Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 8665–8669, November 1990
Biochemistry

An essential arginine residue for initiation of protein-primed DNA replication

(site-specific mutagenesis/DNA-terminal protein/deoxynucleotide binding site)

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Communicated by I. C. Gunsalus, August 6, 1990

ABSTRACT **A group of proteins that act as primers for initiation of linear DNA replication are called DNA-terminal proteins (terminal proteins). We have found a short stretch of conserved amino acid sequence among the terminal proteins from six different sources. The location of this sequence motif is also similar among the different terminal proteins. To determine the functional role of this terminal-protein domain in DNA replication, we have studied the bacteriophage PRD1 system. The PRD1 terminal protein and DNA polymerase genes were cloned into expression vectors, and the recombinant plasmids were used for constructing PRD1 terminal protein mutants. Site-directed mutagenesis and functional analysis showed that one of the two arginines (Arg-174) in the conserved sequence is critical for the initiation complex-forming activity of the PRD1 terminal protein. Replacement of Arg-174 by noncharged amino acids resulted in nonfunctional terminal protein. Phenylglyoxal, an α-dicarbonyl compound that reacts with the guanidino group of arginine, inhibits initiation complex formation between PRD1 terminal protein and dGMP. On the basis of these results, we propose that Arg-174 represents, at least in part, the binding site for phosphate groups of dGTP.**

Because DNA polymerases require template and primer and act only in the 5′ to 3′ direction, linear double-stranded DNA genomes pose the special problem of preserving the 5′ ends of DNA during DNA replication (1). If RNA molecules are used as primers and are removed, gaps will be created at the 5′ ends and will never be filled (1). Several viral genomes use proteins, instead of RNAs, as primers for the initiation of DNA replication (2–6). Such proteins not only function as the primers for DNA synthesis but also remain bound to the 5′ ends of DNA and are encapsulated during viral morphogenesis. These proteins bound to the DNA then can be isolated as covalent DNA-protein complexes from viral particles. Thus, they have been called DNA-terminal proteins or simply terminal proteins.

The "protein-priming" mechanism for the initiation of DNA replication involves in principle the deoxynucleotidylation of a hydroxy amino acid residue within the terminal protein. The hydroxy amino acid can be serine (7, 8), threonine (9), or tyrosine (10). This deoxynucleotidylation is catalyzed by virus-encoded DNA polymerases and is dependent on the presence of Mg^{2+} and the viral DNA–protein complex as a template. The consequent terminal protein-dNMP complex is then used to initiate chain elongation.

Bacteriophage PRD1 is a small lipid-containing phage that infects a wide variety of Gram-negative bacteria (11). The genome of PRD1 is a linear, double-stranded DNA molecule of ≈14.7 kilobase pairs (kb) and has a 28-kDa terminal protein linked covalently to each 5′ terminus (11). The linkage between the terminal protein and DNA is a phosphodiester bond between the hydroxyl group of a tyrosine residue of the terminal protein and a dGMP nucleotide at the 5′ terminus of the PRD1 DNA (10).

The nucleotide sequence of the PRD1 terminal protein gene has been described (12, 13). When the deduced amino acid sequence was compared with those of other terminal proteins previously reported, no substantial amino acid sequence homology was found (12, 13). On the other hand, the PRD1 DNA polymerase showed significant sequence homology not only with the other protein-primed DNA polymerases but also with RNA-primed DNA polymerases. These observations strongly suggest that both protein-primed and RNA-primed DNA polymerases have evolved from a common progenitor (12, 14). Based on these observations, it was anticipated that the terminal proteins from divergent organisms may also be related. Upon further examination of the amino acid sequence of the terminal proteins, we have found a short stretch of conserved amino acid sequences among the terminal proteins from six different sources (see Fig. 1). These conserved amino acid sequences are located at approximately the same position in the terminal proteins, with the exception of adenovirus pre-terminal protein.

Proteins with similar functions, but derived from different sources, commonly share short patches of identical amino acid sequences at corresponding locations of the protein molecules (15, 16). Therefore, it is most likely that these sequence conservations are a consequence of their contribution to the terminal-protein function and that the occurrence of such patches of amino acid sequence homology are a general feature of these terminal proteins. To examine whether these conserved amino acid sequences play an essential role in the function of the DNA-terminal protein, site-directed mutagenesis was performed with phage PRD1 to generate various mutants. Our results show that one of two arginines in the conserved sequence plays a crucial role in the function of the phage PRD1 terminal protein. We propose a possible mechanism by which Arg-174 of the PRD1 terminal protein is involved in dGTP binding, the first step of the protein priming.

MATERIALS AND METHODS

Bacteria, Phage, and Plasmids. *Escherichia coli* RZ1032 ({Hfr KL16 *pro-45* [*lysA* (61-61)]}, *dut-1 ung-1 thi-1 relA1*), kindly provided by C. M. Joyce (Yale University), was used to prepare the uracil-containing DNA template. *E. coli* NM522 {Δ(*lac–proAB*) *thi hsd5 supE* [F *proAB lacI*^{q}*Z*ΔM15]} (17) was used for the transformation of the uracil-containing plasmid DNA. *E. coli* HLB3(pLM2, pLM3), which carries PRD1 genes *1* and *8*, was kindly provided by L. Mindich (18). The phage T7 expression system was a gift of S. Tabor and C. C. Richardson (19). The plasmids pT7-6 in *E. coli* HMS174 (*recA1 hsdR*) and pGP1-2 in *E. coli* strain K38(HfrC λ) (19) were used to obtain overproduction of PRD1 terminal protein and DNA polymerase. The phagemid expression vector pEMBLex3 was

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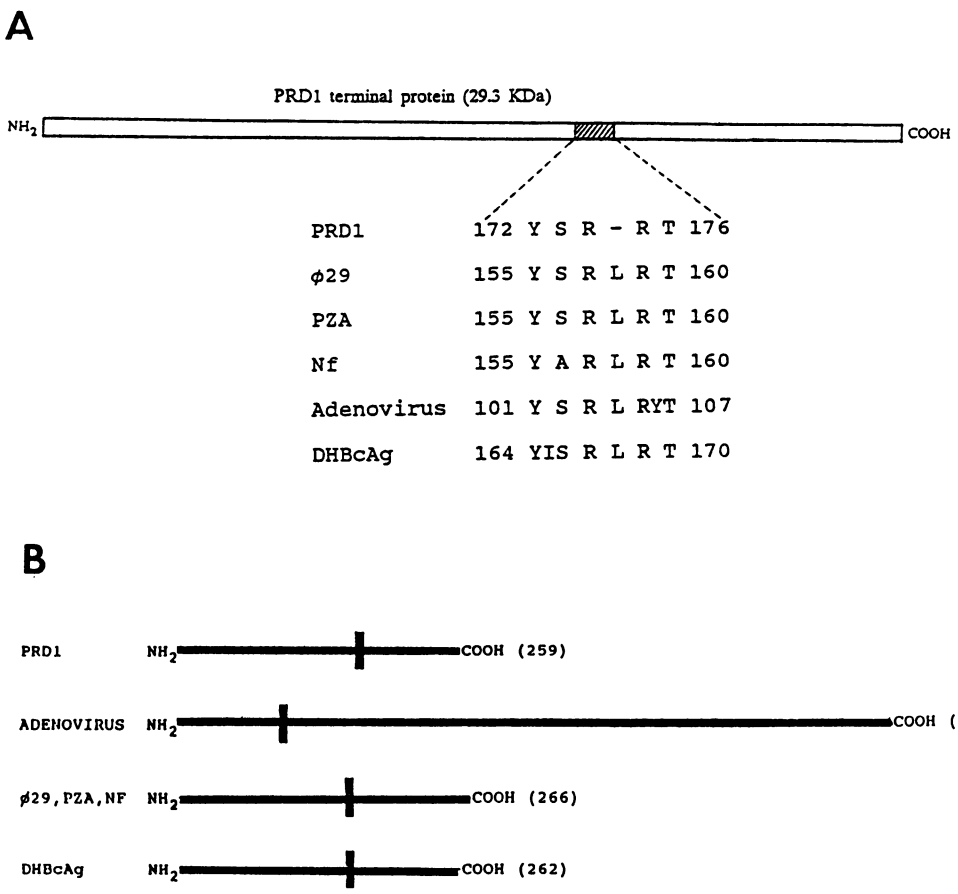


FIG. 1. (*A*) Amino acid similarities among PRD1 terminal protein, the terminal proteins from the viruses ϕ29 (25), PZA (26), Nf (27), and human adenovirus 2 (28), and the core antigen of duck hepatitis B virus (DHBcAg) (29). The single-letter amino acid code is used. (*B*) Spatial location of the conserved amino acid sequence, YSRLRT, among the terminal proteins and DHBcAg. Position of the conserved sequence in each polypeptide is indicated by a vertical bar. Sizes of the proteins are proportional to the lengths of the lines. Number of amino acids in each protein is shown in parentheses.

obtained from G. Cesarevi (20). The helper phage M13K07 (21) was purchased from International Biotechnologies.

Cloning Strategy. A 3-kb DNA fragment containing the PRD1 terminal protein and DNA polymerase genes and the adjacent 90% of the lysin gene was obtained from the recombinant plasmid pLM3 (18) by cleavage with restriction endonuclease *Pst* I. The 3-kb *Pst* I DNA fragment was inserted into the *Pst* I site of pT7-6, which was then used to transform *E. coli* NM522. The plasmid pT7-3k from the resulting clone was then transferred into the second host, *E. coli* K38, a strain harboring pGP1-2 (19).

The 3-kb PRD1 DNA fragment was also inserted into a *Pst* I site of a phagemid, pEMBLex3, that carries the N-terminal portion of the *lacZ* gene. Insertion of the *Pst* I fragment into the cloning sites of this vector results in the loss of β-galactosidase activity. Therefore, a recombinant plasmid can be directly screened on plates containing the substrate 5-bromo-4-chloro-3-indolyl β-D-galactoside (20). The orientation of the insert was verified by DNA sequence analysis.

***In Vitro* Site-Directed Mutagenesis.** Uracil-containing, single-stranded pEMBL3kb DNA was prepared according to Kunkel *et al.* (22). Synthetic oligonucleotides used for site-directed mutagenesis and DNA sequencing were synthesized on a Cyclone DNA synthesizer (MilliGen/Biosearch, Novato, CA). Uracil-containing DNA (1 μg) was mixed with phosphorylated mutagenic oligonucleotides (10 ng), T4 DNA polymerase (1 unit), and T4 DNA ligase (2–5 units). The mixture was incubated at 37°C for 90 min to synthesize the mutated strand. The resulting double-stranded DNA was used to transform *E. coli* NM522 competent cells. Colonies were selected at random and directly sequenced to screen for mutants.

Preparation of Cell Extracts. Cells were grown at 30°C in 500 ml of 2× YT medium supplemented with ampicillin (150 μg/ml). The cultures were shifted to 42°C when the OD_{590} reached 0.45. After 2 hr, cells were harvested by centrifugation at 5°C. Cell extracts were prepared (23) and protein concentrations were determined (24).

Assay for PRD1 Terminal Protein-dGMP Complex Forma-

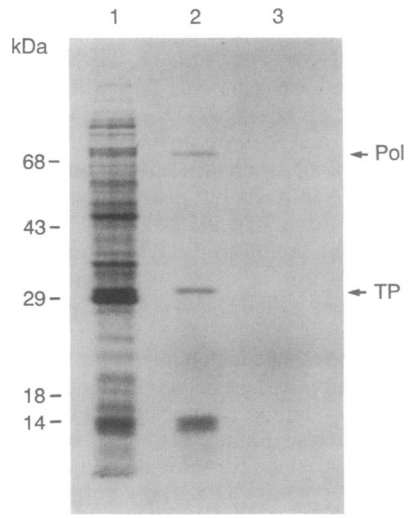


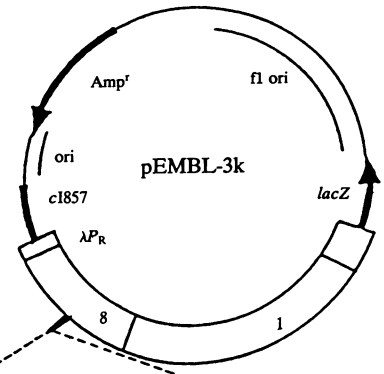
FIG. 2. Exclusive expression of the PRD1 terminal protein and DNA polymerase genes. Conditions for expression of the genes, for cell growth, and for heat induction were essentially as described (19). Cell cultures were shaken at 30°C until OD_{590} reached 0.42, and then the cultures were shifted to 42°C for 30 min with shaking. Rifampicin was added (200 μg/ml) and the cells were left at 42°C for an additional 10 min. Cultures were then shifted down to 30°C. After 20 min, samples were incubated with 10 μCi of [^{35}S]methionine for 5 min. The cells were centrifuged and resuspended in SDS/PAGE sample buffer. After heating at about 95°C, the samples were subjected to SDS/PAGE (23). Lanes: 1, control (no rifampicin was added); 2, specific expression of PRD1 terminal protein (TP) and DNA polymerase (Pol); 3, T7 expression plasmid pT7-6, without inserted DNA.

tion. The standard reaction mixture (50 μl) contained 50 mM Hepes/NaOH (pH 7.5), 5 mM $MgCl_2$, 3 mM dithiothreitol, 2 mM ATP, 0.5 μM [α-^{32}P]GTP (3000 Ci/mmol; 1 Ci = 37 GBq), 1 μg of PRD1 DNA-protein complex, and 100 μg of cell extract. After incubation at 30°C for 30 min, samples were processed as described (23). After centrifugation, the supernatant was discarded and the pellet was dissolved in 30 μl of sample buffer [0.1 M Tris/HCl (pH 6.8) with 2% SDS, 20% glycerol, 10% 2-mercaptoethanol and 0.005% bromphenol blue]. The PRD1 terminal protein-dGMP complex was visualized by autoradiography after SDS/12% PAGE as described (23). In studies with phenylglyoxal, cell extracts were preincubated with 10 mM phenylglyoxal for 30 min at 25°C. The cell extracts were then assayed for complex-forming activity.

RESULTS

Conserved Amino Acid Sequences Among the Terminal Proteins. Comparison of the amino acid sequence of the PRD1 terminal protein with those of other terminal proteins did not reveal any substantial homology (13). However, upon closer examination of the primary sequences of the terminal proteins, a short patch of conserved amino acid sequence was found (Fig. 1*A*). The consensus sequence was Tyr-Ser-Arg-Leu-Arg-Thr. The PRD1 terminal protein lacks the leucine residue from this sequence. A similar amino acid sequence is also present in the core antigen of duck hepatitis B virus (29). All the conserved sequence motifs are similarly located in the central portion of the whole molecule, except for human adenovirus pre-terminal protein, where it is located close to the N terminus (Fig. 1*B*).

Cloning of the PRD1 Terminal Protein and DNA Polymerase Genes in Expression Vectors. To determine the functional role(s) of the conserved amino acid sequence of the terminal protein and to initiate structure–function analysis of the protein-primed DNA replication system, we inserted both the terminal protein gene and the DNA polymerase gene into two expression vectors. First, we chose the T7 expression system developed by Tabor and Richardson (19) to characterize the cloned PRD1 terminal protein and DNA polymerase genes. The T7 RNA polymerase/promoter system consists of two compatible plasmids. One plasmid, pT7-6, was used to clone the structural genes of the terminal protein and the DNA



	170					175					180	Complex Formation
WILD TYPE	Gly GGC	Asn AAC	Tyr TAT	Ser TCC	Arg CGC	Arg CGT	Thr ACC	Tyr TAT	Thr ACG	Ser TCA	Phe TTT	++
Y172Ochre	-	-	Ochre TAA	-	-	-	-	-	-	-	-	-
Y172N	-	-	Asn AAT	-	-	-	-	-	-	-	-	+
S173A	-	-	-	Ala GCC	-	-	-	-	-	-	-	+
R174G	-	-	-	-	Gly GGC	-	-	-	-	-	-	-
R174L	-	-	-	-	Leu CTC	-	-	-	-	-	-	-
R174S	-	-	-	-	Ser AGC	-	-	-	-	-	-	-
R174H	-	-	-	-	His CAC	-	-	-	-	-	-	+
R175G	-	-	-	-	-	Gly GGT	-	-	-	-	-	+++
R175H	-	-	-	-	-	His CAT	-	-	-	-	-	++
T176A	-	-	-	-	-	-	Ala GCC	-	-	-	-	++
Y177FS179T	-	-	-	-	-	-	-	Phe TTT	-	Thr ACA	-	++

FIG. 3. (*Upper*) Diagram of pEMBL-3k used for *in vitro* mutagenesis of the PRD1 terminal protein gene. Gene *1* encodes DNA polymerase; gene *8* encodes the terminal protein. (*Lower*) The enlarged portion of the PRD1 terminal protein gene shows the nucleotide and amino acid sequences where mutations were introduced. Nucleotide and amino acid changes in the mutants are indicated. A summary of the mutants' ability to initiate complex formation is given.

polymerase. The other plasmid, pGP1-2, encodes the T7 RNA polymerase under control of the λ P_L promoter, which is regulated by the cI repressor encoded by the same plasmid (19). The exclusive expression of cloned genes was achieved after heat induction of the T7 RNA polymerase from pGP1-2 and addition of rifampicin to shut off the host *E. coli* RNA polymerase action. The proteins were labeled metabolically with [^{35}S]methionine. SDS/PAGE followed by autoradiography clearly revealed the PRD1 terminal protein and DNA polymerase (Fig. 2, lane 2). The low molecular weight bands may be part of the PRD1 lysin protein and an unknown plasmid-encoded protein. Elevated expression of the PRD1 terminal protein and DNA polymerase was also confirmed by an assay for protein-priming activity (data not shown).

To express proteins carrying site-directed mutations, we subcloned the terminal protein and DNA polymerase genes into the second expression vector, pEMBLex3 (20). Since this phagemid contains an origin of replication for f1 phage in addition to its ColE1 origin of replication, single-stranded circular DNA containing cloned genes can be obtained by superinfection with helper phage (20). This system also contains a strong λ P_R promoter and a temperature-sensitive *c*I857 repressor gene. The PRD1 terminal protein and DNA polymerase were overexpressed by a temperature shift from 30°C to 42°C (data not shown).

Mutational Analysis of the Conserved Amino Acid Sequence in Terminal Protein-dGMP Complex Formation. To evaluate the role of the conserved sequence motif in the initiation of complex formation, a series of mutants were constructed by oligonucleotide-directed mutagenesis (Fig. 3). After the DNA sequence of each mutant was confirmed, we prepared soluble extracts from each mutant clone after heat induction. Extracts were then assayed for covalent complex formation between the terminal protein and [α-^{32}P]dGMP.

The mutant clones Y172N and S173A showed considerably reduced activity in the initiation complex formation, whereas mutant T176A showed normal activity compared to the wild-type terminal protein (Fig. 4*A*). On the other hand, substitution of the noncharged amino acids glycine (R174G), serine (R174S), and leucine (R174L) for Arg-174 completely abolished formation of initiation complex. Interestingly, substitution of glycine (R175G) for Arg-175 resulted in increased activity (Fig. 4*A*). Analysis of crude extracts by SDS/PAGE showed that all mutant clones encoding an amino acid substitution produced a 28-kDa protein that comigrated with wild-type terminal protein. This protein band was absent from an extract of a clone containing the ochre nonsense mutation (Y172Ochre) (data not shown).

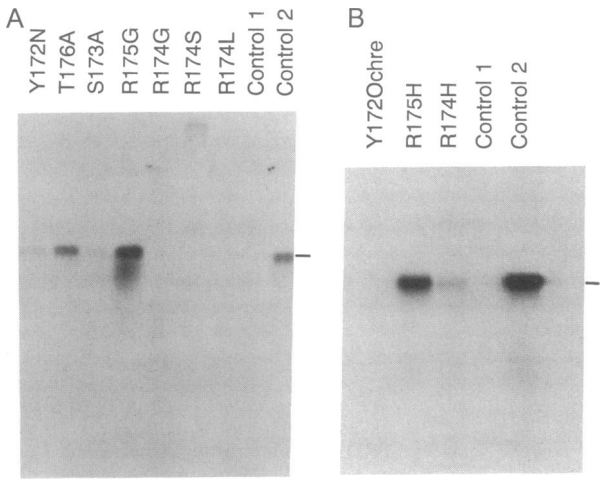


FIG. 4. Mutational effects of PRD1 terminal protein-dGMP complex formation. Complex formation was carried out under the standard reaction conditions with extracts from cells carrying pEMBLex3 (vector without 3-kb insert; Control 1) or pEMBL-3k with insert encoding wild-type (Control 2) or mutant PRD1 terminal protein, as indicated above each lane. Position of the complex is marked at right.

To test whether a positive charge at position 174 is critical for function of the PRD1 terminal protein, we replaced Arg-174 with histidine. As a comparison, Arg-175 was also replaced by histidine. Whereas substitution of histidine for Arg-175 resulted in a nearly normal level of initiation complex-forming activity, substitution of histidine for Arg-174 resulted in greatly decreased activity (Fig. 4*B*). These results suggest that although substitution by a positively charged histidine can partially restore the activity, Arg-174 is an important amino acid for PRD1 terminal-protein function. Since this arginine residue is highly conserved among the various terminal proteins, it would be interesting to examine whether this phenomenon is universal for all protein-primed DNA replication systems.

Phenylglyoxal, an Arginine-Specific α-Dicarbonyl Reagent, Blocks Formation of the PRD1 Terminal Protein-dGMP Complex. An arginine residue can serve as the binding site for anionic phosphorylated ligands as well as for anionic nucleotide coenzymes (30–35). Thus, arginines are critical for substrate binding to a wide range of enzymes. In many enzyme systems, the guanidino groups of functional arginine residues can be specifically modified by α-dicarbonyl compounds such as phenylglyoxal (36). To see whether Arg-174 of the PRD1 terminal protein is a binding site for dGTP, we tested the effect of phenylglyoxal on initiation complex formation. This reagent strongly inhibited PRD1 terminal protein-dGMP complex formation (data not shown). This result is consistent with the hypothesis that Arg-174 of the PRD1 terminal protein is a critical binding site for a phosphate group of dGTP.

DISCUSSION

Protein-primed DNA replication is a mechanism by which the 5′ ends of linear DNA molecules are preserved. Little is known about the molecular details of the protein-priming mechanism, however. In this paper, we have shown that there is a short patch of conserved amino acid sequence common to all DNA-terminal proteins so far studied. A similar conserved sequence was also found in the core antigen of duck hepatitis B virus (29). This core antigen is most likely the terminal protein of this virus (37). The spatial location of this conserved amino acid sequence motif near the center of each polypeptide is also similar except for the human adenovirus pre-terminal protein, where the sequence is located close to the N terminus. The 55-kDa adenovirus terminal protein is derived from the C terminus of the 80-kDa pre-terminal protein (3). This 55-kDa adenovirus terminal protein cannot serve as a protein primer for initiation of adenovirus DNA replication, even though it contains a DNA-linking amino acid, serine.

Our site-specific mutagenesis and functional analysis of PRD1 terminal protein clearly indicated that Arg-174 is a critical amino acid for the initiation of complex formation. Replacement of Arg-174 with glycine, serine, or leucine resulted in a nonfunctional terminal protein. Surprisingly, replacement of the neighboring arginine residue, Arg-175, with glycine resulted in increased activity. Since Arg-175 also appears to be highly conserved among all terminal proteins, this result was unexpected.

It has been well documented that arginine, as a positively charged amino acid, is an important binding site for anionic ligands in a wide variety of enzymes. Examples are the nucleotide binding site of *E. coli* adenylate kinase (38), the ATP-binding site of glutamine synthetase and carbamoyl-

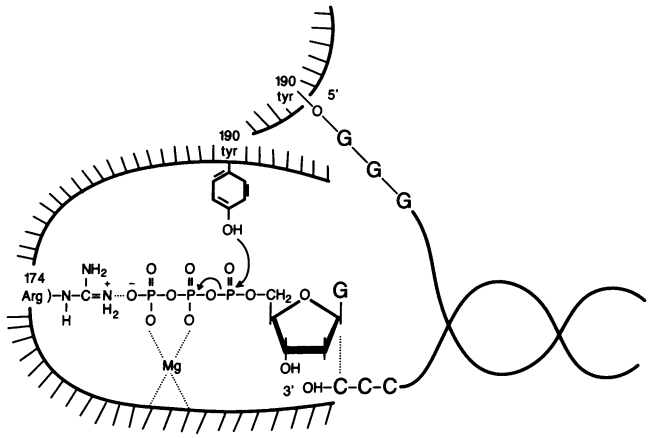


FIG. 5. A plausible mechanism for protein priming of initiation of PRD1 DNA replication. The linking amino acid of the PRD1 terminal protein is Tyr-190 (J.-C.H., Shih Shine, J.I., unpublished work). DNA polymerase, which catalyzes the protein-priming reaction, is not shown.

phosphate synthetase (32), the phosphate binding site of alkaline phosphatase (30), and the pyrophosphate binding site of yeast inorganic pyrophosphatase (39).

Functionally important arginine residues can be specifically modified by α-dicarbonyl compounds such as phenylglyoxal (36). Our results suggest that phenylglyoxal does indeed strongly inhibit PRD1 terminal protein-dGMP complex formation. We noted that once the initiation complex was formed, phenylglyoxal did not inhibit DNA chain elongation (unpublished results). Our results consistent with the hypothesis that Arg-174 participates in binding to dGTP. Why then is Arg-175 not a critical amino acid for initiation complex formation? It seems that a high degree of selectivity in the modification of arginine residues in proteins is a general phenomenon (31, 32). Borders and Riordan (31) showed that only 1 of 18 arginine residues per subunit of creatine kinase was modified by α-dicarbonyl reagents. They compared the rate of modification of the essential arginine residue of creatine kinase by phenylglyoxal with that of free arginine under the same condition. The essential arginine residue reacted $\approx$15 times faster than free arginine. Furthermore, most or all of the 17 other arginine residues reacted at a rate significantly slower than that of free arginine. It is possible that Arg-174 of the PRD1 terminal protein is different from Arg-175 and other arginine residues in terms of reactivity. This can be tested by using radioactive phenylglyoxal and peptide analysis.

In Fig. 5, we present a plausible model for the protein-priming step in the initiation of PRD1 DNA replication. Perhaps the first step of the protein-priming reaction is the association of the terminal protein–DNA polymerase complex with the terminus of the PRD1 genome. The first nucleotide would then be determined by base pairing with the 3'-terminal nucleotide. Thus, dGTP would be chosen as the first nucleotide. The guanidinium group of Arg-174 provides a positive charge for an ionic interaction with the phosphate moiety of dGTP. This electrostatic binding of phosphate group(s) might be important for stabilizing the complex and facilitating deoxynucleotidylation of Tyr-190 of the PRD1 terminal protein, which is catalyzed by DNA polymerase. The resulting covalent complex between the terminal protein and dGMP would then be used as the primer for DNA chain elongation by the same DNA polymerase molecule.

We gratefully acknowledge Drs. H. Bernstein, J. D. Hall, J. Little, A. Cress, and J. Duffy for their advice and critical review of the manuscript. This research was supported by Grant GM28013 from the National Institutes of Health (to J.I.).

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