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Escherichia coli Ribonucleotide Reductase Expression is Cell Cycle Regulated

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The expression of the genes encoding ribonucleotide reductase in *Escherichia coli* was investigated in cultures synchronized by obtaining the smallest cells in a population after sucrose gradient centrifugation. Specific activity of ribonucleotide reductase and DNA initiation were found to increase in parallel, periodically as a function of the cell cycle. The expression of *nrd* was also determined in cells synchronized by periodic repeated doubling in a phosphate limited medium. Antibodies directed against the B2 subunit of ribonucleotide reductase were raised in a rabbit and purified. Immunoprecipitation of the B2 subunit and RNA-DNA dot blot hybridization assays were developed and employed to determine the expression of ribonucleotide reductase translational and transcriptional products during the cell cycle. Both of *nrd*-mRNA and B2 subunit expression were found to increase each generation at approximately the same time DNA synthesis was initiated and then to decrease back to the basal level shortly after DNA initiation. These results provided evidence of cell cycle dependent regulation of ribonucleotide reductase in *E. coli*. When the upstream regulatory region of *nrd* was fused to a promoterless *lacZ* gene on a single copy plasmid, *lac*-mRNA and β-galactosidase were found to be synthesized in parallel to *nrd* expression from the chromosomal operon. When *nrd* sequences surrounding the promoter were removed from this construct, *lac*-mRNA and β-galactosidase synthesis were no longer cell cycle regulated.

INTRODUCTION

Ribonucleoside diphosphate reductase (RR) catalyzes the enzymatic reduction of ribonucleotides to deoxyribonucleotides, the first step in the pathway unique to DNA replication (Thelander and Reichard, 1979). This enzyme is an essential constituent of all living cells and may play a role in the control of DNA synthesis. The *Escherichia coli* enzyme, which serves as a model for most organisms, consists of two nonidentical subunits, B1 and B2. The activity of the holoenzyme is intricately controlled by allosteric effectors binding at sites localized to the large subunit, B1. The smaller subunit, B2, contains a unique stable free radical localized to a tyrosine ring that is required for enzymatic activity (Larsson and Sjoberg, 1986). The *E. coli* enzyme has been well characterized. The genes encoding the two RR subunits (*nrd*A and *nrdB*) have been cloned (Eriksson *et al.*, 1977), sequenced (Carlson *et al.*, 1984), and shown to be regulated in parallel with DNA initiation (Filpula and Fuchs, 1977). In addition, the synthesis of the two subunits of RR is coordinately regulated (Fuchs, 1977) via a 3.2 kb polycistronic mRNA that is transcribed from the *nrd* operon (Hanke and Fuchs, 1983a). The synthesis of RR increases when DNA synthesis is inhibited by thymine starvation, chemical inhibitors (nalidixic acid or bleomycin), or shifts to nonpermissive temperatures in *dna*A, *dna*B, *dna*E, or *dna*G mutants (Filpula and Fuchs, 1977, 1978). The increase in RR activity was also observed in strains containing *recA, recB, recC, recAB,* or a *lexA* mutation and thus the increase is independent of the SOS (error prone repair) response (Filpula and Fuchs, 1977). Because the strains used were not *rnh*, they were not capable of *recA* dependent stable DNA replication. In an exponentially growing culture, an increase in RR specific activity (Filpula and Fuchs, 1977) and *nrd*-mRNA synthesis (Hanke and Fuchs, 1984) was first observed ~20 min after DNA inhibition. When an *E. coli* culture was starved for thymine for various times and thymine was added back to the culture and the amount of DNA synthesized in the absence of protein synthesis was measured, no increase in DNA synthesis was observed for the first 20 min of thymine starvation. After 20 min of starvation, a time dependent increase

in the amount of DNA synthesized in the absence of protein synthesis was observed (Pritchard and Lark, 1964). Because *E. coli* can elongate but not initiate replication forks in the absence of protein synthesis, these results indicate that an *E. coli* culture has increased initiation replication forks only after 20 min of thymine starvation, the same time the culture increases *nrd* expression. Initiation of a new replication fork requires protein synthesis as does the increase in RR expression (Hanke and Fuchs, 1984). When an exponentially growing *E. coli* culture is shifted to better growth conditions, the rate of DNA synthesis does not change for 20 min but then increases to a faster rate. Under these conditions, *nrd* expression also transiently increases 20 min after the shift to better growth conditions (Filpula and Fuchs, 1978) again suggesting a correlation between the timing of new replication forks and *nrd* expression.

Several techniques have been used to obtain synchronized *E. coli* cultures, but the cultures either quickly lose synchrony or the technique produces too few cells for biochemical characterization (Kepes and Kepes, 1980, 1981). In this paper, we used two different methods to synchronize *E. coli* cultures. The first involves collecting the smallest cells from an exponentially growing culture that had been subjected to centrifugation (Gudas and Pardee, 1976). The second uses synchrony induced by repeated doubling in a phosphate limited medium. Although phosphate starvation does not induce cell synchrony, repeated cycling of phosphate starvation does (Kepes and Kepes, 1980, 1981). Cell number and the rate of DNA synthesis were used as parameters to determine cell synchrony. With the use of the centrifugal method to obtain a synchronize culture, Lutkenhaus *et al.* (1979) showed that among 750 polypeptides examined by 2-D gel electrophoresis no polypeptide was synthesized differentially during different parts of the cell cycle. With this method of cell synchrony, we measured specific activity of ribonucleotide reductase at different parts of the cell cycle and found that the specific activity increased significantly at approximately the time that DNA initiation occurred and that specific activity then decreased as expected if protein synthesis continued without further RR synthesis.

To analyze *nrd* expression during the cell cycle, periodic phosphate limitation was used to get better synchrony. Cell-cycle dependent control of *nrd* expression was found for both the *nrd*-mRNA and for the RR B2 protein. These results indicate that the expression of *nrd* is cell cycle dependent. Similar experiments were conducted on a strain with a single copy plasmid containing a translation fusion of the *nrd* promoter and regulatory region directing *lac*-mRNA and β-galactosidase synthesis. β-galactosidase and *lac*-mRNA synthesized from the plasmid in a synchronized culture increased in parallel with protein B2 and *nrd*-mRNA synthesized from the chromosomal genes. When sequences upstream and downstream of the promoter were removed from this construct, *lac*-mRNA and β-galactosidase were no longer cell cycle regulated.

MATERIALS AND METHODS

Bacterial Strain and Growth Medium

E. coli B/5 and K12 derivative strains N110 (*galK, thyA*), BK99 (*pro, his, ile, Δlac*), LS1 (BK99 containing plasmid pLBAR), and strain LS2 (BK99 containing plasmid pLS2) were used. Plasmid pJEL170 is an R1 derivative operon-fusion vector that contains the plasmid origin of replication under control of λpL and contains the λcI857 repressor protein (Larsen *et al.*, 1987). Plasmid pJEL170 has a copy number of one at 30°C. Plasmid pLBAR is a translation fusion derivative of pJEL170. To construct plasmid pLBAR, a minor promoter in the *trpA* sequence upstream of the *lac* operon was deleted, the direction of the polycloning sites was inverted and the *nrd* sequence from −229 to +513 was ligated to the *Bam*HI and *Cla* I sites that are upstream of the *lac* operon (Augustine, personal communication). Thus plasmid pLBAR contains the *lac* operon under control of the *nrd* regulatory sequences as well as *nrd* translational control. Plasmid pLS2 was made by isolation of the *Mnl* I-*Hin*fI fragment of the *nrd* promoter and filling in the *Hin*fI site with DNA polymerase (Klenow fragment) and ligating into pLBAR that had been digested with *Eco*RI and *Bam*HI and the sticky ends filled in by the Klenow enzyme. Light blue colonies were identified as having the correct orientation.

Cultures of B/5 were grown in Davis-Mingioli minimal medium (Davis and Mingioli, 1950) supplemented with 0.4% glycerol or 0.6% sodium acetate. All other cultures were grown in mineral salt medium buffered with 3-(*N*-morpholino)propanesulfonic acid (MOPS)-KOH (5×10^{-2} M, pH = 7.3) at 30°C. The medium contained 4.6 g/l NaCl, 1.5 g/l KCl, 2 g/l $(NH_4)_2SO_4$, 0.2 g/l $MgSO_4$, 0.5 mg/l $FeSO_4$, 50 mg/l proline, 80 mg/l leucine, 30 mg/l histidine, and 4 mg/l thiamine. Glucose was used as the carbon source at a concentration of 2 g/l. The phosphate concentration was 0.2×10^{-3} M in phosphate limiting synchronization medium and 1×10^{-3} M in phosphate nonlimiting batch culture (Kepes and Kepes, 1980, 1981).

Synchronization of Cells

B/5 was synchronized by a modification of the procedure of Gudas and Pardee (1976). An exponentially growing culture (500 ml at optical density [OD] = 0.6) was rapidly chilled at 0°C and concentrated by centrifugation at 3000 g in a Sorvall SS-34 head and was resuspended in 2 ml of medium. One ml was layered on each of two 5–30% linear sucrose gradients and spun for 10 min at 1400 g in a HB-4 swinging bucket rotor in a Sorvall RC2 centrifuge. Approximately the top 5% of the band of cells that had moved two-thirds of the way down the tube was collected through a needle attached to a Buchler micropump. These cells were collected from both gradients and sucrose was removed by spinning at 4340 g in the SS-34 rotor for 10 min. The cells were resuspended in prewarmed medium to begin the experiment. Samples of the culture were collected at various times after synchronization, and permeabilized by ether-treatment, and assayed for RR activity and protein content. Samples were also placed in medium containing ^{3}H-thymidine for 3 min and analyzed for trichloroacetic acid (TCA) insoluble radioactivity to measure the rate of DNA synthesis.

BK99 was grown exponentially in phosphate limiting medium at 30°C for 10 h and then synchronized at 30°C by 16 to 20 successive cycles of doubling followed by phosphate starvation in phosphate limiting medium. At the end of each cycle the culture was diluted two-fold with limiting phosphate medium (2×10^{-4} M) that permitted one mass doubling and one cell doubling before phosphate starvation (Kepes and Kepes, 1980, 1981). After the appropriate number of cycles, the synchronized culture was harvested and diluted for batch culture in nonlimiting phosphate medium (1 mM).

Cell Counting

A 200-μl sample of cells was withdrawn from a synchronously growing culture at 15-min time intervals. The cells in the sample were fixed by addition of 100 μl of 37% formaldehyde and kept on ice until counting. The fixed cells were counted with the use of the Petroff-Houser counting chamber.

Rate of DNA Synthesis

The rate of DNA synthesis was measured by incorporation of [^{3}H]-thymidine into TCA insoluble counts. At 15-min time intervals, a 0.4 ml sample of a culture of synchronously growing cells was incubated with [6-^{3}H]thymidine (2×10^{-6} M, 4 Ci/mmol) and deoxyuridine (5×10^{-5} M) in MOPS medium for 5 min. The reaction was stopped by adding 2.5 ml ice cold 5% TCA containing thymidine (10^{-4} M) and kept on ice for 30 min. Insoluble material was collected on glass fiber filters (0.45 mm pore size), dried, and counted.

Ribonucleotide Reductase Assay

Ten milliliters of cells were removed from the culture, chilled, concentrated 200-fold, and assayed as previously described (Fuchs and Warner, 1975).

Protein Determination

Ether-permeabilized cells were dissolved in 0.1 M NaOH and assayed for protein content by the Hartee modification (Hartee, 1972) of the method of Lowry *et al.* (1951) except that the final volume was reduced to 1 ml.

Antibodies Directed Against B2

Polyclonal antibodies directed against *E. coli* RR B2 subunit were raised in a New Zealand White rabbit by six successive subcutaneous injections (Kabit, 1980). A sample of 1.2 mg B2 protein in 1.5 ml *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer was mixed 1:1 with Freund's complete adjuvant and was injected into the rabbit subcutaneously. Three weeks after the first injection, four successive injections at 2-wk intervals with protein B2 in Freund's incomplete adjuvant were given. The antibody titer was kept high by monthly injections.

To purify the IgG fraction of the polyclonal antibodies, 3 ml of dialyzed antiserum was loaded on the DEAE Affi-Gel Blue (BioRad, Richmond, CA) gel column (20 ml, 30 × 1 cm). The column was washed with five bed volumes of elution buffer (0.02 M tris(hydroxymethyl)aminomethane [Tris]-HCl [pH = 8.0], 0.028 M NaCl, and 0.02% NaN_3), and 3-ml fractions of the eluant were collected. The final purity of IgG fraction was checked by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Enzyme-linked immunosorbent (ELISA) assays were used to determine the titers of antiserum and purified IgG fraction (Engvall and Perlman, 1971; Hudson and Hay, 1980). Preimmune serum and bovine serum albumin (BSA) were used as negative controls for antibody and antigen, respectively. The optimal working dilution of antibody used in immunoprecipitation experiment was determined from the curve of ELISA assay.

Immunoprecipitation

A 5-ml sample of synchronously growing culture from various times after synchrony was pulse labeled by incubating with [^{35}S]-methionine (>800 Ci/mmol, 4 mCi/ml in MOPS minimal medium) for 5 min. Cells were washed three times with phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.2 mM Na_2HPO_4, and 1.4 mM KH_2PO_4 [pH = 7.3]) and lysed by sonication three times for 10 s at 0°C. Insoluble material was removed by centrifugation. For each sample, the amount of protein labeled was determined by adding 10 μl of cell lysate to 1 ml 5% TCA, heating at 100°C for 5 min, and filtering through a glass fiber membrane. The membrane was dried and the radioactivity was quantitated by scintilation counting. This value was used to determine the amount of B2 as a fraction of total labeled proteins. For immunoprecipitation reactions, cell lysates were precleared with protein A-Sepharose (Pharmacia, Piscataway, NJ) at 4°C for 8 h (20 ml 1:1 slurry protein A-Sepharose per 200 ml lysates). Total labeled protein (10^6 cpm) was mixed with 30 ml of a 1:1 slurry of protein A-Sepharose in PBS and 50 ml of a 1000-fold diluted sample of purified antibodies. The final volume was brought to 0.5 ml with dilution buffer (0.01 M Tris-HCl, 0.025% NaN_3, 0.1% Triton X100, and 0.1% bovine hemoglobin) and incubated at 4°C overnight with shaking. The protein A-Sepharose beads were washed twice with dilution buffer, once with TSA buffer (0.01 M Tris-HCl, 0.14 M NaCl, and 0.025% NaN_3) and 0.05 M Tris-HCl buffer, pH = 7.4. Loading buffer (60 μl) was added and the mixture was heated at 100°C for 3–5 min. Samples were subjected to electrophoresis on an 8% SDS-polyacrylamide gel, and the gel was enhanced with 20% 2,5-diphenyloxazole (PPO) (Bonner and Laskey, 1974). The amount of radioactive B2 was determined both by densitometer scanning of an autoradiograph and by scintillation counting of the B2 band that was cut from the gel.

Quantitation of nrd-, lac-, fis-, and bla-mRNA

To standardize for recovery of RNA, the amount of rRNA was quantitated. A 1-ml sample of synchronously growing culture was harvested at each time point by flash freezing in liquid nitrogen and transferring to −70°C until the sample was processed. Total RNA was isolated by the hot-phenol method (Abiba *et al.*, 1981). Briefly, 0.05 ml samples of a solution containing 0.4 M NaAc, 10% SDS, 20 mM EDTA, and 0.5 ml of phenol (65°C) were added to each 1-ml culture, and the mixture was incubated in a 65°C water bath for 5 min with gentle shaking. After centrifugation, the aqueous phase was removed and extracted a second time with phenol followed by a phenol-chloroform extraction and a chloroform extraction. The RNA was precipitated by adding three volumes of ethanol to the aqueous phase and chilling at −70°C for 30 min. The RNA pellet was collected and dissolved in acetate/SDS buffer and subjected to two additional ethanol precipitations. The final RNA pellet was dissolved in 0.5 ml H_2O.

A 40 μl RNA sample from each time point was added to 60 μl H_2O and 0.3 ml 6.15 M formaldehyde containing 10× SSC. RNA was denatured by incubation at 65°C for 15 min. Denatured RNA samples were rapidly chilled on ice and were loaded on the Zeta-Probe (BioRad) membrane with the dot blot apparatus under vacuum. The membrane was dried, prehybridized in a heat sealed plastic bag in 0.5 M NaH_2PO_4 buffer (1% BSA, 1 mM EDTA, and 7% SDS) at 65°C for 2 h. Hybridization was conducted at 65°C for 20 h in the same solution as prehybridization except that the probe (5×10^6 cpm/ml) was added. After hybridization, the membrane was washed with 40 mM NaH_2PO_4 buffer containing 0.5% BSA, 5% SDS, and 1 mM EDTA followed by 40 mM NaH_2PO_4 buffer containing 1% SDS and 1 mM EDTA at 65°C. The membrane was dried and exposed to an X-ray film. The radioactivity of each dot was quantitated by densitometer scanning and direct counting of each dot that was cut from the membrane. The rRNA specific probe was a 3.6 kb *Sac* II fragment from pKK3535 (Brosius *et al.*, 1981) that was labeled with ^{32}P-dATP by random oligonucleotide primed synthesis (Feinberg and Vogelstein, 1983).

For quantitation of *nrd*-mRNA, samples (90–250 μl) from each time point containing the same amount of radioactive rRNA determined by the above hybridization experiment was denatured, hybridized, and quantitated by the same procedures as above except that the 1.3 kb *Bam*HI-*Kpn* I fragment from pPS2 (Carlson *et al.*, 1984) and the 4.8 kb *Cla* I-*Bst*EII fragment from the *lac* operon were used as *nrd*-mRNA and *lac*-mRNA specific probes, respectively. The *fis* probe was a 250 bp *Xmn* I-*Hin*cII fragment from plasmid pRJ753 (Johnson *et al.*, 1988), and the *bla* probe was a 340 bp *Ssp* I-*Sca* I fragment from pUC18.

Northern and Southern Hybridization Analysis

To analyze the specificity of probes used in quantitation of *nrd*-mRNA and *lac*-mRNA, northern hybridization was employed as described

by Hassonna *et al.* (1984). Total RNA was isolated from log phase cultures of BK99, LS1, and LS2 with the hot phenol method as described above. RNA samples (30 μg) were run on a 1.2% formaldehyde agarose gel and transferred to a Zeta-Probe membrane (BioRad) with 20× SSC for 24 h. Hybridization conditions and washing stringency were the same as the dot blot hybridization assay used to quantitate the mRNA.

To verify that plasmid pLBAR was replicated randomly during the cell cycle, plasmid DNA was isolated from synchronously growing cultures of LS1 at 15-min intervals by the alkaline miniprep method (Sambrook *et al.*, 1990). DNA from an equal optical density of cells was digested with *Eco*RI and run on an 0.8% agarose gel. After electrophoresis, the DNA in the gel was denatured with a solution of 0.5 M NaOH and 0.15 M NaCl for 1 h, and the gel was neutralized with a solution of 0.5 M Tris-HCl (pH = 7.7) and 0.15 M NaCl for 1 h. The gel was dried onto Whatman 3M filterpaper at 60°C under vacuum for 2–3 h. The DNA in the gel was hybridized to a probe that was labeled with ^{32}P-dATP by random oligonucleotide primed synthesis with linearized pLBAR as template. Hybridization was in 5× SSPE, 0.1% SDS, 20 μg/ml *E. coli* tRNA, and 20 μg/ml sheared and denatured salmon sperm DNA at 55°C overnight followed by lower to higher stringent washes (6× to 0.1× SSC containing 0.1% SDS). Bands were visualized after overnight exposure at −70°C and quantitated by scintillation counting. Restriction enzymes were purchased from Boehringer Mannheim (Indianapolis, IN). Chemicals were bought from Sigma (St. Louis, MO) and radioactive nucleotide were purchased from Amersham (Arlington Heights, IL).

RESULTS

Quantitation of Ribonucleotide Reductase Specific Activity in a B/5 Culture Synchronized by Centrifugation

An *E. coli* B/5 culture growing exponentially at 37°C with 0.6% sodium acetate as the sole carbon and energy source was synchronized and grown under the same conditions; samples were harvested at various times after synchrony. DNA synthesis and RR specific activity were determined in these samples (Figure 1). If perfect synchrony had been achieved, the rate of DNA synthesis should change only when cells initiate or complete rounds of DNA replication. Because a culture grown with acetate as a carbon source (118-min generation time) will require less time to replicate DNA than the generation time, there should be a gap between rounds of DNA replication. Although complete synchrony was not obtained in this experiment, it is still possible to determine RR synthesis as a function of the cell cycle. The data in Figure 1 indicates that there appears to be an increased synthesis of RR early in the experiment followed by a decrease in specific activity of the enzyme that would be expected if protein synthesis continues without additional synthesis of RR. At ~100 min, both RR specific activity and the rate of DNA synthesis increased rapidly. After a short rapid increase of RR activity, the specific activity again slowly decreased. From this experiment, it would appear that RR is synthesized once per cell cycle over a rather short interval that seems to correlate with the timing of DNA initiation. This experiment was repeated several times with similar results. It is surprising that synchrony of RR activity appears to

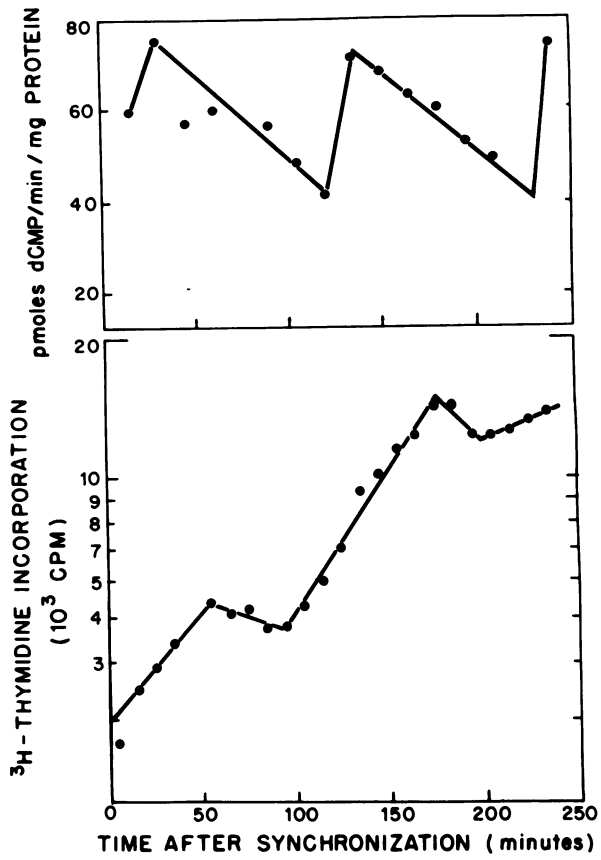

Figure 1. Ribonucleotide reductase specific activity and DNA synthesis in synchronized *E. coli* growing with acetate as the carbon source. An exponentially grown culture of B/5 (118 min generation time) was synchronized by harvesting the smallest cells from a band on a sucrose gradient, resuspending in prewarmed medium, and collecting samples at various times to measure ribonucleotide reductase specific activity. Samples of the culture were added to [^{3}H]-thymidine containing medium for 3 min to quantitate DNA synthesized per 3-min pulse.

be better than the synchrony of DNA initiation. This may reflect the fact that DNA initiation is very complex and that even if both processes responded to the same signal, DNA initiation would appear to be less synchronous.

To investigate further the timing of RR synthesis in the cell cycle, we repeated the above experiment with cells growing at a different rate. A culture of *E. coli* B/5 growing with glycerol as a sole source of carbon and energy and growing with a generation time of 68 min was synchronized by centrifugation and treated as above. The data in Figure 2 indicate that RR is synthesized during a 25-min period early after synchrony and again 50 min later. Both of these times correspond to initiations of DNA synthesis.

The data in Figure 1 indicate that most of the smallest cells had initiated DNA synthesis and had synthesized RR before they were harvested. A culture exponentially

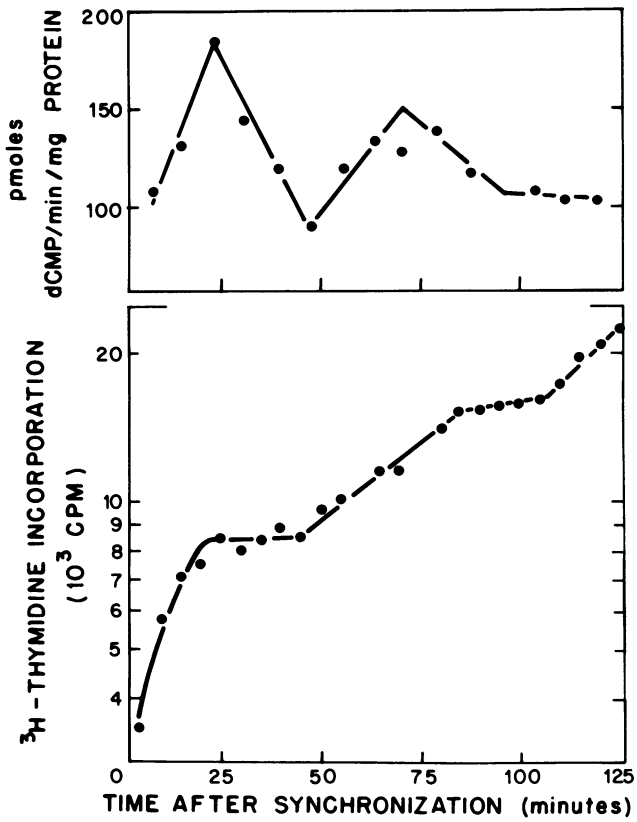

Figure 2. Ribonucleotide reductase specific activity and DNA synthesis in synchronized *E. coli* culture growing with glycerol as the carbon source. Cells exponentially growing with glycerol as the carbon source (68 min generation time) were harvested and analyzed as described in the legend for Figure 1.

grown with acetate as a carbon source was treated with 50 μg/ml of chloramphenicol for 110 min before the cells were harvested and treated as in Figure 1. Because this treatment would stop all protein synthesis, there should not be a change in the size or shape of any cell, but the treatment would allow any DNA replication forks that were initiated to complete replication. A comparison of Figures 1 and 3 indicates that the treatment with chloramphenicol prevents the early increase in DNA synthesis and causes RR specific activity to drop rather than to increase. At ~100 min DNA synthesis was initiated, and there was an increase in RR specific activity. This corresponds to the second synchronized DNA initiation observed in Figure 1. This experiment suggests that conditions that alter DNA initiation also affect the synthesis of RR.

Because the technique we used here as well as techniques using membrane elution work only on a few very specific strains (mostly B strains) and may give a distorted view of the cell cycle for more commonly used K-12 strains of *E. coli*, we switched to a technique of synchrony that is more generally useful for any *E. coli* strain. This technique uses periodic phosphate starvation to achieve synchrony (Kepes and Kepes, 1980, 1981). The mechanism used by this technique to achieve synchrony is not understood, but the results suggest that very good synchrony can be achieved.

To improve the resolution of RR quantitation, direct quantitation of protein made during short intervals was used rather than relying on enzymatic activity. Likewise, because the half-life of *nrd*-mRNA is 2.5 min, (Hanke and Fuchs, 1983b) direct quantitation of *nrd*-mRNA was used to assay *nrd* expression during the cell cycle. We therefore not only changed the method of synchrony but also changed the methods of quantitating *nrd* expression in the following experiments.

Quantitation of Ribonucleotide Reductase Subunit B2

To verify that the B2 protein could be specifically quantitated, the following experiment was conducted. An

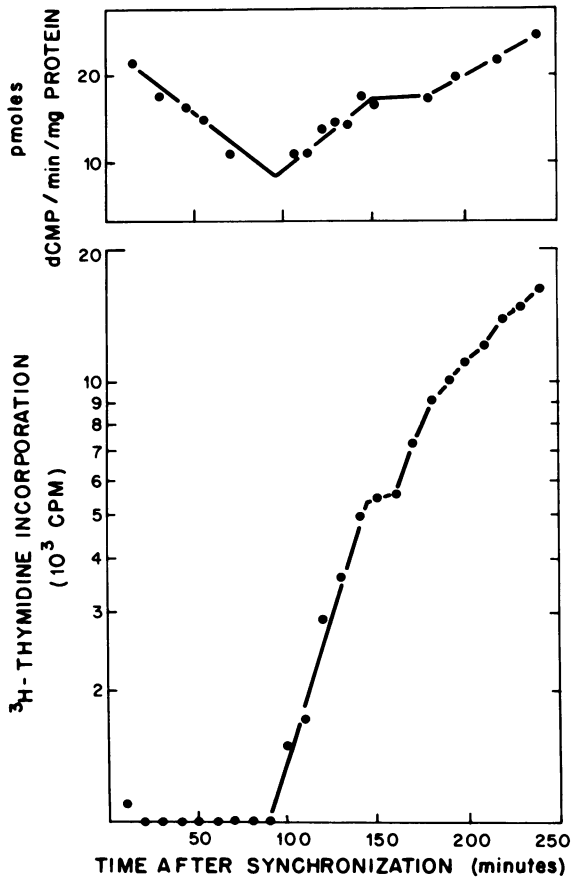

Figure 3. Ribonucleotide reductase specific activity and DNA synthesis in a synchronized *E. coli* culture that had been growing with acetate as the carbon source and were treated for one generation with chloramphenicol to stop cell growth but allow rounds of DNA replication to be completed. The smallest cells from the sucrose gradient were collected and analyzed as described in Figure 1 legend. If the chloramphenicol step had been omitted, the results should be identical to Figure 1.

exponentially growing culture was labeled with [^{35}S]-methionine, and the total extract was run in lane 4 (Figure 4A) of an SDS gel. Although it appears that B2 can be quantitated without purification, gels were not always of sufficient quality to make this possible. Pure B2 protein was run on the gel in a separate lane that was removed and stained (lane 1). Lane 5 (Figure 4A) contains the supernatant after immunoprecipitation and indicates that the band corresponding to B2 is not visible, whereas lane 3 contains the immunoprecipitate from ten times the volume of extract used in lanes 4 and 5. Figure 4B lanes 5–8 contain an equal amount of radioactive protein obtained from cultures of different densities and indicate that the amount of labeled B2 is a constant fraction of the labeled protein independent of the culture density. An exponentially growing culture was starved for thymine for various times before labeling and immunoprecipitation. Lanes 1–3 (Figure 4B) represent cells starved for 0, 180, or 240 min. These cells contain differing amounts of B2 per total amount of protein. The amount of B2 detected is similar to that predicted from earlier work (Tuggle and Fuchs, 1986, 1990) indicating that B2 can be measured accurately in cultures with differing B2 content.

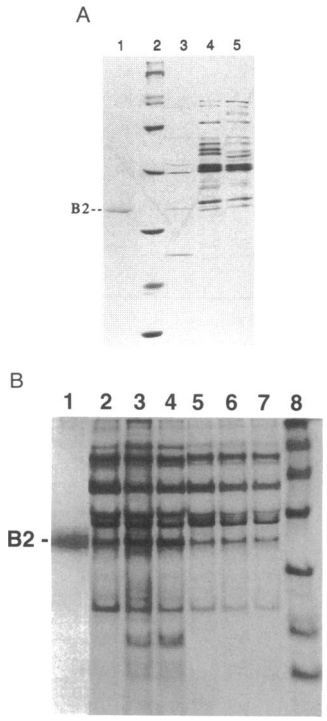

Figure 4. Quantitation of ribonucleotide reductase B2 subunit followed immunoprecipitation and SDS-PAGE. (A) an exponentially growing culture of N110 was labeled with [^{35}S]-methionine, harvested, and subjected to immunoprecipitation. Lane 1 contains pure B2 protein that was stained with coomassie blue. Lane 2 contains labeled protein markers of molecular weights (from top to bottom) of 200K, 92K, 69K, 46K, 30K, 21.5K, and 14.3K. Lane 4 contains cell lysate before immunoprecipitation whereas lane 5 contains the supernatant after immunoprecipitation. Lane 3 contains the immunoprecipitate from a volume of extract 10-fold larger than that used in lanes 4 and 5. (B) lane 1 contains pure B2 treated as in A. Lane 2 contains extract from an exponentially grown culture not starved for thymine. Lane 3 contains extract from a culture starved for thymine for 180 min. Lane 4 contains an extract from a culture starved for thymine for 240 min. Lanes 5–7 contain extracts from cultures at optical density (OD_{660}) of 0.10, 0.25, and 0.50, respectively. Lane 8 contains labeled protein markers (same as in A).

Synchronization of E. coli Cells

E. coli strain BK99 growing in supplemented MOPS minimal medium at 30°C had a generation time of ~100 min. A phosphate limited culture was allowed to grow for 150 min before one-half of the culture was removed and replaced with an equal volume of fresh medium containing limited phosphate (2×10^{-4} M), a concentration that allowed only one mass doubling and one cell doubling before the culture was limited by depletion of phosphate. This cycle was repeated 20 times to synchronize the culture of BK99. The culture was diluted into minimal medium containing a nonlimiting amount of phosphate. Cell number, DNA synthesis, synthesis of RR B2 subunit, and *nrd*-mRNA synthesis was quantitated at 15-min intervals. Figure 5A indicates that cell division occurred synchronously for the 500 min that the culture was followed, doubling over a period of 15 min every 120 min. DNA synthesis measured by incorporation of [^{3}H]-thymidine into TCA insoluble material appeared to be less well synchronized with initiation of new DNA replication forks occurring over a significant portion of the 120-min generation time but with a rate of synthesis leveling off indicating that no new forks were initiated and that some degree of synchrony had been achieved. These results are similar to the results that had previously been obtained with this method (Kepes *et al.*, 1980, 1981; Joseleau-Petit *et al.*, 1990; Robin *et al.*, 1990).

Quantitation of B2 Synthesis During the Cell Cycle

Figure 5B indicates the B2 content in samples of the synchronized culture used in Figure 5A. Insufficient protein was present in samples for the first 100 min after synchrony for accurate analysis. As well, analysis was not carried out after 400 min. As can be seen in Figure 5B, B2 synthesis appears to be periodic with burst of synthesis occurring 120 min apart and coinciding with a burst of DNA initiation. From the autoradiograms, it was obvious that other bands seen in Figure 4 did not vary with the cell cycle as would be expected from the results of Lutkenhaus *et al.* (1979), if these represent typical proteins.

Quantitation of nrd mRNA During the Cell Cycle

As an independent method to confirm the cell cycle dependent control of *nrd* expression, total RNA was isolated from the synchronously growing culture at 15-

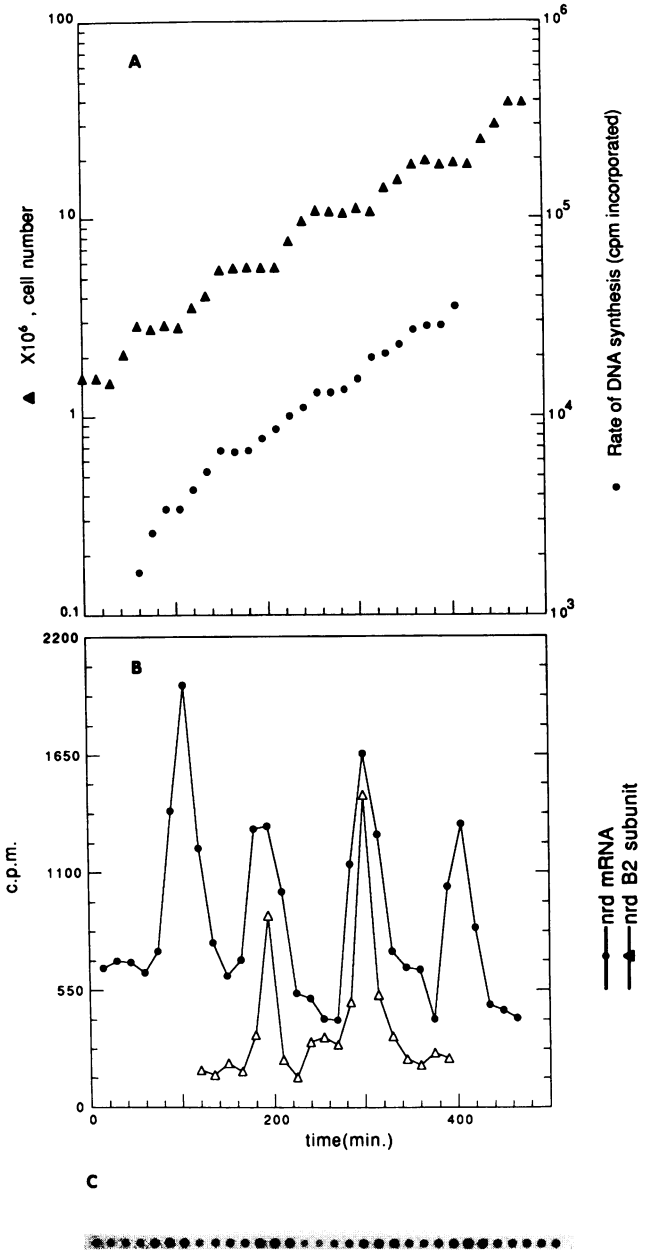

Figure 5. Quantitation of the cell number, rate of DNA synthesis, rate of ribonucleotide reductase B2 subunit synthesis, and *nrd*-mRNA synthesis during the cell cycle. A culture of BK99 was synchronized as described in the text. Cell numbers, rate of DNA synthesis, rate of protein B2 subunit synthesis, and rate of *nrd*-mRNA synthesis were analyzed at 15-min intervals and were plotted as a function of time after synchrony. (A) (△) cell number and (●) rate of DNA synthesis. (B) (△) rate of synthesis of *nrd* B2 subunit determined after immunoprecipitation; (●) rate of synthesis of *nrd* mRNA. (C) Autoradiograph of dot blot hybridization of *nrd* mRNA during the cell cycle.

min intervals. An exponential curve of rRNA was obtained as function of time indicating that the synchronized culture was increasing exponentially as expected. The synthesis of rRNA paralleled protein synthesis and increased in cell mass. To quantitate the amount of *nrd*-mRNA, the same amounts of radiolabeled rRNA of each sample were loaded on Zeta-Probe membrane with a dot blot apparatus. Figure 5C is an autoradiogram of the dot blot of *nrd*-mRNA. Figure 5B presents the quantitation of the dot blot shown in Figure 5C of *nrd*-mRNA during the cell cycle. The four peaks of *nrd*-mRNA synthesis occur at the same time that DNA initiation was observed, and the second and third peaks correspond to the increase observed for protein B2 synthesis. Because the half-life of *nrd*-mRNA is 2.5 min (Hanke and Fuchs, 1983b), *nrd*-mRNA should be found only in cells actively expressing *nrd*. The *nrd*-mRNA increased each generation at approximately the time of DNA initiation and decreased to a basal level. These results indicate that transcription of *nrd* is cell cycle dependent.

Quantitation of β-Galactosidase Expression Driven by the nrd Regulatory and Promoter Sequences During Cell Division Cycle

The expression of *lacZ* reporter gene under control of *nrd* regulatory and promoter sequences was determined during cell cycle. Both β-galactosidase protein and *lac*-mRNA were analyzed during the cell cycle by immunoprecipitation of β-galactosidase and dot blot assays of *lac*-mRNA with the same methods that were used for quantitating RR B2 protein and *nrd*-mRNA described above, except that a monoclonal antibody against β-galactosidase and a *lac* specific probe were used in immunoprecipitation and dot blot analysis, respectively. Northern analysis was used to show that the *lac*-mRNA could be specifically determined. The plasmid content was determined during synchronous growth to determine whether cell synchrony affected copy number. Plasmid DNA was isolated and separated from chromosomal DNA by gel electrophoresis, subjected to Southern analysis, and the radioactive spots were counted. No difference in copy number was found. Figure 6 indicates that both β-galactosidase protein and *lac*-mRNA exhibit increases in synthesis that parallels the increase and decrease of *nrd* gene expression from the chromosome during the cell cycle. These results further confirmed that *nrd* expression is cell cycle regulated. These results also indicate that all the information required for cell cycle dependent regulation of *nrd* expression is included in the *nrd* regulatory and promoter sequences from −229 to +513 that was characterized (Tuggle and Fuchs, 1986, 1990) to be necessary to regulate the response of *nrd* expression to treatments that interrupt DNA synthesis.

To determine whether cell cycle regulation was due to a unique promoter or to regulatory sequences other than the promoter, the construct pLS2 was used. This construct contains the −34 to +1 region of the *nrd* promoter fused to the +93 region of *nrd* but otherwise is identical to pLBAR. This plasmid has the *nrd* promoter

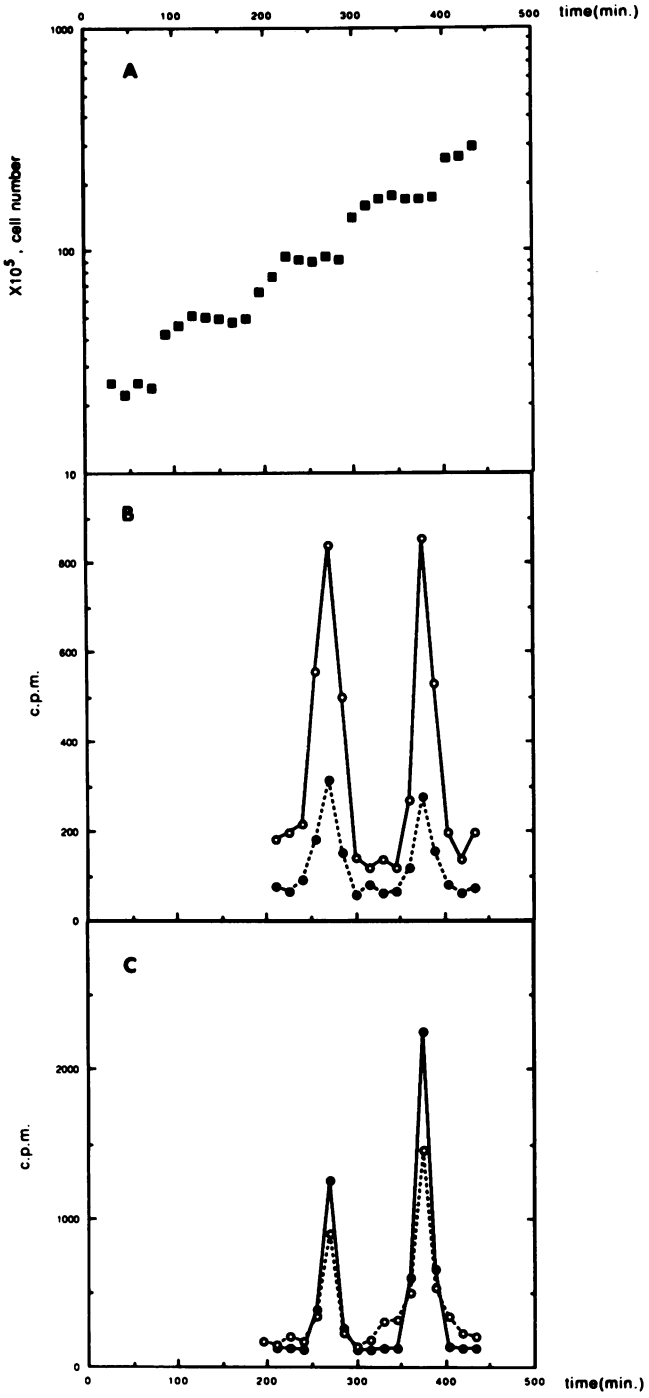

Figure 6. Comparison of the expression of *nrd* from the chromasomal gene with *lacZ* expression from a plasmid borne *nrd-lacZ* fusion. A culture of LS1 was synchronized as described in the text. Cell number, the amount of β-galactosidase and ribonucleotide reductase B2 subunit synthesized during a 5-min pulse, and the rate of *lac*-mRNA and *nrd*-mRNA synthesis were analyzed at 15-min intervals and were plotted as a function of time after synchrony. (A) (■) cell number. (B) (— O —) rate of synthesis of *nrd*-mRNA and (- - - ● - - -) rate of synthesis of *lac*-mRNA. (C) (- - - O - - -) rate of synthesis of *RR* B2 subunit and (— ● —) rate of synthesis of β-galactosidase determined by immunoprecipitation.

but is missing upstream as well as 94 bp of downstream DNA. Strain LS2 was synchronized and RNA was isolated at various times and analyzed. The data in Figure 7A indicate that the culture was synchronized. The data in Figure 7B indicate that the chromosomal encoded *nrd*-mRNA was cell cycle regulated whereas the *lac*-mRNA synthesized from the plasmid was not cell cycle regulated indicating that *nrd* sequences other than the promoter are required for cell cycle regulation. Figure 7B also gives two additional controls indicating that neither the chromosomal gene *fis* nor the plasmid encoded *bla* gene are affected by the cell cycle as one would expect.

DISCUSSION

In *E. coli* the requirement for deoxyribonucleotide triphosphates is limited to the need for DNA replication. DNA repair requires deoxyribonucleotide triphosphates but triphosphates used in DNA repair can be regenerated from the deoxyribonucleotide monophosphates generated during repair. Thus, the amount of RR, the sole enzyme that is capable of generating deoxyribonucleotides in *E. coli* grown aerobically, required is directly proportional to the rate of DNA synthesis. The rate of DNA synthesis is determined by the rate of DNA initiation. Thus it is not surprising that there is a burst of RR synthesis at approximately the time DNA initiation occurs. A regulatory mechanism that could generate a controlled burst of RR synthesis every time a new replication fork is initiated would ensure the appropriate amount of enzyme under any growth condition. Cooper in his book (Bacteria Growth and Division, 1991) strongly argues that no gene in *E. coli* is cell cycle regulated and that reports to the contrary are artifacts. He argues that the need for enzymes is independent of the cell cycle and that cell cycle regulation of a gene would only complicate the regulation of that gene. Although this may be true of most genes, RR seems to be an exception because the requirement of RR parallels the amount of DNA synthesis and DNA synthesis is cell cycle regulated. In an *E. coli* cell growing with a generation time longer than the time required for replication of the chromosome, the need for RR goes from zero to a maximum as DNA initiation occurs.

Previous work (Filpula and Fuchs, 1977; Hanke and Fuchs, 1984) has indicated that there is a correlation between the increase in ribonucleotide reductase and DNA initiation. When DNA synthesis is inhibited in *E. coli* for varying lengths of time before synthesis is restored, the original replication fork continues to be replicated, but after 20 min of inhibition a new fork is also initiated. This response only occurs if protein synthesis occurs during the 20 min of DNA inhibition. Approximately 20 min after inhibition of DNA synthesis, *nrd* expression begins to increase and continues to increase for several hours. The increase in *nrd* mRNA synthesis

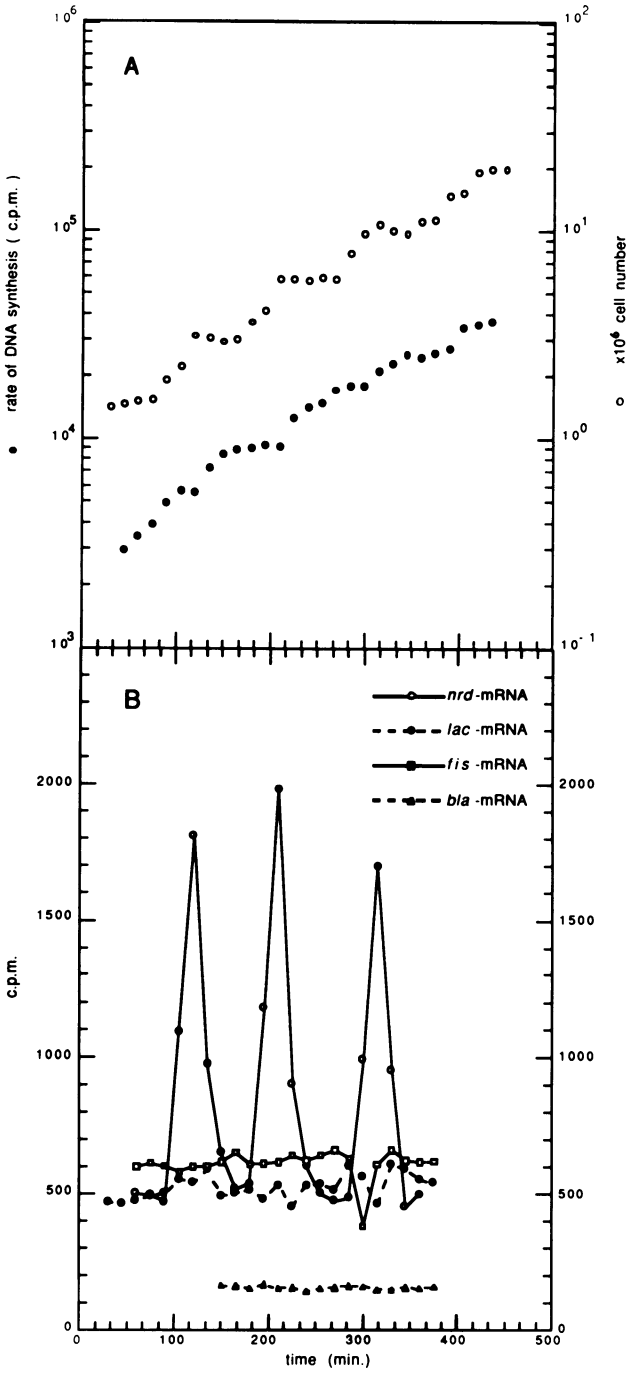

Figure 7. Comparison of the plasmid encoded *lac*-mRNA expressed from a *nrd* promoter lacking regulatory sequences fused to *lac* to the expression of the chromosomal *nrd* and *fis* genes as well as the plasmid encoded *bla* gene during the cell cycle. A culture of LS2 was synchronized and the cell number, rate of DNA synthesis, and the rates of synthesis of *lac*-mRNA, *nrd*-mRNA, *fis*-mANA, and *bla*-mRNA were analyzed at 15-min intervals as described in the text and were plotted as a function of time after synchrony. (A) (○) cell number and (●) rate of DNA synthesis. (B) (○) rate of synthesis of *nrd*-mRNA, (●) rate of synthesis of *lac*-mRNA, (■) rate of synthesis of *fis*-mRNA, (△) rate of synthesis of *bla*-mRNA.

occurs only if protein synthesis occurs during the period of DNA synthesis inhibition (Hanke and Fuchs, 1984).

When a culture of *E. coli* is shifted up to a faster growth rate, protein synthesis and RNA synthesis immediately increase to a new rate whereas DNA synthesis continues at the previous rate for ~20 min before it rapidly increases and then levels off to a new steady level. The increased rate is due to increased initiation of DNA replication forks. RR expression also increases several fold transiently ~20 min after a shift-up and then decreases to a new steady level (Filpula and Fuchs, 1978).

The upstream regulatory region of the *nrd* operon has been dissected by deletion analysis (Tuggle and Fuchs, 1986, 1990). Removal of the positive site greatly lowers expression and prevents induction by DNA inhibition.

The regulation of ribonucleotide reductase synthesis reported here is similar to that found in eucaryotic organisms except that in eucaryotes the B1 and B2 protein equivalents are encoded by separate transcripts (Engstrom *et al.*, 1985, 1988; Fernandez-Sarabia and Fantes, 1990). Furthermore, the mouse has two different transcripts encoding the B2 equivalent protein (Engstrom and Rozell, 1988) whereas *Saccharomyces cerevisiae* has two different transcripts encoding the B1 equivalent (Andrews and Herskowitz, 1990). However, in each case at least one transcript for each subunit appears to be cell cycle regulated (Andrews and Herskowitz, 1990; Elledge and David, 1990).

The only procaryotic organism in which the synthesis of an enzyme involved in DNA replication has been shown to be synthesized in a cell-cycle dependent manner is *dnaC* in *Caulobacter crescentus* (Ohta *et al.*, 1990). *C. crescentus* is an unusual organism that divides asymmetrically to produce two different cell types, a motile swarmer cell and a nonmotile stalked cell. DNA synthesis in a swarmer cell is delayed ~60 min after division. During this period, a swarmer cell loses its flagellum and forms a stalk before DNA synthesis begins. This system was used to study the control of the synthesis of a gene (*dnaC*) that is required for DNA elongation. The gene encoding *dnaC* was found to be cell cycle regulated. Ten to fifteen percent of *C. crescentus* proteins examined were also found to be synthesized in a cell-cycle dependent manner (Milhausen and Newton, 1981). Many *C. cresentus* proteins are synthesized only during the portion of the cell cycle for which they are needed (Newton, 1989; Shapiro and Gober, 1989; Gober *et al.*, 1991). In contrast, in *E. coli* where the patterns of synthesis of 750 individual polypeptides were examined during cell cycle, no polypeptide with cell-cycle regulation was observed. Presumably, the polypeptides encoded by *nrdA* and *nrdB* were not detected in this experiment (Luthausen *et al.*, 1979).

We have previously investigated the ribonucleotide reductase regulation in a variety of bacteria including several that contain a very different enzyme that re-

quires B-12 to form an organic radical rather than tyrosine radical used by the *E. coli* type enzyme and found that all increased their ribonucleotide reductase when DNA synthesis was inhibited. We would predict that these bacteria all exhibit cell cycle regulation of ribonucleotide reductase and that DNA inhibition causes increased DNA initiation and increased ribonucleotide reductase synthesis.

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