IDENTIFICATION OF A PROTEIN FROM ESCHERICHIA COLI WHOSE SYNTHESIS APPEARS TO BE TRIGGERED BY THE INITIATION OF DNA REPLICATION

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ABSTRACT: Using a DNA temperature sensitive initiation mutant to synchronize the replication and cell division cycle, we have compared proteins which are synthesized during a period of DNA arrest with those synthesized after return to permissive temperature. This work has led to the identification of a DNA-binding protein of 60-65,000 molecular weight (SDS-gel electrophoresis) whose synthesis appears to be triggered by the initiation event.

INTRODUCTION

Lark and Renger have described a sequence of synthetic events that appear necessary during the replication cycle in order to achieve the next initiation,[1] It has also been previously demonstrated that after a period of time where DNA synthesis is blocked and protein synthesis allowed to continue, upon release of the DNA block, DNA synthesis will resume and a "premature initiation" of replication will take place at the chromosomal origin[2-10]. Schwartz and Worcel have studied the conditions required to produce premature initiation using a temperature-sensitive dnaB mutant of Escherichia coli. Their work indicates that protein synthesis is required both during the period of DNA arrest (at non-permissive temperature) and during a 15 min period immediately after return to permissive conditions in order to achieve the premature initiation[11]. Other workers have also observed that a period of 15-20 minutes of protein synthesis appears necessary after the release of dnaA mutants from non-permissive conditions in order to observe premature initiation[10].

Using a temperature sensitive initiation mutant to synchronize the replication and cell division cycles, we have sought to identify the protein(s)

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which may have a role in the regulation of initiation. The results reported below indicate that there is a protein of about 60,000-65,000 molecular weight, as determined by SDS acrylamide gel electrophoresis which appears to be made immediately after release from non-permissive temperature, and whose synthesis appears to be triggered by the initiation event.

MATERIALS AND METHODS

Bacterial strains. E. coli 165/120/6, dnaA, Thy^-, Leu^-, B_1^-, Pro^- (isolated by Bonhoeffer), and a revertant for the temperature-sensitive dnaA character were used in this study[10].

Growth and labeling of cells. The cells were grown routinely in M9 medium[12], supplemented with 4 µg/ml thymine, 20 µg/ml L-leucine, 2 µg/ml thiamine hydrochloride, 20 µg/ml proline and 1% Casamino acids (Difco). During experiments where the cultures were labeled with radioactive L-amino acid mixes (New England Nuclear) at ^{14}C (3 µCi/ml) and ^{3}H (10 µCi/ml) the cells were grown in the presence of 500 µg/ml Casamino acids. When labeling with [^{3}H-Methyl] thymine (New England Nuclear), the label was present at 2 µCi/4 µg/ml.

DNA cellulose chromatography. DNA cellulose chromatography was carried out essentially as described by Alberts and Herrick[13]. Homogenous DNA-cellulose was packed in columns of 1 cm (i.d.) to a height of 6 cm.

After the cells had been labeled, the cultures were merged, collected by centrifugation, resuspended in 3 ml sonication buffer[13] and broken by sonication. The soluble proteins of the cell were separated by centrifugation at 40,000 rpm for 30 min in a Beckman SW 50.1 rotor. Pancreatic DNase (Worthington) at 20 µg/ml was added to the supernatant followed by incubation for 15 min at 20°C, as described by Alberts and Herrick[13]. The supernatant was then dialyzed against three five-hour changes of dialysis buffer at 4°C and loaded on a DNA-free cellulose pre-column which fed into a homologous DNA-cellulose column 1 cm in diameter, with a bed volume of 4.7 ml. The proteins were loaded at 0.1 M NaCl concentration and washed with wash buffer containing the same concentration of NaCl for approximately 7 column volumes. The pro-

teins were then eluted step-wise with 2-3 column volumes at 0.2 M, 0.4 M, 0.6 M, 0.8 M and 2.0 M NaCl concentrations. Columns were run at 3-4 ml/hr. Aliquots were removed from each fraction and precipitated with cold 5% trichloroacetic acid (TCA). The precipitated material was collected on glass fiber filters (Reeve Angel), washed with cold H_2O, dried and counted in 5 ml of toluene-Liquifluor in a Beckman LS-250 scintillation counter.

SDS-acrylamide gel electrophoresis. Samples containing high amounts of radioactivity in each elution were pooled and dialyzed against a sample buffer[14] containing 0.1% SDS and 0.01 M sodium phosphate pH 7.0, followed by incubation at 45°C for 60 min. Bromphenol blue was added as a tracking dye and samples were run on either 8% or 9% SDS acrylamide gels. Molecular weight standards were included in each run. The gels were stained in Coomasie blue, and then fractionated on a Savant autogeldivider. Fractions were collected every 15 sec. The gel fractions were dissolved in 0.5 ml of 30% H_2O_2 overnight at 42°C. 10 ml of Aquasol was then added and samples were counted 3 days later, after chemiluminescence had disappeared. The ^{14}C overlap into the ^{3}H channel was subtracted, using a ^{14}C standard, and samples were normalized using a portion of the proteins that had undergone dialysis and the other above procedures as a reference. Molecular weights were determined from a standard curve according to the method of Weber and Osborn[14].

RESULTS

When Hfr 165/120/6, a dnaA temperature sensitive initiation mutant, is held at 42°C for a period of 100 min followed by release to 30°C, DNA synthesis starts almost immediately and what appear to be synchronous initiation events take place at about 20, 40, and 60 min[10]. However, as we can see in Fig. 1, if 150-200 µg/ml CAP is added to 0, 5, 10, or 20 min after return to permissive temperature, there appears to be a little less than one round of synthesis completed, as measured by this residual synthesis experiment. If CAP is added after 20 min, however, the cells appear to be able to achieve somewhat less than two rounds. These results have been interpreted to suggest that a

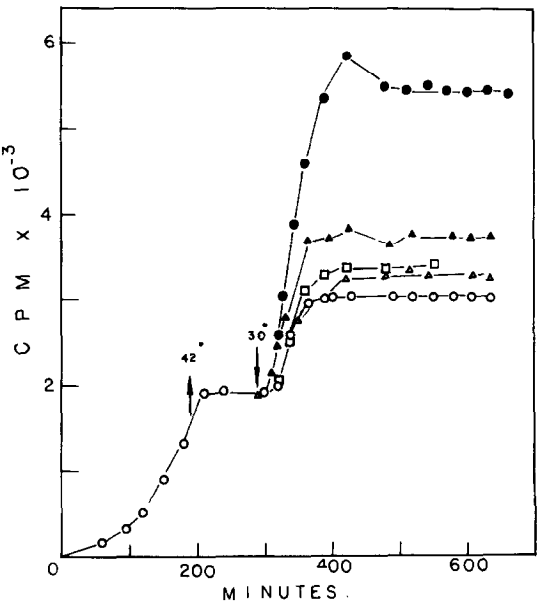

Figure 1. The effect of protein synthesis inhibition on subsequent DNA synthesis. Cells of Hfr 165/120/6 dnaA were grown at 30° in the presence of 2 µg/4 µg/ml ^{3}H-thymine, transferred to 42° for 100 min, then transferred back to 30°. Chloramphenicol (200 µg/ml) was added at 0 (○), 5 (△), 10 (□), 20 (▲) and 30 (●) min after return to 30°.

period of protein synthesis from 0-15 min is needed for the next initiation to occur[9].

In order to detect unique protein(s) synthesized during the first 15-20 min after return to permissive temperature, Hfr 165/120/6 was grown at 30°C, transferred to 42°C for a period of 100 min and one-half of the culture labeled with ^{3}H-L-amino acids during the last 15 minutes of the 100 min period (a time when one would not expect the proteins to be made). The other half of the culture was transferred back to 30°C and labeled with ^{14}C-L-amino acids for 15 min (a time when one would expect the protein to be made). The cultures were merged, and the soluble proteins were prepared for DNA-cellulose chromatography. The results of a typical DNA-cellulose column can be seen in Fig. 2. The 0.4 M NaCl fractions were pooled and run on SDS acrylamide gels to separate the individual proteins and the results of the SDS gel separations can be

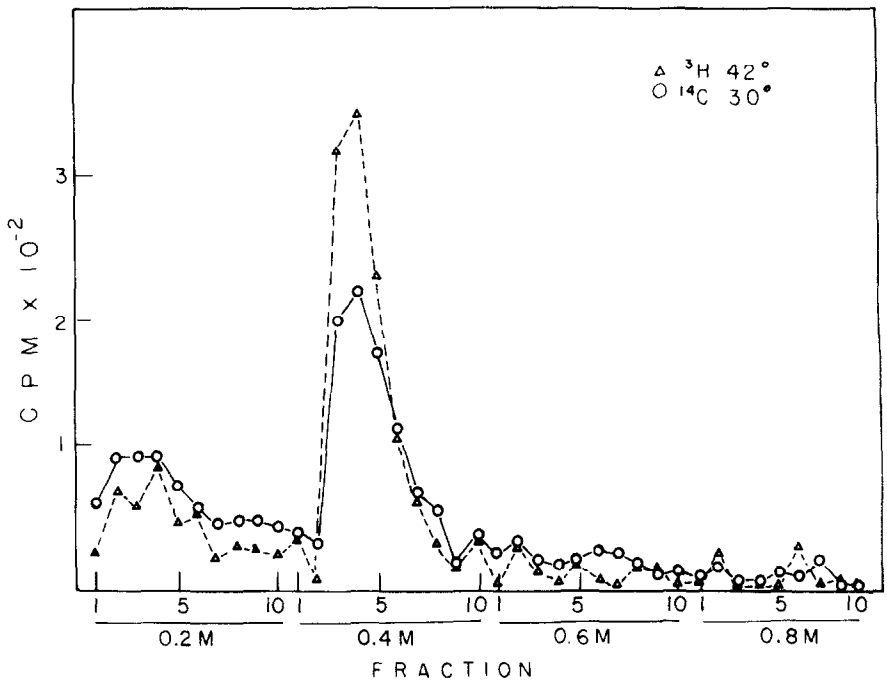

Figure 2. DNA-cellulose chromatography of proteins labeled with ^{3}H-AA for last 15 min at 42° (△) and with ^{14}C-AA for first 15 min after return to 30° (○). The counts were normalized to the value of the unfractionated sample before loading on the column.

seen in Fig. 3. These results indicate that there is a protein of about 60,000-65,000 molecular weight which is greatly elevated in the 30°C cells and a peak at 100,000 M.W. which is high in the 42°C cells.

In order to determine whether this protein, which was elevated in the cells labeled at 30°C, might be synthesized in response to the reinitiation of DNA synthesis, or whether it represented some differential synthesis or inactivation caused by the 42°C treatment, a revertant of this dnaA mutant, which appears to replicate DNA normally at 42°C, was subjected to the same analysis as described above. Since DNA synthesis does proceed normally in the revertant at 42°C, one would expect a random population of cells, with respect to the replication cycle both during the 42° and 30° periods. Thus, the differences seen at 42° vs. 30° which are reflections of cell cycle differences

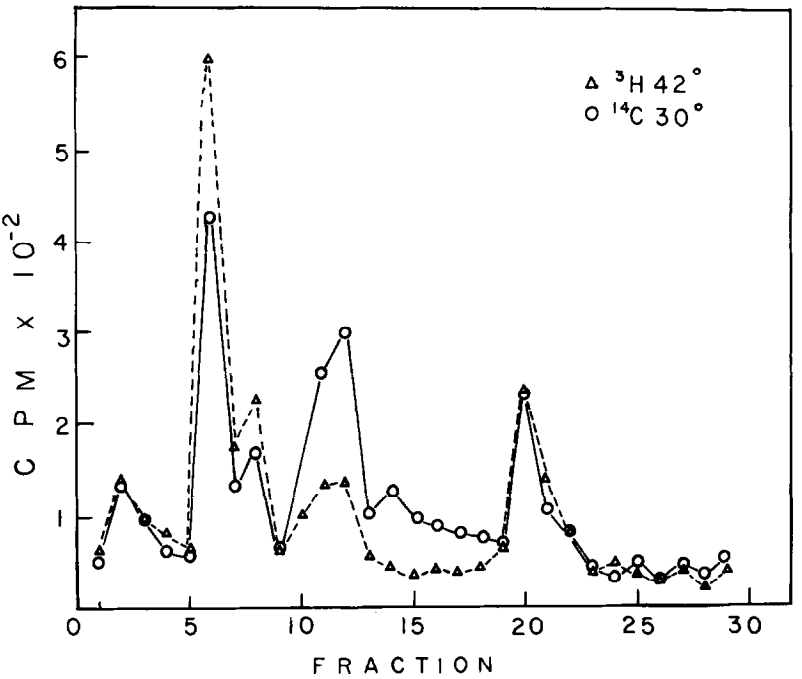

Figure 3. SDS polyacrylamide gel electrophoresis of the proteins from the 0.4 M NaCl step of the DNA-cellulose column. The counts were normalized to 0.4 M pooled fraction before loading on the gel.

should disappear but differences due to the temperature effects alone should remain.

The results of separating the 0.4 M NaCl DNA-cellulose fractions from the revertant by SDS-acrylamide gel electrophoresis can be seen in Fig. 4.

The 100,000 molecular weight peak is slightly elevated in this revertant experiment, but the slight variations in this peak from experiment to experimant make any conclusions about its significance doubtful. However, the 60,000 molecular weight peak, which is consistently higher at 30°C in the mutant experiments now appears equally labeled, suggesting that the synthesis of this protein may be triggered by the first initiation event and may have some significance in the DNA replication cycle.

DISCUSSION

At present, we do not know the function of this protein, nor whether it

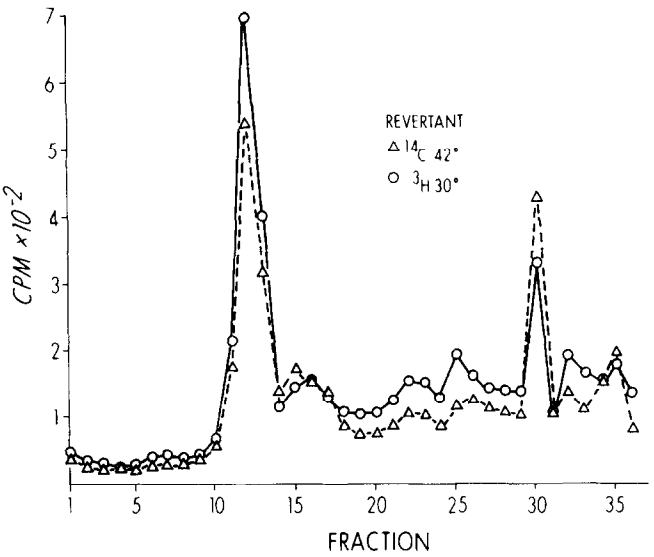

Figure 4. SDS polyacrylamide gel electrophoresis of the proteins from the 0.4 M NaCl step after DNA-cellulose chromatography of proteins from a revertant of Hfr 165/120/6 which shows normal DNA synthesis at 42°. The counts were normalized to the 0.4 M pooled fraction before loading on the gel.

is one of the known gene products required for DNA replication, but its synthesis does appear to be specifically triggered by initiation. Preliminary experiments also suggest that its synthesis may be limited to a brief period just after initiation.

Other work in our laboratory (unpublished results) suggests that the apparent requirement for protein synthesis to achieve the premature initiation at 20 min may be an artifact of premature chain termination caused by protein synthesis inhibition and a misinterpretation of residual synthesis experiments. If this is true, then this protein may not be required for reinitiation but may have some other function necessary for timing some event in the replication cycle.

We are currently trying to determine more precisely the timing of the synthesis of this and other proteins which appear to show differential synthesis during replication cycle, and hope that it will be possible to determine whether they are known gene products necessary for replication by in vitro DNA synthesis complementation tests.

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