nalidixic acid. After centrifugation, cells were resuspended in 3 volumes of buffer E (10 mM Tris-HCl, pH 8.0, 50 mM NaCl, 0.1 mM EDTA, 0.1 mM β-mercaptoethanol, 10% glycerol) and then eluted with a 0–0.4 M NaCl gradient in 50 mM Tris-HCl, pH 7.0, 20 mM NaCl, 5 mM MgCl₂, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 1 mM ferrous ammonium sulfate (buffer D). Cells were disrupted by sonic disruption, and nucleic acids were precipitated from the clear supernatant by adjustment to 0.5% streptomycin sulfate. Ammonium sulfate was added to 40% of saturation and the precipitated protein was dissolved in buffer D and desalted into the same buffer. Chromatography was carried out in a Mono-Q anion-exchange column in the FPLC apparatus, using a gradient of 40–200 mM NaCl in buffer D. This column separated the R1R2 holoenzyme from its constituent unassociated R1 and R2 subunits. A typical 1.0-liter culture yielded about 1 mg of free R1 subunit, 2.5 mg of free R2 subunit, and 4.5 mg of R1R2 holoenzyme. For complete details, see Hanson (1994).

Affinity Chromatography—Methods for immobilization of protein, radiolabeling of T4 proteins, elution of proteins from affinity columns, and identification of retained proteins after two-dimensional gel electrophoresis were generally as described previously (Wheeler et al., 1989). Briefly, T4 proteins were labeled by incorporation of [35S]methionine from 3 to 8 min after infection of E. coli at 37°C. An extract was applied in column buffer to a column prepared by immobilizing 2–5 mg of purified recombinant enzyme on Affi-Gel-10 (Bio-Rad). Column buffer was 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM β-mercaptoethanol, 5 mM MgCl₂, 0.025 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride, and 10% glycerol. After thorough washing to remove unbound, “flow-through” proteins, bound proteins were eluted in three steps: first, by 0.2 M NaCl in column buffer, next by 0.6 M NaCl, and finally by 2.0 M NaCl.

During the course of this work, we found that applying extracts to affinity columns in the presence of a “physiological buffer” led to binding of higher quantities of protein; the amount of each protein bound was changed but not the ensemble of bound proteins. The physiologic

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2 Y. Shamo, personal communication.

3 S. Gerrard and C. K. Mathews, unpublished observations.
buffer contains potassium and glutamate, reflecting the principal intracellular small ions in E. coli (Richey et al., 1987). That buffer contains 0.1 M potassium glutamate, pH 8.0, 10% glycerol, 0.5 mM magnesium acetate, 1.0 mM β-mercaptoethanol, and 0.2 mM phenylmethylsulfonyl fluoride. Elution involved addition of NaCl to this modified column buffer in steps of 0.1, 0.5, and 2.0 M. We identify the conditions used for chromatography in each of the respective figure legends.

**RESULTS**

Two-dimensional Gel Electrophoretic Analysis of Bound Proteins—Formosa, Burke, and Alberts (1983) originally used protein affinity chromatography to identify protein-protein interactions in T4 phage infection. That study used gene 32 protein as the affinity ligand, and each interacting protein was identified by two criteria: 1) retention on the column at 0.05 M NaCl and elution at 0.2 M NaCl and 2) failure to be similarly retained by a column of immobilized bovine serum albumin. We have also used bovine serum albumin as a negative control; in addition, we have found that the same set of nonspecific proteins binds to a column of immobilized T4 lysozyme (data not shown).

In our earlier study of protein interactions with immobilized dCMP hydroxymethylase (gp42), we considered as significant only proteins that were retained on the column in 0.2 M NaCl and eluted at 0.6 M NaCl or higher. Thus, our criterion for significance of binding was more stringent than that of Formosa et al. (1983). Nevertheless, we identified 13 proteins that were retained by immobilized gp42 under these conditions (Wheeler et al., 1992). Subsequently, we identified six T4 proteins that bind similarly to immobilized T4 lysozyme (data not shown).

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Figs. 1–5 show two-dimensional electrophoretic patterns of T4 proteins strongly retained by five other enzymes in the T4 dNTP synthetase complex: E. coli nucleoside diphosphokinase (NDP kinase; Fig. 1), aerobic ribonucleotide reductase (Fig. 2), deoxyribonucleotide monophosphate kinase (dNMP kinase; Fig. 3), dCTPase-dUTPase (Fig. 4), and dihydrofolate reductase (Fig. 5). Each enzyme behaves similarly to the other three we have analyzed in binding a half dozen or more T4 proteins specifically and fairly strongly.

The results with NDP kinase are particularly noteworthy, because this enzyme is of bacterial origin, yet a small amount of NDP kinase is evidently sequestered within the dNTP synthetase complex by specific protein associations (Reddy and Mathews, 1978; Allen et al., 1983). Even more phage proteins are bound to this host-cell enzyme if we consider those eluted by 0.2 M NaCl (Fig. 6). These observations are all the more remarkable when we consider that the immobilized NDP kinase retains very few E. coli proteins. Fig. 7 illustrates this, showing one-dimensional SDS-polyacrylamide gel electrophoresis analysis of E. coli and T4 proteins bound to immobilized NDP kinase. Fig. 7C shows one additional observation. Note from Fig. 1 that a prominent bound protein is one that we have identified as the product of gene uvsY, a protein involved in DNA repair and recombination (Yonesaki et al., 1985). Using...
one-dimensional gel analysis, we analyzed T4 proteins in an extract of E. coli infected by a uvsY amber mutant. Several of the most tightly bound proteins were missing in this pattern (Fig. 7C, far right lane, showing proteins retained at 0.6 M NaCl but eluted by 2.0 M NaCl). It seems likely that these proteins do not bind directly to E. coli NDP kinase, but are retained by the column because they associate with bound gp uvsY.

In our analysis of proteins bound to T4 ribonucleotide reductase, we observed the effects of including an allosteric ligand, ATP, in the eluting buffers. This ligand strongly affects subunit associations in the heterotetrameric holoenzyme (Hanson and Mathews, 1994). The lower panel of Fig. 2 shows an electrophoretic pattern from an experiment identical to that of the upper panel, except for the presence of 1 mM ATP in the column buffer and all of the eluting buffers. This low molecular weight ligand increased the number and amounts of retained proteins, suggesting that protein-protein interactions in this complex are mediated in substantial measure by low molecular weight substrates and regulatory molecules.

A Direct Interaction between gp32 and T4 Thymidylate Synthase—Table II lists all of the T4 proteins that we have identified in the 0.6 M NaCl eluates as being bound specifically to each of the eight immobilized enzymes that we have examined. Remarkably, one protein retained by all eight affinity ligands is gp32, the single-strand DNA-binding protein. This finding is particularly noteworthy, because gp32, a protein of moderate size, has already been shown to interact with numerous proteins of DNA replication, recombination, and repair (Formosa et al., 1983; Krassa et al., 1991; Hurley et al., 1993). It seems unlikely that gp32 binds directly to each of the eight enzymes we have examined. Rather, some of these proteins probably associate indirectly by binding to a protein that binds directly to gp32. In fact, our earlier study (Wheeler et al., 1992) presented evidence supporting a direct association between gp32 and T4 thymidylate synthase. We tested that idea by asking whether purified gp32 is retained on a column of immobilized T4 thymidylate synthase. As shown in Fig. 8, some of the gp32 was found in the flow-through fractions, suggesting that the binding capacity of the immobilized dTMP synthase column
The original aim of this study was not, as implied by the title of this paper, to characterize interactions between the proteins that copurify as components of the T4 dNTP synthetase complex and gp32. The experiment of Fig. 10, which showed that the three proteins, dNMP kinase, dCTPase-dUTPase, and thymidylate synthase, interacted, was carried out in potassium glutamate buffer, with stepwise NaCl additions as indicated. Three fractions were collected in each step. Analysis of eluates was by one-dimensional SDS-polyacrylamide gel electrophoresis and Coomassie Blue staining. M, molecular weight markers; gp32, purified protein applied to the column; FT, flow-through fraction.

had been exceeded. However, of that protein that did bind, most was retained at 0.2 M NaCl and eluted at 0.6 M, indicating a strong interaction between these proteins in the absence of other proteins. When the same experiment was repeated with immobilized E. coli dTMP synthase instead of the T4 enzyme, no retention of gp32 was seen, indicating the specific nature of the interaction between the two T4 proteins (data not shown).

Other Protein-Protein Associations in the dNTP Synthetase Complex—The original aim of this study was not, as implied by the title of this paper, to characterize interactions between the dNTP synthetase complex and gp32. Rather, our goal was (and remains) to identify as many protein-protein interactions as possible, using a range of experimental approaches. Several other interesting associations have been discovered recently, and they are described in this section.

**DISCUSSION**

Table II summarizes the principal results of our affinity chromatography experiments with eight purified recombinant proteins that copurify as components of the T4 dNTP synthetase complex and gp32. Each identified association results from detection of the indicated protein among those proteins retained at 0.2 M NaCl and eluted at 0.6 M NaCl, either in this paper or in our previous studies (Wheeler et al., 1992).
The gene 32 protein exists at about 10,000 copies per cell, and it plays a stoichiometric role in supporting replication; thus, if the dNTP synthetase complex facilitates the transfer of dNTPs to replication sites, several complexes must serve each replication site. How might this occur? On the other hand, genetic evidence indicates that dNTPs used for replication are drawn from pools that are much smaller than those determined by biochemical analysis (Ji and Mathews, 1993), as expected if growing DNA chains draw precursors from dNTPs synthesized in the immediate vicinity. To date, ten enzymes have been reported as components of the purified dNTP synthetase complex: the eight that we have immobilized, plus T4 thymidine kinase and E. coli adenylate kinase (Allen et al., 1983; Moen et al., 1988). From the molecular mass of the purified complex (about 1.5 MDA), it seems that no more than one or two copies of each enzyme molecule can exist in each complex. However, the turnover numbers for enzymes in the complex are at least an order of magnitude lower than the rate of replicative DNA chain growth. Also, the enzyme molecules themselves exist in considerable molar excess (a few thousand molecules per cell) over the number of replication forks or 120 growing DNA chains (Werner, 1968). Hence, each replicating DNA strand has not been involved, and the base composition of T4 DNA and assuming that dNTPs used for replication are drawn from pools that are much smaller than those determined by biochemical analysis (Ji and Mathews, 1993), as expected if growing DNA chains draw precursors from dNTPs synthesized in the immediate vicinity. To date, ten enzymes have been reported as components of the purified dNTP synthetase complex: the eight that we have immobilized, plus T4 thymidine kinase and E. coli adenylate kinase (Allen et al., 1983; Moen et al., 1988). 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Thus, if the dNTP synthetase complex facilitates the transfer of dNTPs to replication sites, several complexes must serve each replication site. How might this occur? The physical and functional relationships between the T4 dNTP synthetase complex and the DNA replication complex in vivo have long been obscure. Evidence for existence of the complex was originally sought as a means to explain how DNA precursors could be delivered to replication sites at rates sufficient to sustain chain growth rates of 500 s\(^{-1}\) or more. However, isolation of the complex provided little evidence for its association with replication proteins, although DNA polymerase was observed to cosediment with dNTP-synthesizing enzymes in gradient analysis of T4-infected cell extracts (Chiu et al., 1982). 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The gene 32 protein exists at about 10,000 copies per cell, and it plays a stoichiometric role in supporting replication; reducing the number of gp32 molecules per cell reduces the replication rate proportionately (for reviews, see Karpel (1990) and Kornberg and Baker (1992)). The number of gp32 molecules associated with each replicating DNA strand has not been
enzyme complexes, then this implies that a great deal of protein must be moving along with the DNA polymerase holoenzyme. However, because T4 DNA replication is associated with the bacterial membrane (Miller, 1972), it is likely that replication involves movement of DNA chains past a stationary replicative complex. The data of this paper suggest that this complex could also include DNA precursor-synthesizing complexes.

The suggestion that gp32 helps to organize dNTP synthetase complexes just ahead of DNA growing points is speculative. However, it explains several observations in addition to the affinity chromatographic analysis described here. More important, the model sets the stage for future experiments.

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FIG. 11. A speculative model for the association of T4 dNTP synthetase with DNA replication machinery. For simplicity, only a leading strand complex is shown. gp43 is DNA polymerase, and gp45 is the processivity-enhancing protein. Whether the other polymerase accessory proteins, gp44 and gp62, travel with the polymerase is not yet determined, but it must be considerable, because 10,000 molecules divided by 60 forks divided by two strands per fork gives about 80 molecules per strand. Because some gp32 molecules are associated with recombination, DNA repair, and probably other functions, the number 80 represents an upper limit.

However, this semi-quantitative discussion suggests that the associations between gp32 and the dNTP-synthesizing enzymes might provide a route for attracting several dNTP synthetase complexes to the vicinity of each fork. Moreover, because gp32 is bound to DNA single strands created by action of the primosome in advance of the movement of the DNA polymerase holoenzyme, an association between gp32 and multiple dNTP synthetase complexes could maintain high dNTP concentrations in the precise region where they are needed: just in front of a rapidly moving DNA polymerase holoenzyme. Fig. 11 suggests a simplified and speculative form of this model.

Note from Table II that several other replication proteins are bound by enzymes in the dNTP synthetase complex, including gp61 (primase) and gp45 (processivity-enhancing protein). Because these proteins were also shown to be bound by immobilized gp32 (Formosa et al., 1983), the associations with dNTP-synthesizing enzymes may be indirect; that hypothesis can be tested experimentally. The associations with gp45X and gp45Y suggest that the dNTP synthetase complex may be associated also with the complex that carries out recombination-dependent replication, because both of those proteins are involved in that process (Kreuzer and Morrical, 1994). However, the significance of the associations involving gp45G is difficult to discern at this stage, because the reaction catalyzed by the gene product, glucosylation of DNA hydroxymethylcytosine residues, occurs away from the replication fork.

If gp32 moves along a replicating DNA strand by "treadmilling" (simultaneous association and dissociation of individual gp32 molecules), then the model proposed here is untenable. Association of a 35-kDa protein with a 1.5-MdA complex would seem to fatally hinder the mobility of a protein that must be in such continuous motion. However, structural studies on gp32 (Shamoo et al., 1995) indicate that each molecule of the protein contacts only two or three DNA nucleotide residues. This finding plus kinetic analysis of gp32 dissociation from DNA in vitro (Lohman, 1984) are consistent with the idea that the several gp32 molecules associated with one DNA polymerase holoenzyme slide as a unit in front of that polymerase. Even so, if gp32 molecules at a replication fork are associated with large
