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# Effects of the Bacteriophage T4 Gene 41 and Gene 32 Proteins on RNA Primer Synthesis: Coupling of Leading- and Lagging-Strand DNA Synthesis at a Replication Fork†

Tai-An Cha‡ and Bruce M. Alberts*
*Department of Biochemistry and Biophysics, University of California, San Francisco, San Francisco, California 94143*
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ABSTRACT: We have demonstrated previously that the template sequences 5′-GTT-3′ and 5′-GCT-3′ serve as necessary and sufficient signals for the initiation of new DNA chains that start with pentaribonucleotide primers of sequence pppApCpNpNpN or pppGpCpNpNpN, respectively. Normally, the complete T4 primosome, consisting of the T4 gene 41 (DNA helicase) and gene 61 (primase) proteins, is required to produce RNA primers. However, a high concentration of the 61 protein alone can prime DNA chain starts from the GCT sites [Cha, T.-A., & Alberts, B. M. (1986) *J. Biol. Chem. 261*, 7001–7010]. We show here that the 61 protein can catalyze a single-stranded DNA template-dependent reaction in which the dimers pppApC and pppGpC are the major products and much longer oligomers of various lengths are minor ones. Further addition of the 41 protein is needed to form a primosome that catalyzes efficient synthesis of the physiologically relevant pentaribonucleotides that are responsible for the de novo DNA chain starts on the lagging strand of a replication fork. The helicase activity of the 41 protein is necessary and sufficient to ensure a high rate and processivity of DNA synthesis on the leading strand [Cha, T.-A., & Alberts, B. M. (1989) *J. Biol. Chem. 264*, 12220–12225]. Coupling an RNA primase to this helicase in the primosome therefore coordinates the leading- and lagging-strand DNA syntheses at a DNA replication fork. Our experiments reveal that the addition of the T4 helix-destabilizing protein (the gene 32 protein) is required to confine the synthesis of RNA primers to those sites where they are used to start an Okazaki fragment, causing many potential priming sites to be passed by the primosome without triggering primer synthesis.

An accurate and efficient DNA replication process has been reconstituted using purified components specified by bacteriophage T4 (Alberts et al., 1983; Nossal & Alberts, 1983; Alberts, 1984, 1987). A mixture of seven T4-encoded proteins—the products of T4 genes 43 (DNA polymerase), 45 and 44/62 (DNA polymerase accessory proteins), 41 (DNA helicase), 61 (primase), and 32 (helix-destabilizing protein)—catalyzes rapid DNA replication fork movement. These replication forks closely resemble those formed by the T4 replication apparatus in vivo, having a similar speed, geometry, and fidelity of DNA synthesis (Hibner & Alberts, 1980; Sinha et al., 1980).

At a DNA replication fork, the leading strand is synthesized continuously, and the lagging strand is synthesized discontinuously. Leading-strand DNA synthesis requires the concerted action of helix unwinding by the 41 protein and DNA synthesis by the DNA polymerase holoenzyme (the gene 43 DNA polymerase and its three accessory proteins (Cha & Alberts, 1989). Lagging-strand DNA synthesis, on the other hand, requires the periodic synthesis of RNA primers that are efficiently utilized by a lagging-strand DNA polymerase holoenzyme to initiate successive Okazaki fragments. Previous studies have shown that the 41 and 61 proteins form the primosome that is responsible for RNA primer synthesis (Liu & Alberts, 1980, 1981; Nossal, 1980) and that the major products synthesized are pentaribonucleotides both in vivo

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‡ Present address: Chiron Corp., 4560 Horton St., Emmeryville, CA 94608.

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(Kurosawa & Okazaki, 1979) and in vitro (Liu & Alberts, 1980, 1981; Nossal, 1980). Whereas RNA primers with the sequence pppApCpNpNpN are the major products on single-stranded T4 DNA [which contains naturally modified (hydroxymethyl)cytosine residues], additional RNA primers that start with pppG are found when any cytosine-containing DNA is used as the template (Liu & Alberts, 1981).

We have previously determined the specific template sequences that serve as the RNA primer start sites by mapping the sites where RNA-primed DNA fragments begin on a single-stranded M13 DNA template (Cha & Alberts, 1986). These data reveal that the DNA sequences 5′-GTT-3′ and 5′-GCT-3′ are both necessary and sufficient to specify the efficient synthesis of RNA primers by the 41 and 61 proteins. The RNA primers are initiated by the pairing of a complementary nucleoside triphosphate to the center nucleotide in these trinucleotide sequences, and thus start with either pppApC or pppGpC.

When its concentration is raised to a high level, the 61 protein alone is capable of priming DNA chain starts, although only at the GCT sequences (Cha & Alberts, 1986). Therefore, the 61 protein appears to be a primase that requires the 41 protein to work effectively.

The 41 protein DNA helicase (Liu & Alberts, 1981; Venkatesan et al., 1982) is both necessary and sufficient to establish a high rate and processivity of DNA synthesis on the leading strand (Cha & Alberts, 1989). In this report, we characterize the role of the 41 protein in RNA primer synthesis and discuss its significance in coupling the leading- and lagging-strand DNA syntheses. We also examine the role of the gene 32 protein, the T4 helix-destabilizing (SSB) protein, in these processes.

Recent independent studies (Hinton & Nossal, 1987; Nossal & Hinton, 1987) have reported that 61 protein alone possesses a weak DNA template-dependent, oligonucleotide-synthesizing activity and that pppApC and pppGpC are the major products. Addition of 41 protein greatly stimulates oligomer synthesis, especially the synthesis of pentaribonucleotides. These workers also demonstrated that the primers used for DNA synthesis in the presence of a high concentration of 61 protein when 41 protein is absent are not the major dinucleotide products, but rather trace quantities of longer oligonucleotides which begin predominantly with pppGpC. The results reported in this paper agree with these aspects of their findings, while extending them to examine primer utilization at the replication fork.

## Materials and Methods

*DNAs and Enzymes.* Single-stranded closed-circular M13 and M13mp8 DNAs were purified from phage particles as described previously (Cha & Alberts, 1986, 1989). (Hydroxymethyl)cytosine-containing and cytosine-containing T4 DNAs (HMC-T4 and C-T4 DNAs) were prepared according to Liu and Alberts (1981). The synthetic dinucleotides ApC and GpC and nucleoside triphosphates were purchased from Pharmacia P-L Biochemicals. Radioactively labeled nucleoside triphosphates were obtained from Amersham.

The bacteriophage T4 replication proteins were prepared as described elsewhere (Cha & Alberts, 1989). Calf intestine alkaline phosphatase was from Boehringer Mannheim Biochemicals, and *Escherichia coli* RNA polymerase was the generous gift of Dr. Michael Chamberlin at the University of California, Berkeley.

*Oligoribonucleotide Synthesis and Characterization.* Standard oligoribonucleotide synthesis reactions were carried out in 20 μL of replication buffer (33 mM Tris–acetate, pH 7.8, 66 mM potassium acetate, 10 mM magnesium acetate, and 0.5 mM dithiothreitol) that contained ribonucleoside triphosphates (133 μM each of the four rNTPs), DNA template (single-stranded M13 DNA at 7.5 μg/mL), and priming proteins (61 protein at 40 μg/mL when used alone, or at 2.4 μg/mL when used together with the 41 protein at 76 μg/mL). In addition, [$\alpha$-$^{32}$P]CTP at 7.5 Ci/mmol was included to label the reaction products. This mixture was incubated at 30 °C for 15 min and stopped by heat inactivation at 90 °C for 5 min. When T4 DNA was used in place of the M13 DNA template, the C-T4 or HMC-T4 DNA was heat-denaturated at 90 °C for 5 min at 11 μg/mL and then chilled in ice–water immediately before use. Variations from the standard reaction conditions are noted where appropriate.

Our previous analysis of the RNA primers synthesized by the priming proteins employed a laborious two-dimensional separation scheme (Liu & Alberts, 1980, 1981) that fails to resolve dimeric product molecules. In our recent experiments, we have instead used one-dimensional polyacrylamide gel electrophoresis after treating the RNA products with alkaline phosphatase (Hillenbrand et al., 1979). Because their 5′-terminal triphosphate group has been removed, the RNA primers move slowly enough in the gel to be separated from the unincorporated radioactive ribonucleotides and their contaminants. The oligoribonucleotide synthesis reactions are stopped, and calf intestine alkaline phosphatase (0.038 unit) is added for 30 min at 37 °C. The phosphatase-treated samples are then mixed with an equal volume of loading buffer (80% formamide and 0.1% each of bromophenol blue and xylene cyanol dyes) and applied directly onto a urea-containing 20% polyacrylamide gel (Hillenbrand et al., 1979). After electrophoresis in Tris–borate–EDTA buffer (TBE: 89 mM Tris, 89 mM boric acid, and 2 mM $Na_3EDTA$, pH 8.2), autoradiographs were obtained by transferring the gels to Whatman 3MM filter paper and exposing them to Kodak X-Omat AR film. To locate the dimeric molecules, PEI–cellulose thin-layer chromatography was performed on radioactive molecules purified from individual gel slices using commercial ApC and GpC markers as reference standards (Hillenbrand et al., 1979). Presumably because of their low charge-to-mass ratio (one phosphate per two nucleosides), dinucleotides migrate more slowly than pentaribonucleotides (but faster than large RNA molecules) during polyacrylamide gel electrophoresis.

*RNA-Primed DNA Synthesis.* DNA synthesis was carried out on a single-stranded M13 DNA template as described previously (Cha & Alberts, 1986). The products were radioactively labeled by the incorporation of radioactive ribo- or deoxyribonucleoside triphosphates and separated by electrophoresis through 0.7% agarose gels in TBE buffer containing ethidium bromide (0.5 μg/mL). The gels were dried onto Whatman DE-81 filter paper before exposing them to Kodak X-Omat AR film for autoradiography.

RNA-primed DNA fragments (Okazaki fragments) were synthesized on the lagging strand of a DNA replication fork [see Cha and Alberts, (1989)]. The RNA primers were labeled by the incorporation of [$\alpha$-$^{32}$P]CTP in the presence of unlabeled deoxyribonucleoside triphosphates. The reaction mixture was stopped at various time intervals by heat-inactivation at 90 °C for 5 min and then electrophoresed after alkaline phosphatase treatment, as described above.

## Results

*Comparison of the Oligoribonucleotides Synthesized by the 61 Protein Alone with Those Synthesized by the Primosome.* An analysis of the RNA products synthesized on a single-

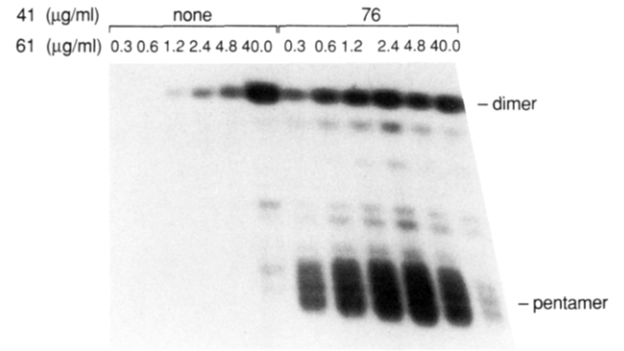


FIGURE 1: Oligoribonucleotide synthesis catalyzed by various concentrations of the 61 protein, alone and in combination with the 41 protein. Polyacrylamide gel electrophoresis was performed after alkaline phosphatase treatment of the radioactive RNA products, as described under Materials and Methods. This treatment removes the 5′-terminal triphosphate and causes the pentaribonucleotides ("pentamer") to migrate faster than dinucleotides such as ApC and GpC ("dimer"), as indicated (Hillenbrand et al., 1979). Larger oligoribonucleotides migrate slower than the dimers (see Figure 2), but only the bottom part of the gel is shown here. The template was single-stranded M13 bacteriophage DNA. [This figure has previously appeared in a preliminary communication (Cha & Alberts, 1988).]

stranded M13 DNA template in the presence of increasing concentrations of 61 protein is presented in Figure 1, with and without an excess of 41 protein present. Without 41 protein, the 61 protein catalyzes inefficient oligoribonucleotide synthesis with dinucleotides as the major products. Synthesis increases in proportion to the 61 protein concentration, and a minor fraction of oligomers larger than the dimer can be detected at a concentration of 40 μg/mL (a level of about one 61 protein molecule per potential primer start site in the M13 DNA). The 41 protein by itself produces no oligoribonucleotides even at high concentrations (data not shown). However, when added to the 61 protein, the 41 protein greatly increases the quantity of oligoribonucleotides synthesized by the 61 protein; by far the largest effect is on the pentamers, which now become the major product. However, even the synthesis of dimers is also enhanced (Figure 1). With 41 protein present, excess 61 protein becomes inhibitory, maximal synthesis occurring at a 41 protein to 61 protein concentration ratio of about 30. We routinely use 61 protein at 40 μg/mL when present alone and at 2.4 μg/mL when present with the 41 protein (76 μg/mL).

With a very long exposure of a gel autoradiograph, a small proportion of longer RNA molecules with lengths up to at least 50 nucleotides can be seen to be produced by the 61 protein alone; the synthesis of these products is blocked by the presence of the 41 protein, suggesting that they are made by the 61 protein and not by RNA polymerase contamination in the 61 protein preparation (Figure 2). As a further test, we examined the sensitivity of their synthesis to rifampicin. By comparing the synthesis in lanes 1 and 6 of Figure 2 (in which the proteins were preincubated with rifampicin before addition of the DNA template to start the oligoribonucleotide synthesis) with that in lanes 2 and 7 (in which no rifampicin was added), we conclude that a level of rifampicin that completely blocks the activity of *E. coli* RNA polymerase has no significant effect on the activities detected in our assay.

The above data show that the 61 protein contains the catalytic domain for phosphodiester bond formation, and thus is a primase in agreement with previous studies. By itself, this enzyme mainly synthesizes dinucleotides. In contrast, only oligoribonucleotides that are four nucleotides or more in length function as RNA primers in the T4 system (Cha & Alberts, 1986). This explains why the further addition of the 41 protein is so strongly required for synthesis of the biologically active

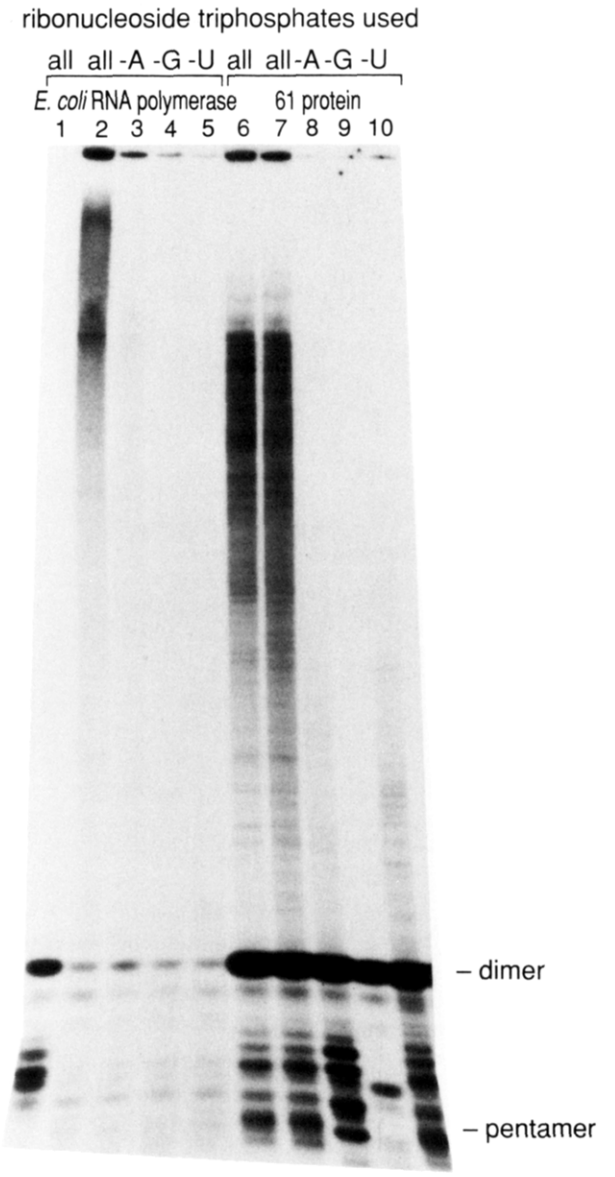


FIGURE 2: Rifampicin inhibits *E. coli* RNA polymerase but not the oligoribonucleotide synthesis activity of the 61 protein. A gel identical with that in Figure 1 was run and an autoradiograph produced after a long exposure time that renders minor species visible. Rifampicin (40 μg/mL) was incubated with *E. coli* RNA polymerase (3.4 μg/mL) (lane 1) or the 61 protein (40 μg/mL) (lane 6) for 10 min at 30 °C before addition of the DNA template to start RNA synthesis. Rifampicin was not added to the experiments presented in the other lanes. The template was single-stranded M13 bacteriophage DNA.

primers that start new DNA chains.

*Recognition of GTT and GCT Primer Start Sites by the Primase and the Primosome.* Previous studies have shown that the primosome uses both GTT and GCT sequences on a single-stranded M13 DNA template to prime the synthesis of DNA fragments, while the 61 protein alone only primes DNA chain starts at the GCT sites (Cha & Alberts, 1986). The 41 protein therefore seems to enhance GTT sequence recognition by the 61 protein. Is this selectivity due to selective primer synthesis? We can determine the relative usage of the GTT and GCT sites for primer formation by omitting either GTP or ATP from the reaction and measuring the amount of oligoribonucleotide synthesized. Figure 3 compares the oligoribonucleotide synthesis catalyzed by the 61 protein alone (Figure 3B) and by the primosome (Figure 3A) on three different DNA templates (single-stranded M13, C-T4, and HMC-T4 DNAs) in reactions containing various mixtures of

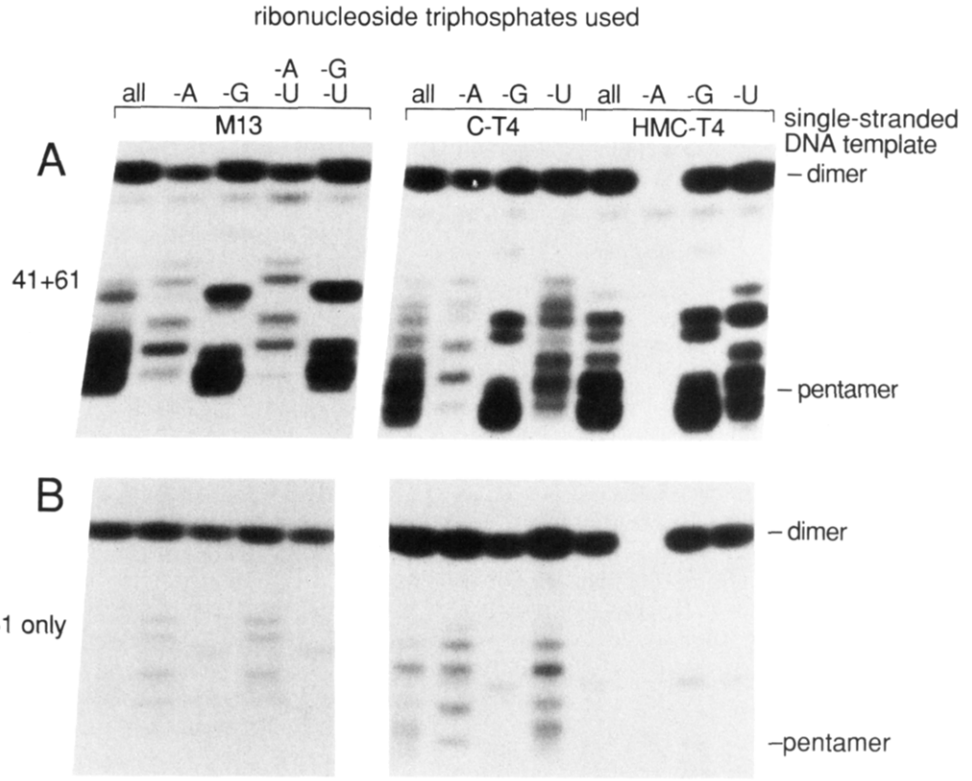


FIGURE 3: Comparative analysis of the oligoribonucleotide synthesis observed on different DNA templates. These templates were single-stranded M13 bacteriophage DNA, heat-denatured C-T4 bacteriophage DNA, and heat-denatured HMC-T4 bacteriophage DNA, as indicated. Experimental conditions are described under Materials and Methods. (A) Both 41 protein (76 μg/mL) and 61 protein (2.4 μg/mL) were present; (B) only 61 protein was present (40 μg/mL).

ribonucleoside triphosphates. Indistinguishable results are obtained on both templates that contain unmodified cytosine (M13 and C-T4 DNAs). Judging by the relative intensities of the dimeric RNA products in the "minus A" lane (in which only GpC is produced) and the "minus G" lane (in which only ApC is produced), the 61 protein prefers to use the GCT primer start site when present alone, but prefers the GTT site when present with the 41 protein. However, both the 61 protein alone and the primosome can use both of these sites.

The different affinities of the 61 protein and the primosome toward GTT and GCT sites have a larger effect on the production of longer oligoribonucleotides. In Figure 2, where a longer exposure of an autoradiogram is displayed, the omission of *either* ATP or GTP from the reaction (lanes 8 and 9) completely blocks the synthesis of the most slowly migrating group of oligoribonucleotides (those much larger than a pentamer); this is presumably a trivial result due to chain termination at the missing nucleotide. However, comparison of lanes 8 and 9 (see also Figure 3B) also shows that omitting GTP—which leaves only the GTT primer start sites available for use—is unusual in that it also drastically reduces the amount of the oligoribonucleotides produced of intermediate size (those migrating between the dimer and pentamer positions). Since only oligonucleotides that are four or more residues long can act as primers, this result presumably explains why the RNA-primed DNA synthesis catalyzed by the 61 protein alone requires GTP, but not ATP (Cha & Alberts, 1986; see also Figure 5). When all rNTPs are present, the DNA synthesis observed in the presence of 61 protein alone (no 41 protein) is presumably also primed by the heterogeneous group of very long oligomers made, since without 41 protein the level of their synthesis is comparable to that of the tetramers and pentamers (lane 7 in Figure 2).

On the natural template for T4 DNA replication (HMC-T4 DNA), the synthesis of G-start primers is completely abolished (i.e., the GCT start site is not used, and therefore no oligonucleotides are synthesized when ATP is omitted; see Figure 3). Therefore, the modification of cytosine residues in natural T4 DNA explains the production of only the A-start primers in vivo [Kurosawa & Okazaki, 1979; see also Liu and Alberts (1981)].

*Deoxyribonucleotides Can Be Incorporated Only as the Initial Nucleotide of RNA Primers.* Since deoxyribonucleotides are readily incorporated in place of ribonucleotides in the primers synthesized by the *E. coli* primosome (Kornberg, 1980, 1982), we tested for the incorporation of dNTPs into the oligoribonucleotides synthesized by the T4 bacteriophage primase and primosome. The reaction was monitored by the incorporation of [$\alpha$-$^{32}$P]CTP into oligonucleotides, and the results are shown in Figure 4. In lane 6, where the [$\alpha$-$^{32}$P]CTP is the only ribonucleoside triphosphate present with all four dNTPs, the only products are deoxyribonucleotide-containing dimers (position "b") that migrate slightly faster in the borate buffer used for electrophoresis than the dimers that contain only ribonucleotides (position "a"). If each of the four dNTPs is added individually to reactions containing the four rNTPs, only dATP and dGTP are seen to form these position "b" dimers (see lanes 2–5). Since all of the radioactive dimers in Figure 4 must contain ribo-C as their second nucleotide, the relative amount of position "a" and position "b" dimers shows that dATP and dGTP can be readily substituted for their ribonucleotide counterparts at the 5′ end of the synthesized oligonucleotides. The absence of trimers or larger oligomers in lane 6 of Figure 4 is taken to mean that dNTPs cannot be incorporated in the subsequent elongation steps of oligonucleotide synthesis.

In a separate experiment (compare lanes 5–8 and 9–12 of Figure 6 below), a partial inhibition of RNA primer synthesis is observed when all dNTPs and rNTPs are present together (dNTPs at 500 μM each, ATP and GTP at 400 μM each,

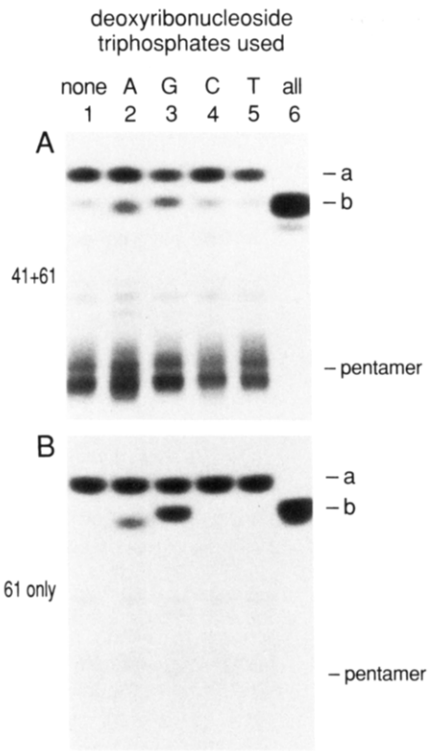


FIGURE 4: Attempts to substitute deoxyribonucleotides for ribonucleotides during oligonucleotide synthesis catalyzed by the 61 protein alone and by the primosome (61 protein plus 41 protein). Radioactive rCTP was incorporated into oligonucleotides on a single-stranded M13 DNA template and analyzed as described under Materials and Methods. A single deoxyribonucleoside triphosphate was added together with all four ribonucleoside triphosphates in lanes 2–5, as indicated. Lane 1 is a control, with only the ribonucleoside triphosphates present, whereas lane 6 contains the four dNTPs with [$\alpha$-$^{32}$P]CTP as the only ribonucleotide. The concentrations of rNTPs were 133 $\mu$M each and of dNTPs were 200 $\mu$M each. "a" labels the position of ApC and GpC, while "b" labels the position of these dimers with a deoxyribonucleotide at their 5′ end. Note that the relative incorporation of dATP and dGTP at GTT and GCT sites mimics the incorporation found for ATP and GTP at these sites. Thus, the amount of dG-start dimers is greater than the amount of dA-start dimers when the 61 protein is present alone, whereas the reverse is true when the primosome is present (see lanes 2 and 3).

UTP and CTP at 80 $\mu$M each). This inhibitory effect requires ratios of dNTPs to NTPs that are unlikely to occur in vivo, where the ribonucleotides are at much greater concentrations than the deoxyribonucleotides.[1]

*61 Protein Alone Requires GTP To Prime DNA Synthesis.* We have previously shown that, at very high concentrations, the 61 protein alone can prime a high level of DNA synthesis by the DNA polymerase holoenzyme on a single-stranded M13 DNA template; this synthesis would be predicted to require the presence of GTP, because only the GCT RNA primer sites are utilized (Cha & Alberts, 1986). The expected dependence of DNA synthesis on GTP is observed in the agarose gel electrophoresis assay for DNA synthesis in Figure 5, where both deoxyribonucleotide- (A) and ribonucleotide (B)-labeled DNA product molecules are analyzed. Although a trace amount of DNA synthesis is seen at high 61 protein concentrations in the "minus G" lane (lane 12), we find that even these RNA-primed DNA fragments all start from GCT sites. This was determined by using the mapping methods described earlier (Cha & Alberts, 1986) to locate the RNA-primed DNA chain start sites in the RFII product molecules purified from such agarose gels (data not shown). Thus, the very small amount of DNA synthesis observed in the absence of GTP must start with primers made either by misincorporation of the initial nucleotide or by an incorporation of dGTP as this nucleotide.

*RNA Primers Made on the Lagging Strand of a Replication Fork Are Efficiently Utilized.* Since GTT sequences occur in T4 DNA about once per 55 nucleotides on average and the Okazaki fragments are about 1200 nucleotides long, there is only a low probability that the replication apparatus will start an Okazaki fragment at any particular GTT sequence encountered by the primosome on the lagging strand. Is a primer made but not utilized at most sites, or are primers only made when they are needed for priming an Okazaki fragment? In an attempt to answer this question, we have carried out DNA synthesis in the presence of a radioactive ribonucleoside triphosphate and then analyzed all of the radioactive products (both free primers and Okazaki fragments) by polyacrylamide gel electrophoresis.

Figure 6 compares RNA primer synthesis on a single-stranded M13 DNA template with or without accompanying DNA synthesis. In these reactions, strand displacement DNA synthesis is prevented by the omission of the 32 protein, so that the production of both RNA primers and DNA stops after one complete single strand is synthesized on the circular DNA template. Thus, whereas the synthesis of RNA primers increases with time in the absence of accompanying DNA synthesis (Figure 6B, lanes 5–8), RNA synthesis ceases in less than 5 min when DNA is made (lanes 1–4). The DNA that contains radioactive primer appears at the top of the gel (Figure 6A). The ratio of the label in DNA to that in free RNA primers (the primers that are not used to prime DNA synthesis) reveals that the primers are very efficiently utilized on this single-stranded DNA template.

In order to determine what fraction of RNA primers are used to prime Okazaki fragment synthesis on the lagging strand of a replication fork, we have carried out an experiment similar to that in Figure 6 on a tailed-duplex DNA template (see Figure 7A). On this template, strand displacement DNA synthesis requires the 41 helicase plus the DNA polymerase holoenzyme, and it occurs with or without the 32 protein present; in both cases, lagging-strand DNA synthesis ensues when the 61 protein and rNTPs are included in the reaction (Cha & Alberts, 1989).

Figure 7B presents a tracing of a gel autoradiograph from an experiment carried out with all replication proteins present, including the 32 protein. In this experiment, more than 60% of the radioactive label incorporated into the RNA primers synthesized after 5 min of strand displacement synthesis is attached to long DNA strands, indicating that the majority of the primers have been used to prime the initiation of an Okazaki fragment; in fact, if one ignores the dimers (which are too short to prime DNA synthesis), about 90% of the primers have been used.

*32 Protein Has a Major Effect on Primer Synthesis at a Fork.* The amount of DNA made on both leading and lagging strands is essentially the same whether or not the 32 protein is present [data not shown; see Cha and Alberts (1989)].

[1] When the concentrations of the dNTPs are raised to levels higher than those of the rNTPs, the average size of the Okazaki fragments increases during the strand displacement DNA synthesis reactions described elsewhere (Cha & Alberts, 1989). These artificially high concentrations of dNTPs presumably inhibit the normal RNA primer synthesis needed for priming an Okazaki fragment. When an RNA primer fails to be made on the lagging strand, the leading-strand polymerase continues its translocation and produces an unusually large amount of single-stranded DNA template for the next round of lagging-strand synthesis, which leads to longer Okazaki fragments (see Discussion).

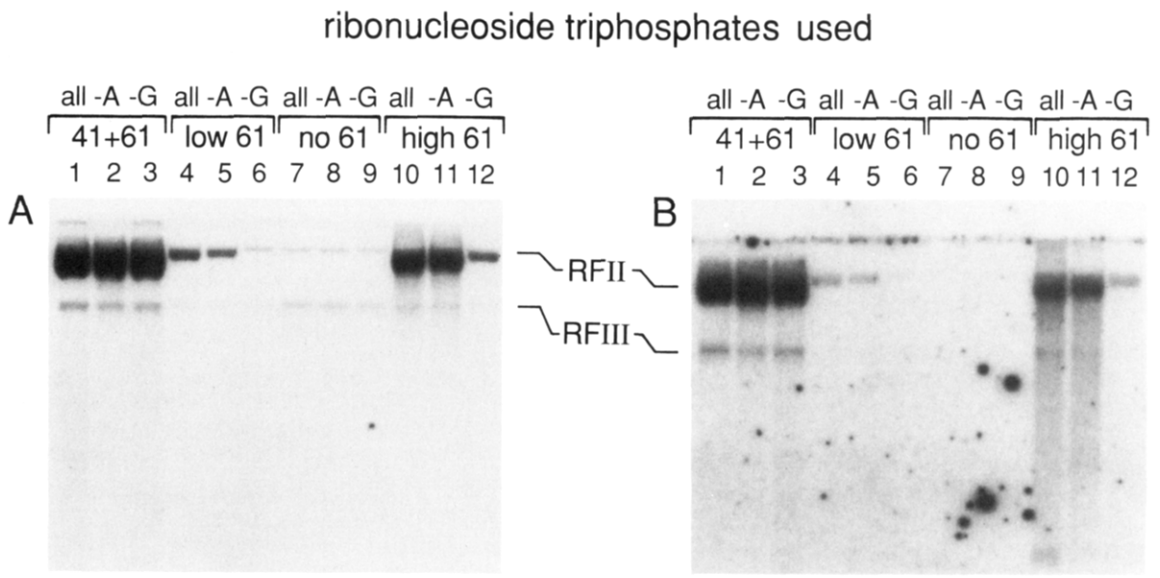


FIGURE 5: RNA-primed DNA synthesis catalyzed by the 61 protein alone and by the primosome: dependence on ATP and GTP. The DNA was synthesized and analyzed by agarose gel electrophoresis followed by autoradiography as described under Materials and Methods; incubation was for 15 min at 37 °C. In (A), the DNA products are radioactively labeled whereas only the RNA portions of the RNA-primed DNA fragments are radioactively labeled in (B). The 61 protein concentrations used were 2.4 μg/mL for lanes 1–6 and 40 μg/mL for lanes 10–12; the 41 protein was present at 76 μg/mL in lanes 1–3.

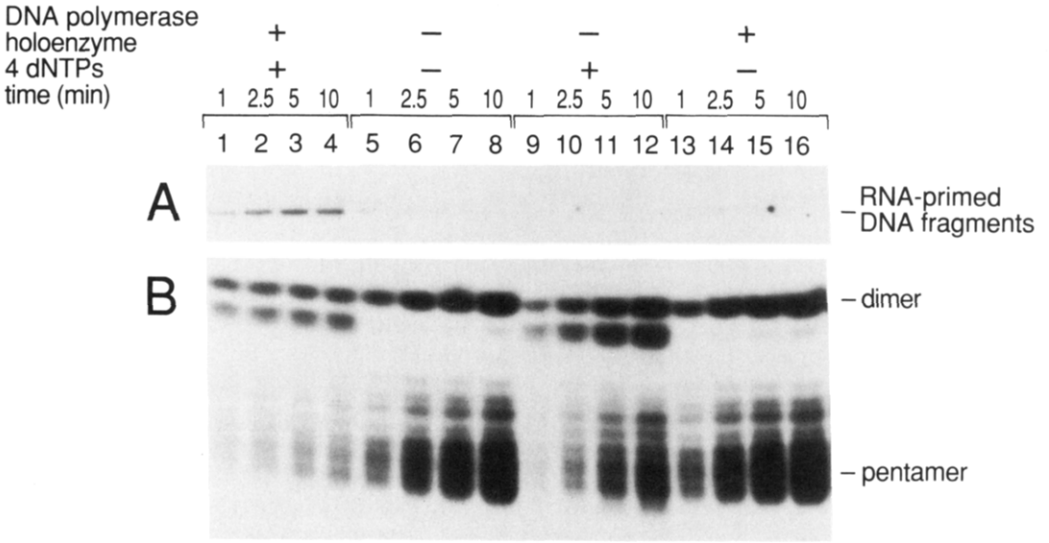


FIGURE 6: RNA primers synthesized by the primosome on a single-stranded M13 DNA template are utilized efficiently by the DNA polymerase holoenzyme. The primers were labeled with radioactive rCTP as described under Materials and Methods, and the dNTPs were unlabeled. The DNA polymerase holoenzyme was added where indicated; it is composed of the DNA polymerase (gene 43 protein, 3.4 μg/mL) and the gene 45 (34 μg/mL) and 44/62 (28 μg/mL) polymerase accessory proteins. Where added, the dNTPs were present at 500 μM each. In all reactions, ATP and GTP were present at 400 μM each, and CTP and UTP were present at 80 μM each. In (A), an autoradiograph of the top portion of a gel where the RNA-primed DNA fragments migrate during electrophoresis is displayed, while the lower part of this same autoradiograph is shown in (B). Lanes 1–4 show an experiment with DNA synthesis, while lanes 5–8 show an experiment without DNA synthesis. As controls, in lanes 9–12 the four dNTPs are present without the DNA polymerase holoenzyme, and in lanes 13–16 the DNA polymerase holoenzyme is present without the nucleotides required for DNA synthesis. Note that a faster migrating dimer band containing a deoxyribonucleotide at the 5′ end appears whenever the dNTPs are present (see Figure 4).

Figure 8 compares the free primers produced during strand displacement DNA synthesis without (Figure 8A) or with (Figure 8B) 32 protein present. Quantitation of these gels reveals that about 40-fold more free primers are produced without 32 protein present than in the complete replication system. Since the amount of labeled primers attached to DNA is about the same in (A) and (B) (data not shown), not more than a few percent of the primers synthesized end up attached to Okazaki fragments when the 32 protein is omitted. Thus, the presence of 32 protein on the lagging strand seems to block wasteful primer synthesis by the primosome. Recalling that slightly more than one pentaribonucleotide primer is synthesized per Okazaki fragment with 32 protein present (see Figure 7), a 40-fold increase in primer synthesis means that the number of primers made is about equal to the total number of GTT and GCT sequences passed by the primosome on the lagging strand. It therefore seems as though a primer is produced at nearly every GTT and GCT on the lagging strand without 32 protein, whereas primers are only made when needed for priming an Okazaki fragment in the presence of 32 protein.

The patterns of oligoribonucleotide formation observed in Figure 8 when one of the four ribonucleoside triphosphates is omitted (the minus A, minus G, and minus U reactions) resemble those found previously on a single-stranded DNA template (compare with Figure 3). This result suggests that

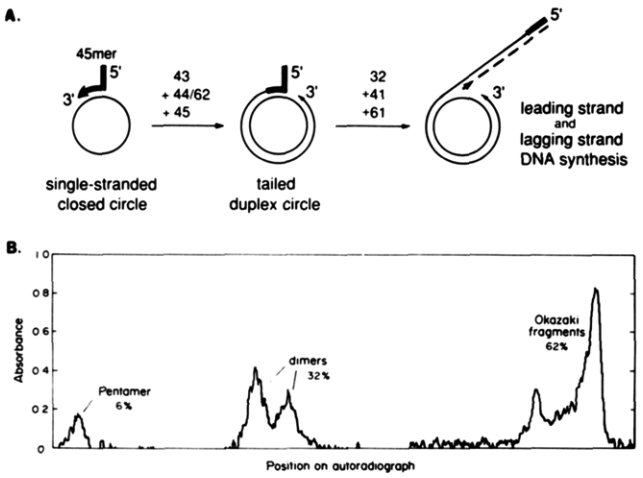


FIGURE 7: Quantitation of the fraction of the RNA primers synthesized at the T4 replication fork that are used to start Okazaki fragments. In vitro DNA synthesis was carried out on a tailed-duplex DNA template as described elsewhere (Cha & Alberts, 1989) in the presence of the DNA polymerase holoenzyme (see Figure 6 legend) plus 65 $\mu$g/mL 41 protein, 2.1 $\mu$g/mL 61 protein, and 170 $\mu$g/mL 32 protein (A). The RNA primers were labeled by addition of [$\alpha$-$^{32}$P]CTP after 5 min of DNA synthesis at 37 °C in the absence of radioactivity. The reactions were stopped after another 5 min and treated with alkaline phosphatase and then analyzed by electrophoresis through a 5% polyacrylamide gel. After autoradiography, the relevant portion of the gel was scanned with a Bio-Rad 620 video densitometer (B). These scans revealed that 62% of the total RNA oligonucleotide label was present in Okazaki fragments. This is a minimal estimate, since controls showed that a trace contamination of RNase H activity was present in the preparation of 32 protein used, which degraded some of the RNA in Okazaki fragments, but not the free RNA oligonucleotides (data not shown).

the mechanism of RNA primer synthesis by the primosome at a replication fork is unchanged from that observed on simpler templates.

## Discussion

Our main conclusions can be summarized as follows. (1) The 61 protein is a primase that produces mainly dinucleotides when present without the 41 protein; these products are too short to prime DNA synthesis. Adding the 41 DNA helicase to this primase forms the primosome component of the T4 DNA replication apparatus, which catalyzes efficient synthesis of the pentaribonucleotides responsible for de novo DNA chain starts under physiological conditions. (2) In agreement with in vivo measurements (Kurosawa & Okazaki, 1979), only the A-start primers are expected to be made in a T4-infected cell, because the naturally occurring modification of cytosine residues in T4 DNA completely abolishes the ability of 61 protein to recognize GCT primer-start sites. (3) At a DNA replication fork, it appears that an RNA primer is only made by the primosome when it is required for priming an Okazaki fragment, and it is utilized by the DNA polymerase holoenzyme very efficiently once it is produced.

On average, the primosome passes more than 20 GTT sites on the lagging strand (plus an equal number of GCT sites on a cytosine-containing DNA) before each initiation of Okazaki fragment synthesis. The DNA synthesis rate of about 600 nucleotides/s on a single-stranded DNA template (Mace & Alberts, 1984) is much faster than the rate of the leading strand that we have observed, which is about 250 nucleotides/s (Cha & Alberts, 1989). Therefore, the lagging-strand DNA polymerase molecule should move faster than the leading-strand DNA polymerase molecule at a replication fork. A timing mechanism based on the release of a recycled lagging-strand DNA polymerase molecule has been proposed to govern the initiation of Okazaki fragment synthesis (Selick et al., 1987), as schematically illustrated in Figure 9. The 32 protein is needed to speed up the DNA polymerase on the lagging strand (Cha & Alberts, 1989) and to prevent the synthesis of large numbers of RNA primers by the primosome. In the absence of 32 protein (a situation not occurring in vivo), primers can be made at nearly every GTT (and GCT) site (Figure 8A). However, according to the Figure 9 model, the excess primers made before the release of DNA by the lagging-strand DNA polymerase molecule will be wasted, as observed in the absence of 32 protein.

The 41 DNA helicase must be loaded onto a replication fork from the onset of the DNA replication process to attain the in vivo DNA synthesis rate and processivity (Cha & Alberts, 1989). Inside a cell, single-stranded DNA regions can be created through recombination and repair processes as well as by replication. Presumably, therefore, this DNA helicase has evolved so that it can be put onto DNA only at a DNA replication origin inside the cell, and it remains with the same fork until DNA synthesis is complete. The requirement that the 61 protein interact with the 41 protein in order to synthesize pentaribonucleotide primers guarantees that Okazaki fragments will start only at a fork. From this perspective, the analogies among prokaryotic DNA replication systems are striking. In the *E. coli* DNA replication system, the 41 protein is replaced by the dnaB protein (LeBowitz & McMacken,

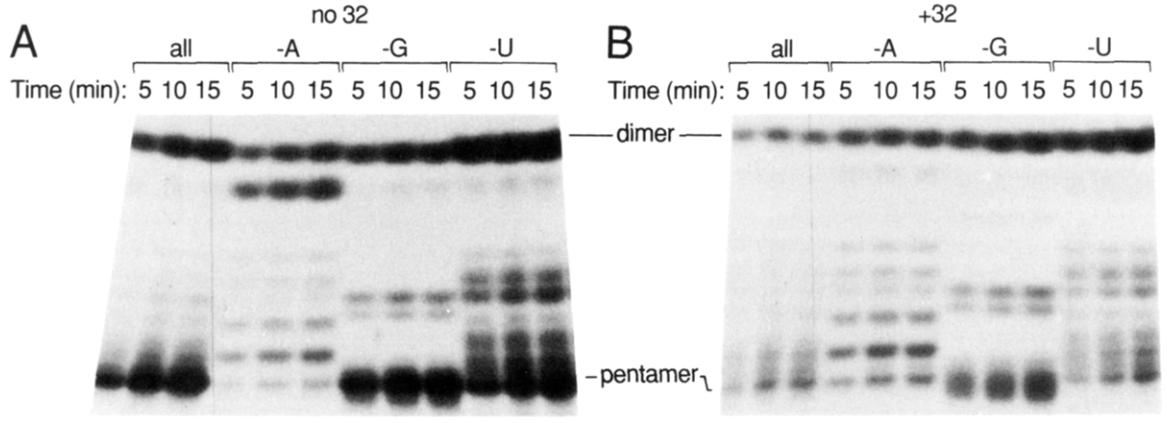


FIGURE 8: Striking effect of the 32 protein on the free (unused) RNA primers produced by the primosome during strand displacement DNA synthesis reactions. A replication fork was established on a tailed-duplex DNA template, as described elsewhere (Cha & Alberts, 1989). Aliquots taken during the time course of DNA synthesis in the presence of radioactive rCTP were processed as described under Materials and Methods. Quantitation of the free radioactive primers in the "all" lanes (all four ribonucleoside triphosphates present) without (A) and with (B) 32 protein present reveals a 40-fold decrease in free primers when this protein is added.

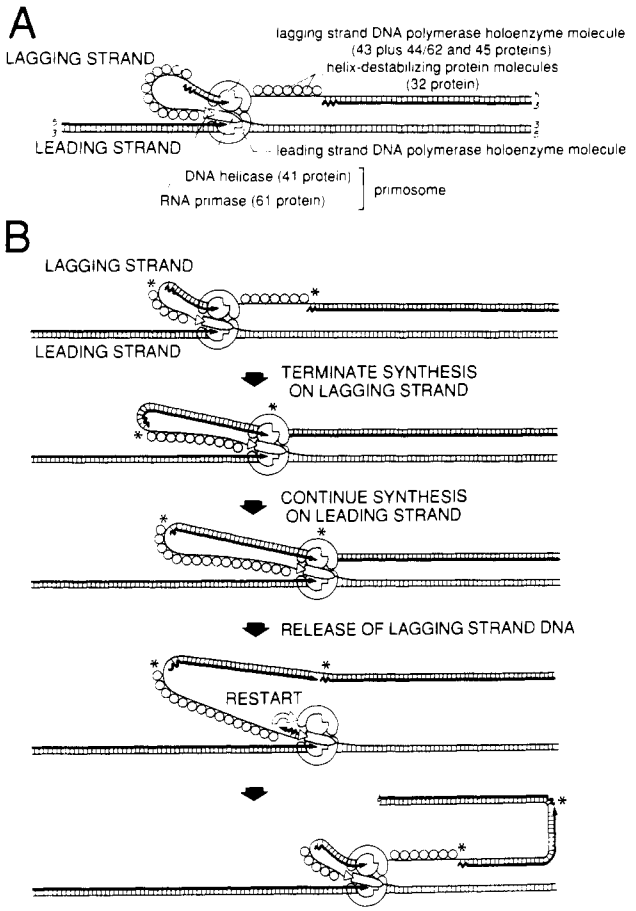


FIGURE 9: (A) Schematic representation of the T4 replication protein complex at a DNA replication fork. As indicated, a large multiprotein complex composed of a dimeric DNA polymerase holoenzyme linked by the primosome is thought to catalyze concerted leading- and lagging-strand DNA synthesis at the replication fork. In this complex, the lagging-strand DNA is folded in a way that brings the start site for the next Okazaki fragment in close juxtaposition to the site where the previous Okazaki fragment will end. (B) A model for DNA synthesis at a replication fork that allows the same DNA polymerase molecule to be repeatedly recycled to synthesize all of the Okazaki fragments on the lagging strand. Once the lagging-strand DNA polymerase reaches the 5′ end of a previously synthesized Okazaki fragment, it pauses before releasing its DNA template (note that the polymerase holoenzyme by itself cannot penetrate the double-stranded DNA). During this pause, the leading-strand polymerase continues its translocation, displacing an additional amount of single-stranded template for the next round of lagging-strand synthesis. Finally, the release of the DNA by the lagging-strand polymerase signals the associated primosome to synthesize an RNA primer at the next available primer site, and a refolding of the lagging-strand DNA hands off this primer to the lagging-strand DNA polymerase molecule, beginning the next cycle of Okazaki fragment synthesis. This aspect of the model is needed to explain the efficient use of RNA primers in the Figure 7 experiment. On the basis of this mechanism, the length of each Okazaki fragment is equivalent to the distance that the leading-strand DNA polymerase molecule travels during the time required to complete the recycling of the lagging-strand DNA polymerase molecule. This time is the sum of the travel time and the pause time for the lagging-strand DNA polymerase molecule. If we assume that the leading-strand synthesis rate and the pause time on the lagging strand are fixed quantities, reducing the lagging-strand synthesis rate should cause longer Okazaki fragments to be made [see Selick et al. (1987) and Cha and Alberts (1989)].

1986), and the dnaG protein is the counterpart of the 61 protein (Kornberg, 1980, 1982). In the T7 bacteriophage system (Richardson et al., 1987), the gene 4 protein possesses both helicase and primase activities. However, a truncated form of the gene 4 protein is also produced from the same gene that is a helicase but defective in primer synthesis (Bernstein & Richardson, 1988).

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**Registry No.** 5′-ATP, 56-65-5; 5′-GTP, 86-01-1.

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