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Expression of *Escherichia coli dnaA* and *mioC* Genes as a Function of Growth Rate

ANNE E. CHIARAMELLO AND JUDITH W. ZYSKIND*

Department of Biology and Molecular Biology Institute, San Diego State University, San Diego, California 92182

Received 21 December 1988/Accepted 2 May 1989

The synthesis of specific cellular components related to the initiation process of DNA replication was correlated with changes in growth rate. The concentrations of DnaA protein and *mioC* mRNA were determined for cells grown at six different growth rates; both increased relative to either total protein or total RNA, respectively, as the growth rate increased. Expression from the chromosomal *mioC* promoter, which contains a DnaA protein-binding site, was not repressed when the DnaA protein concentration was increased and was not derepressed in a *dnaA46* mutant at 42°C. The *mioC* transcript had a characteristic mRNA-type half-life of 1.51 min.

The bacterial cell regulates the rate of DNA synthesis by controlling the initiation frequency of chromosomal replication. Initiation is at a fixed site, *oriC*, located at 84 min on the *Escherichia coli* genetic map (Fig. 1) (for recent reviews, see the following: A. C. Leonard and C. E. Helmstetter, *in* E. W. Adolph, ed., *Chromosomes: Eukaryotic, Prokaryotic, and Viral*, vol. 2, in press; D. W. Smith and J. W. Zyskind, *in Chromosomes: Eukaryotic, Prokaryotic, and Viral*, vol. 2, in press; and J. W. Zyskind, *in* M. Riley and K. Drlica, ed., *The Bacterial Chromosome*, in press). Initiation from *oriC* is thought to occur when a certain cell mass is reached called the initiation mass, which is the cell mass per origin at the time of initiation (18). Because the protein/origin ratio remains constant at most growth rates (8), an assumption is that the major macromolecular synthesis contributing to the initiation mass is protein synthesis. One candidate for such a protein is the DnaA protein, because it is positively required for initiation of DNA replication at *oriC* in *E. coli* cells (27, 30) as well as in crude (23) and purified (41) enzyme systems. A primary question we address here is whether the ratio of DnaA protein to total protein remains unchanged with respect to growth rate. An invariant ratio would suggest that DnaA protein could be the contributing factor in the initiation mass. Rather than being constant, this ratio varied directly with growth rate.

An RNA polymerase synthetic event appears to be required for initiation to occur (Zyskind, in press), and the single transcription event that to date has been linked to initiation function originates from the *mioC* promoter. Many modifications of *oriC* minichromosomes that involve deletions, insertions, or single-base mutations in the *mioC* promoter region cause a decrease in copy number and increase in instability, properties that are the result of a decrease in initiation frequency (17, 34, 55). Transcripts that enter *oriC* in the counterclockwise direction (Fig. 1) originate mainly from this promoter, as demonstrated in this study and by others (25a). The 3′ ends of *mioC* transcripts are located at and near the RNA-DNA transitions that most likely result from priming of the leading strand (31, 45, 46, 48).

The *mioC* promoter is stringently controlled (45, 46). Most stringently controlled promoters are also under growth rate control, and the same nucleotide sequence in rRNA operon promoters appears to be responsible for both stringent control and growth-rate-dependent regulation (13). We have examined here whether the *mioC* promoter is growth rate regulated and whether transcripts originating only from the *mioC* promoter enter the origin or whether transcripts from the *asnC* promoter also reach *oriC*. Our approach was to use S1 nuclease protection by in vivo-generated transcripts of a probe that contained the 5′ ends of both transcripts so that the intracellular concentration of each transcript in cells grown at different growth rates as well as relative to each other could be measured by densitometric analysis. Using total RNA isolated from *E. coli* cells grown at six different growth rates, we determined that most transcription reaching *oriC* originates from the *mioC* promoter and that the *mioC* transcript is growth rate regulated. The stability of the *mioC* transcript was examined and found to have a half-life of 1.51 min.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *E. coli* K-12 strain used for the growth rate regulation studies, EMG2 F^+ λ^+, was provided by B. Bachmann. Strains EC559 F^- *argE3 his-4 leu-6 proA2 thr-1 ara-14 galK2 lacY1 thi-1 supE44 rpsL31*, EC558 (EC559 containing the temperature-sensitive mutation *dnaA46*; 29), and LR59 (EC559 containing two plasmids, F′ *proA*$^+$*B*$^+$ *lacI*^{q} *lacZ*ΔM15 and pLAR13) were used to determine the effect of DnaA protein on the *mioC* promoter. Plasmid pLAR13 (46) contains the *dnaA* gene under control of the *tac* promoter. Plasmid pAC3 contains the *mioC* promoter on the *Alu*I fragment from *E. coli oriC* coordinates +1018 to +660 inserted into the *Sma*I site of pLAR5 (29) in an orientation such that transcription from the *mioC* promoter enters the *galK* gene. Plasmids pAC1 (29) and pLAR7 (46) were used as sources of DNA for probes.

Media and growth conditions. In the studies of growth rate regulation, medium supplementation was used to modify the rate of growth. An overnight culture of strain EMG2 was grown in M9 minimal medium (38) containing 0.4% glycerol. This culture was diluted to an optical density at 450 nm (OD_{450}) of 0.025 into M9 medium supplemented with one of the following: 0.4% glycerol (doubling time [τ], 109 min), 0.4% glucose (τ, 75 min), 0.4% glucose–0.015% Casamino Acids–0.015% yeast extract (τ, 58 min), 0.4% glucose–0.05% Casamino Acids–0.05% yeast extract (τ, 45 min), 0.4% glucose–0.09% Casamino Acids–0.09% yeast extract (τ, 38 min), or 0.4% glucose–0.2% Casamino Acids–0.2% yeast

* Corresponding author.

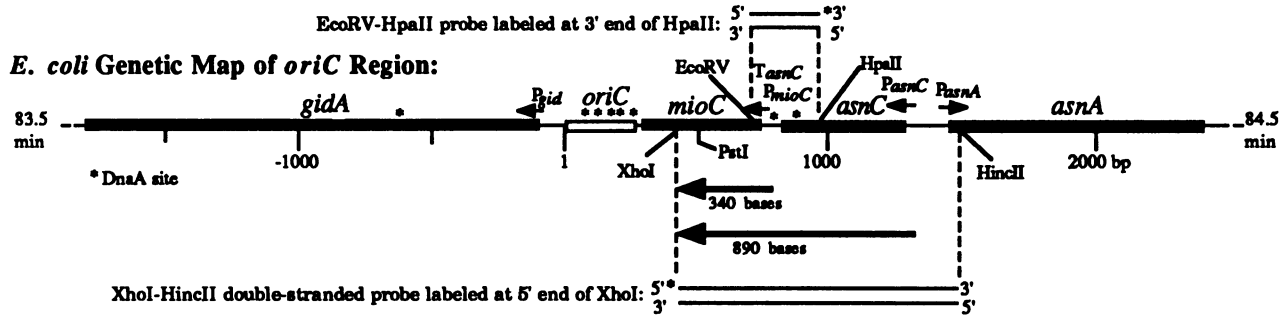

FIG. 1. *E. coli* replication origin region and probes. Assignments of protein-coding regions (■) are based on maxicell analysis of λ transducing phage (28) and nucleotide sequence data (10, 56). The size of the minimal origin (□) is based on deletion data (42a).

extract (τ, 30 min). The cells were grown in the six different media to an OD_{450} of 0.41 to 0.48, quickly chilled on ice, and then centrifuged. The number of cells per milliliter was determined with a Petroff-Hauser counter, total RNA was extracted, cells were treated with trichloroacetic acid for immunoblot analysis, and RNA and protein concentrations were determined. Cell mass per milliliter of culture was determined at OD_{450} with a 1-cm light path. For other experiments, cells were grown in M9 minimal medium supplemented unless indicated otherwise with 0.2% glucose, 0.1% Casamino Acids, and 40 μg of the required amino acids per ml. The antibiotics ampicillin and chloramphenicol were added at 50 μg/ml for plasmid selection where necessary.

Enzymes, DnaA protein, and anti-DnaA protein antibody. Restriction endonucleases were obtained from New England BioLabs, Pharmacia Fine Chemicals, Boehringer Mannheim Biochemicals, or Bethesda Research Laboratories, Inc. T4 DNA ligase and S1 nuclease were obtained from Bethesda Research Laboratories, avian reverse transcriptase was from Dupont, NEN Research Products, DNA polymerase I large fragment and calf intestinal alkaline phosphatase were from Boehringer Mannheim, and T4 polynucleotide kinase was from Pharmacia. For these enzymes, the reaction conditions recommended by the manufacturers were used. DnaA protein was generously provided by Arthur Kornberg, Stanford University, and anti-DnaA protein antibody was kindly provided by Judith Campbell, California Institute of Technology.

Isolation of plasmid DNA. Plasmid DNA was extracted by the cleared-lysate method (14) and isolated with two successive isopycnic ethidium bromide-cesium chloride gradient centrifugations. For routine examination of plasmid DNA, the alkaline lysis method (5) was used.

Protein determinations. To determine the concentration of protein in cells, 2 ml of cells was centrifuged and washed three times with M9 salts (38). The cells were suspended in 100 μl of 1 N NaOH, and total proteins were determined by using the Pierce BCA protein assay reagent (53). The conditions were as described by the manufacturer (Pierce Chemical Co.). Bovine serum albumin, fraction V (Boehringer Mannheim), was used as a standard, and 1 μg of protein was assumed to be equivalent to 5.6×10^{15} amino acid residues.

Immunoblot analysis of DnaA protein. A 2-ml sample of cells (OD_{450}, 0.41 to 0.48) was centrifuged, suspended in 0.5 ml of cold 10% trichloroacetic acid, and then pelleted. The protein pellets and purified DnaA protein were mixed with 50 μl of loading buffer (4% sodium dodecyl sulfate, 2% 2-mercaptoethanol, 0.2 M Tris base, 20% [vol/vol] glycerol) and heated for 10 min at 95°C. The samples were loaded onto a 10% sodium dodecyl sulfate–polyacrylamide gel, and electrophoresis was continued until the bromophenol blue dye had migrated 10.5 cm. The proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell Co.) with an ABN polyblot apparatus as recommended by the manufacturer. Prestained molecular weight markers (Sigma Chemical Co.) were included so that transfer and retention could be followed. In all cases, the prestained markers transferred completely to the nitrocellulose during electrophoresis. Transfer was also confirmed by staining the gel after transfer with Coomassie brilliant blue R-250 (Bethesda Research Laboratories). After transfer, the membrane was incubated with polyclonal anti-DnaA protein antibody overnight at 4°C. The filter was washed four times with a phosphate buffer (0.02 M KPO_4 [pH 7.5], 0.5 M NaCl, 0.05% Tween-20 [Bio-Rad Laboratories]) and incubated with ^{125}I-labeled protein A (Amersham Corp., 30 mCi/mg) for 1 h at room temperature followed by several washes of the phosphate buffer. The filter was exposed to Kodak XRP-1 film with a Cronex Lightning-Plus screen at −70°C for different periods of time. Autoradiograms with low but readable exposures were scanned with an LKB Ultrascan XL laser densitometer, and the area of peaks was integrated by using the LKB Gelscan XL software package.

Cellular concentration of RNA. The intracellular RNA concentration was determined as described previously (9). The OD_{260} of the sample was measured, and the values were converted to nucleotides by assuming that 1 OD_{260} unit of RNA at pH 2 corresponds to 5.4×10^{16} ribonucleotides (8).

Isolation and purification of total RNA. Total RNA was isolated from cells after lysis in hot (90°C) 1% sodium dodecyl sulfate and purified by pelleting through CsCl as described previously (20), except that, after precipitation with ethanol, the pellet was suspended in 0.2 mM EDTA and 2 volumes of 100% ethanol. The samples were stored at −70°C, and the concentration of RNA in the samples was measured by OD_{260}.

Probes. In the isolation of the *Xho*I-*Hin*cII probe (coordinates +418 to +1497, Fig. 1), 10 pmol of pLAR7 was digested with *Xho*I. The 5′ ends were dephosphorylated with calf intestinal alkaline phosphatase. After heat denaturation at 68°C followed by phenol-chloroform extraction, the DNA was precipitated with ethanol. The *Xho*I fragment 5′ ends were labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP (5000 Ci/mmol; Amersham). After phenol extraction and ethanol precipitation, the DNA was digested with a second-restriction enzyme, *Hin*cII. The fragment containing the 1,070-nucleotide labeled probe was separated on a 1% agarose gel and then moved by electrophoresis onto an NA45 DEAE membrane (pore size, 0.45 μm; Schleicher & Schuell). The fragment was eluted from the membrane in a

high-salt buffer containing 1.0 M NaCl–0.1 mM EDTA–20 mM Tris chloride (pH 8.0) by incubation at 68°C for 2 h (19).

The *Eco*RV-*Hpa*II probe (coordinates +726 to +977, Fig. 1) was isolated after digesting 10 pmol of pAC1 with *Hpa*II and then labeling the *Hpa*II fragment 3′ ends with the large fragment of DNA polymerase I and with [α-^{32}P]dCTP and [α-^{32}P]dGTP (both at 3,000 Ci/mmol; Amersham). After phenol extraction and ethanol precipitation followed by digestion with *Eco*RV, the fragment containing the 252-nucleotide probe was separated by electrophoresis from the other fragments on an 8% polyacrylamide gel, electroeluted into 0.5 M TBE (36) buffer for 18 h, and purified with an Elutip-d column (Schleicher & Schuell).

S1 nuclease protection. Hybridizations were performed as described previously (1) in 100% formamide at 48°C for 6 h. The total concentration of RNA in each hybridization reaction was brought to 150 mg with yeast tRNA (Bethesda Research Laboratories) when the amount of sample RNA was less. The conditions of hybridization were chosen to optimize RNA-DNA hybridization, and the choice of hybridization temperature was determined from the G+C content of the probe (12). The hybrids were treated with 150 U of S1 nuclease at 37°C for 30 min, followed by phenol-chloroform extraction and ethanol precipitation. The pellets were suspended in distilled water and lyophilized overnight. The pellets were dissolved in loading buffer (8 M urea, 0.1% xylene cyanole), denatured at 100°C for 2 min, and loaded onto either 8 or 10% polyacrylamide gels containing 8 M urea in 0.8 × TBE. After electrophoresis, the gels were exposed to AGFA-Curix RP2 film with a Cronex Lightning-Plus screen at −70°C for different periods of time. Densitometric measurements were performed on autoradiograms with low but readable exposures. For quantitative analysis of transcripts, dilutions of the probe were included, and the intensity of the band for different probe dilutions was related to the number of probe molecules in each dilution. Using this information, the number of protected probe fragments in a band could be determined from the band intensity. A one-base correction was taken into consideration when determining transcript length from Maxam and Gilbert (36) sequencing reactions.

Primer extension. The *Xho*I-*Pst*I primer was isolated after digesting 10 pmol of pLAR7 with *Xho*I. The 5′ ends of the fragments were dephosphorylated with calf intestinal alkaline phosphatase and then labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP (5,000 Ci/mmol; Amersham). After phenol extraction and ethanol precipitation, the DNA was digested with *Pst*I. The fragment containing the 75-nucleotide primer (coordinates +418 to +492) was separated on an 8% polyacrylamide gel and isolated from the acrylamide by treatment of the gel slice with 5 ml of 500 mM ammonium acetate–1 mM EDTA–0.1% sodium dodecyl sulfate–10 μg of yeast tRNA per ml. After incubation overnight at 40°C, polyacrylamide was removed by centrifugation, and the fragment was precipitated with ethanol.

The *Xho*I-*Pst*I primer was hybridized to 150 μg of cellular RNA as described above for S1 nuclease protection and then precipitated with ethanol and dissolved in 20 μl of 0.05 M Tris chloride (pH 8.0)–0.01 M $MgCl_2$–0.06 M NaCl–5 mM dithiothreitol–1.5 mM each dATP, dCTP, dGTP, and TTP. The primer was extended with 10 U of avian myeloblastosis virus reverse transcriptase at 37°C for 90 min. The products of the reaction were separated by electrophoresis on a denaturing 8% polyacrylamide gel and exposed to film as described above for S1 nuclease protection.

Determination of *mioC* transcript stability. Rifampin (Sigma) was added at a concentration of 0.2 mg/ml to a culture (OD_{450}, 0.35) of strain EMG2 growing exponentially at 37°C with doubling time of 30 min. At selected time intervals, 250-ml samples were withdrawn from the culture and rapidly chilled by pouring into 100 ml of crushed ice. The cells were centrifuged and then lysed with hot (90°C) 1% sodium dodecyl sulfate and RNA was purified as described previously (19). S1 nuclease protection analysis was used to determine the amount of transcript present (37). The rate of decay was calculated by the method of least squares of the percentage of RNA remaining versus time.

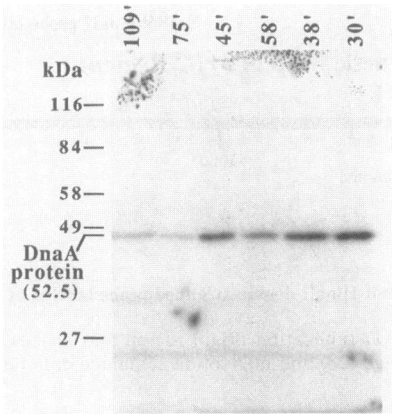

FIG. 2. Quantitative determination of DnaA protein in cells growing with different growth rates. Cells (2 ml) growing at six different doubling times (indicated in minutes above each lane) were pelleted and treated as described in Materials and Methods. The amounts of protein in samples were 77.2 μg (109 min), 78.1 μg (75 min), 67.5 μg (58 min), 67.1 μg (45 min), 68.9 μg (38 min), and 69.6 μg (30 min). The samples were loaded onto a 10% sodium dodecyl sulfate–polyacrylamide gel along with known amounts of DnaA protein and prestained molecular weight markers (Sigma). Samples were run until the bromophenol blue reached 10.5 cm. The proteins were transferred to a nitrocellulose membrane and incubated with anti-DnaA protein antibody and ^{125}I-labeled protein A. The membrane was exposed to film for 14 h at −70°C. Note that the samples corresponding to 45- and 58-min doubling times were loaded out of sequence. The gels in Fig. 2 and Fig. 5 were of the same size, run simultaneously, and exposed to the same film in the same cassette. The DnaA protein standards shown in Fig. 5 were used to determine the concentration of DnaA protein in the samples in the gel of Fig. 2 at an exposure time of 14 h. The molecular weight of DnaA protein is 52,551.

RESULTS

DnaA protein is growth rate regulated. The growth rate of strain EMG2, an original K-12 wild-type strain, was manipulated by varying the source and concentration of carbon and energy as described in Materials and Methods. Exponentially growing cells were isolated, and a variety of cell parameters were measured, including cell number, cell mass, and concentrations of protein and RNA. The amount of DnaA protein present in these cells was determined by immunoblot analysis with anti-DnaA antibody (Fig. 2). Note that the samples corresponding to 45- and 58-min doubling times were loaded out of sequence. The amount of DnaA protein increased with respect to protein as the number of doublings per hour (μ) increased (Table 1, Fig. 3).

DnaA protein and the *mioC* promoter. To examine the effect of the intracellular concentration of DnaA protein on the *mioC* promoter while residing in the chromosome, we

TABLE 1. Concentration of DnaA protein at different growth rates[a]

τ (doubling time, min)	μ (doublings/h)	Cells/mass (10^8 cells/OD_{450})	Mass (10^{-10} OD_{450})/cell	DnaA monomers/cell[b]	Protein		% DnaA protein of total protein
					Total (μg/2 ml)	DnaA[b] (ng/2 ml)	
109	0.55	12.89	7.76	74	77.23	7	0.009
75	0.80	5.83	17.15	144	78.13	6	0.008
58	0.97	5.10	19.61	234	67.52	10	0.015
45	1.33	3.72	26.88	579	67.12	16	0.024
38	1.58	3.17	31.55	803	68.92	20	0.029
30	2.00	2.27	44.05	1,319	69.64	24	0.034

[a] See Materials and Methods for medium supplementation to vary growth rate.
[b] The concentration of DnaA protein in the cell culture and the number of DnaA monomers per cell were estimated by comparing the density of bands in the immunoblot shown in Fig. 2 with band densities of dilutions of purified DnaA protein shown in Fig. 6 but at the same exposure time.

isolated total RNA from the DnaA-overproducing strain LR59, with and without induction by isopropyl-β-D-thiogalactopyranoside, and from the isogenic strain EC559. RNA was also extracted from an isogenic *dnaA46* temperature-sensitive mutant, EC558, grown at the permissive temperature to an OD_{450} of 0.3 and then shifted to 42°C for either 15 min or 1 h. The amount of *mioC* mRNA was assayed by two methods, S1 nuclease protection and primer extension (Fig. 4). The high concentration of DnaA protein in LR59 exposed to IPTG led to the elimination of *mioC* promoter activity on the plasmid as assayed by galactokinase (Table 2), but the *mioC* transcript originating from the chromosomally located *mioC* promoter was still present, although at reduced levels (Table 2, Fig. 4). When the expression of the chromosomally derived *mioC* transcript was examined by S1 protection in the DnaA-overproducing strain LR59, only a 50% decrease was observed even though the DnaA protein concentration had increased by 173-fold over the amount obtained with EC559 (Fig. 4 and 5, Table 2). The lowered amount of available RNA polymerase molecules caused by the induction of the *tac* promoter may be partly responsible for this decrease. A greater effect of DnaA protein was seen when the amount of *mioC* mRNA was assayed by primer extension (Fig. 4B). Because reverse transcriptase is not highly processive, this method probably underestimates the amount of transcript present. The concentration of DnaA protein was also increased 25-fold above wild-type levels in the uninduced cells containing pLAR13, which has the *dnaA* gene under control of the *tac* promoter (Fig. 5, Table 2). The increased concentration of DnaA protein in this strain in the absence of IPTG eliminated the activity of the *mioC* promoter when measured in the *galK* transcription fusion (pAC3; Table 2) but did not inhibit the activity of the *mioC* promoter located in the chromosome as measured by S1 protection; only a 50% reduction was obtained when assayed by primer extension (Table 2).

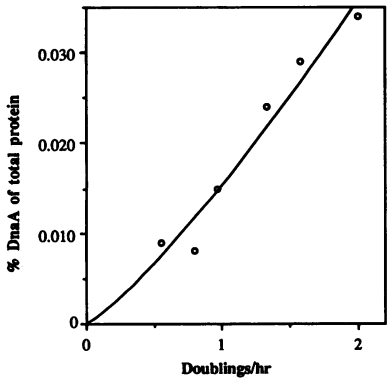

FIG. 3. DnaA protein concentration relative to total protein concentration.

A similar difference was observed between the activity of the *mioC* promoter on the chromosome and on the plasmid in the temperature-sensitive *dnaA46* mutant EC558 (Table 2). Strains containing the *dnaA46* mutation appear to be derepressed for expression from the *dnaA* promoter at 42°C (2). This mutant contained an increased amount of DnaA protein at 30°C as well as at 42°C, suggesting that the mutant DnaA protein is not as effective at repressing *dnaA* promoter activity as wild-type DnaA protein (Fig. 5, Table 2). Increased DnaA protein instability at 42°C may account for the very slight increase over the amount of DnaA protein seen at 30°C. When assayed with transcription fusions, the *mioC* promoter is stimulated in a *dnaA46* mutant at 42°C (34, 35) (Table 2); but when the concentration of the *mioC* transcript originating from the chromosomally located *mioC* promoter was examined by S1 protection, a reduction in the amount of mRNA was observed with the *dnaA46* mutant at the restriction temperature (46) (Fig. 4A, Table 2). This was confirmed by primer extension analysis (data not shown). The results described here of varying the DnaA protein concentration in vivo by two different conditions suggest that the activity of the *mioC* promoter is much less sensitive to changes in DnaA protein concentration when *mioC* is located in the chromosome than when it is on a plasmid, and the activity may even be stimulated by DnaA protein at certain concentrations.

Response of *mioC* promoter activity to changes in growth rate. The concentrations of transcripts originating from the *mioC* promoter and transcripts originating from the *asnC* promoter that pass through the *asnCt* terminator (see below) are determined by using S1 nuclease protection (4) of the *Xho*I-*Hin*cII probe, which includes the 5′ ends of both transcripts (Fig. 1). Expected lengths for transcripts originating from the *mioC* promoter (48) and the *asnC* promoter (32) are 340 and 890 ± 10 nucleotides, respectively. RNA was isolated from the same cultures used for the DnaA protein studies (Fig. 2, Table 1). Probe protection by *asnC* mRNA was very low to undetectable compared with protection by *mioC* mRNA, so very little *asnC* transcription appears to reach *oriC*. The intensity of each band was quantified by densitometric scanning of the autoradiogram. Serial dilutions of the probe were also run on a denaturing 8% polyacrylamide gel under the same conditions used in the S1 nuclease protection experiment, from which the number of molecules of probe protected by the RNA could be estimated (data not shown).

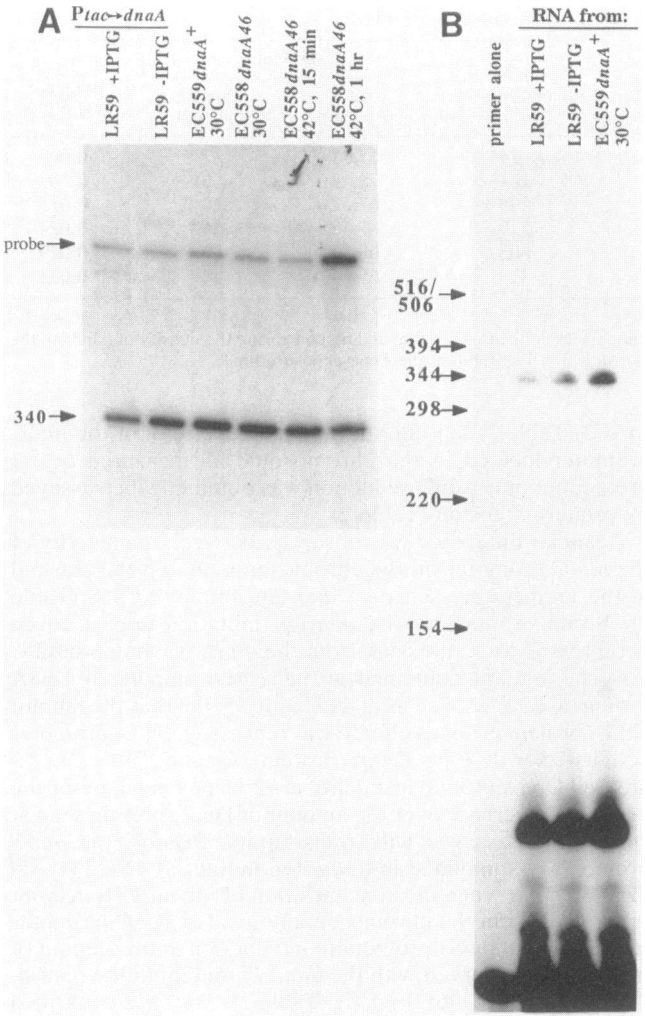

FIG. 4. Effect of DnaA protein on *mioC* promoter activity. (A) Abundance of the *mioC* transcript was determined by S1 nuclease protection of the *Xho*I-*Hin*cII probe. Total RNA was isolated from LR59 cells (containing pLAR13 with the *dnaA* gene under control of the *tac* promoter) grown for 1 h at 37°C in the presence or absence of IPTG. Total RNA was also isolated from the control strain, EC559, grown at 30°C and the *dnaA46* temperature-sensitive mutant, EC558, grown at 30°C, 42°C for 15 min, or 42°C for 1 h. All cells were grown to an OD_{450} of 0.4 before the addition of IPTG or the shift to 42°C. The probe (100 fmol, 1,200 cpm/fmol) was heat denatured, hybridized to 150 μg of total RNA, and treated with S1 nuclease as described in Materials and Methods. Half of the reaction products were loaded on an 8% polyacrylamide–8 M urea gel and run until the xylene cyanole dye reached 20 cm. The gel was exposed to film for 50 h. (B) Abundance of the *mioC* transcript was determined by primer extension analysis with the *Xho*I-*Pst*I primer. Total RNA (150 μg) isolated from the DnaA-overproducing cultures described above was hybridized with 100 fmol of primer (1,420 cpm/fmol) and treated with avian myeloblastosis virus reverse transcriptase. One-tenth of the primer along with half of the primer extension reactions and the 1-kilobase ladder (Bethesda Research Laboratories), labeled by filling in the ends with DNA polymerase I large fragment, were loaded on an 8% polyacrylamide–8 M urea gel. The samples were run until xylene cyanole dye reached 20 cm, and the gel was exposed to film for 24 h at −70°C.

The concentration of *mioC* transcripts as a function of growth rate is shown in Fig. 6 and Table 3. The ratio of *mioC* transcripts to total RNA increased with respect to the number of doublings per hour, suggesting that expression of the *mioC* promoter increases with growth rate in a manner similar to that for stable RNA promoters.

Stability of the *mioC* transcript. The stability of the *mioC* transcript was investigated by following the decay of *mioC* mRNA after rifampin treatment by using S1 nuclease protection analysis (4, 37). The S1 nuclease-resistant RNA-DNA hybrids were separated on a denaturing 8% polyacrylamide gel, and the resulting autoradiogram (Fig. 7) was scanned. A half-life of 1.51 min, similar to those of other mRNAs of *E. coli*, was estimated from the rate of decay, calculated by least-squares determination of the slope of the data.

Transcription termination of *asnC* transcription in the *mioC* promoter region. Little if any *asnC* transcription reaching the *Xho*I site at coordinate +418 (Fig. 1) was detected when the *mioC* transcript was analyzed. The possibility that *asnC* transcripts terminate between the reading frames of the *asnC* and *mioC* genes was investigated by using a 3′-labeled probe with S1 nuclease protection analysis of the 3′ ends of *asnC* mRNA. RNA was isolated from *E. coli* EC559 and hybridized to the *Eco*RV-*Hpa*II probe labeled at the *Hpa*II 3′ end (Fig. 1). The sizes of the protected fragments, determined on sequencing gels by comparing the resulting bands with probe products of the A+G and G chemical reactions, indicated that these transcripts terminate at five sites in and near the *mioC* promoter (Fig. 8, lane C). These sites were at nucleotides 819, 818, 779, 754, 738, and 732, with the nucleotide number representing the nucleotide at the 3′ end of the transcript. The relative strengths of these sites as determined by densitometric scanning of lane C in Fig. 8 were 738 > 732 > 818 and 819 > 779 > 754. Predominant termination events occur at position 738, with 47.6% of the transcripts terminating at this site. These termination sites have been designated termination region *asnCt*. The *mioC* transcription unit begins at nucleotide 757, so termination of *mioC* transcription may also occur at sites 754, 738, and 732. The level of *asnC* transcripts passing through *asnCt*, determined by comparing the amount of full-length probe protected by RNA to bands resulting from protection by transcripts terminating within *asnCt*, was 19%.

DISCUSSION

Five different patterns of response to increasing growth rates were observed when the abundance of 140 individual proteins was measured in cells grown in different media (44). The patterns observed for the amount of a specific protein per total protein as growth rate increased were linear decrease, no change, linear increase, and nonlinear responses. DnaA protein exhibited a linear increase (Table 1 and Fig. 2). Most proteins that have been identified to exhibit a linear increase, such as the ribosomal proteins, are involved in protein synthesis.

Mechanisms responsible for growth rate-dependent regulation of gene expression have been demonstrated to involve the control of promoter activity as well as to act posttranscriptionally. The growth rate-dependent regulation and stringent control of rRNA promoters appears to be regulated by the concentration of ppGpp, which varies inversely with growth rate (3) and is a growth rate-determining factor (47), although a feedback regulation mechanism may be involved as well (16).

The synthesis of ribosomal proteins is also growth rate dependent and under stringent control. Regulation, however, appears to be via a translational feedback mechanism that either operates posttranscriptionally (15) or regulates

TABLE 2. Effect of DnaA protein concentration on *mioC* promoter expression[a]

Strain	Treatment	% *mioC* mRNA		GalK U (pAC3, *mioCp→galK*)[d]	DnaA monomers/cell[e]	Fold increase of DnaA[f]
		S1 protection[b]	Primer extension[c]			
LR59(pLAR13)	1 mM IPTG	48	17	<5	28,957	173
LR59(pLAR13)	No IPTG	98	49	6	4,093	25
EC559	30°C	100	100	98	167	1
EC558 *dnaA46*	30°C	94	ND[g]	210	402	2.4
EC558 *dnaA46*	42°C, 15 min	73	ND	ND	649	3.9
EC558 *dnaA46*	42°C, 1 h	62	ND	353	574	3.4

[a] Cells were grown in M9 medium supplemented with 0.08% Casamino Acids, 0.4% glucose, and required amino acids. The doubling times were 65 min (EC559 at 30°C), 80 min (EC558 at 30°C), and 68 min (LR59 at 37°C, no IPTG). The same cell cultures were used to determine amounts of *mioC* transcripts (Fig. 4) and DnaA protein (Fig. 5).

[b] Values are comparisons of the band densities shown in Fig. 4A, which resulted from protection of the *Xho*I-*Hin*cII probe by the *mioC* transcript, to the band density of EC559.

[c] Values are comparisons of the band densities shown in Fig. 4B, which resulted from synthesis primed by the *Xho*I-*Pst*I primer hybridized to the *mioC* transcript, to the band density of EC559.

[d] GalK assays and units are as previously described (29). Similar results were obtained by others (34, 35).

[e] The number of DnaA monomers per cell was estimated by comparing the density of bands in the immunoblot shown in Fig. 5 with band densities of dilutions of purified DnaA protein.

[f] The number of DnaA monomers per cell for the *dnaA46* mutant and the DnaA protein-overproducing strain, LR59, is compared with that of EC559.

[g] ND, Not determined.

both transcription and translation (22). Control of another growth rate-regulated protein, 6-phosphogluconate dehydrogenase, also operates posttranscriptionally, and the regulation appears to involve a sequence within the structural gene (11). Another mechanism operating posttranscriptionally and affecting growth rate-dependent regulation involves transcript stability (39). For example, the stability of *ompA* mRNA is growth rate dependent, and action by site-specific endonucleases in the 5′-noncoding region of this mRNA may regulate the growth rate response (37). The growth rate-regulated expression of DnaA protein also may operate posttranscriptionally, because the specific activity of β-galactosidase in a translational fusion of the *dnaA* promoter and the first 22 amino acids of the *dnaA* gene to *lacZ* (7) remained constant except for cells grown in glycerol as the growth rate was changed (A. Wright, personal communication).

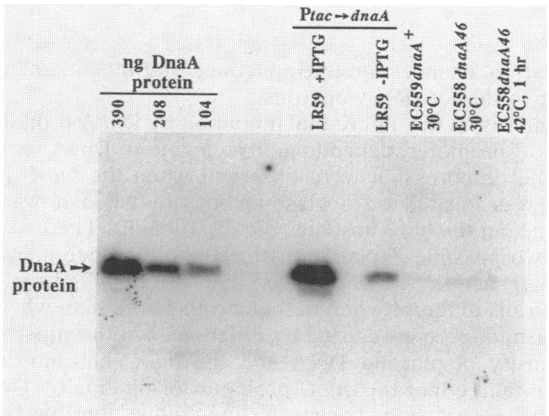

FIG. 5. Amount of DnaA in the DnaA-overproducing strain LR59 and the temperature-sensitive *dna46* mutant EC558. The DnaA protein content of the same cell cultures used for the experiment in Fig. 4 was determined by immunoblotting. Cells (2 ml) were pelleted, and proteins were separated by electrophoresis as described in Materials and Methods. The proteins were transferred to a nitrocellulose membrane and incubated with anti-DnaA protein antibody and ^{125}I-labeled protein A. The photograph is of film exposed for 5 h at −70°C.

DnaA protein is autoregulated, acting as a repressor of the two *dnaA* gene promoters (2, 7, 33, 40, 57). The concentration of an autoregulated protein has been predicted to be invariant with respect to growth rate (54). That DnaA protein

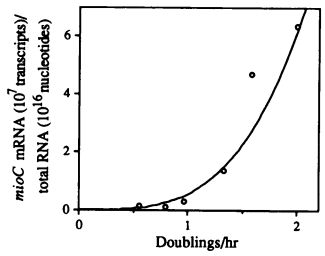

FIG. 6. Growth rate-dependent synthesis of the *mioC* transcript. The number of *mioC* transcripts was measured at different growth rates by S1 nuclease protection (Table 3). It is expressed here as a function of the abundance of total RNA.

TABLE 3. Concentration of *mioC* transcripts at different growth rates[a]

τ (doubling time, min)	μ (doublings/h)	RNA/mass (10^{16} nucleotides/OD_{450})	*mioC* transcripts/mass (10^7 molecules/OD_{450})	*mioC* transcripts/total RNA(10^7 transcripts/10^{16} nucleotides)
109	0.55	5.39	0.785	0.15
75	0.80	8.22	0.81	0.099
58	0.97	8.38	2.56	0.31
45	1.33	8.81	12.06	1.37
38	1.58	9.14	43.16	4.72
30	2.00	10.98	70.15	6.39

[a] Total RNA was isolated from *E. coli* EMG2 grown at six different growth rates. The *Xho*I-*Hin*cII probe (210 fmol, 17,390 cpm/fmol) was hybridized to RNA and then treated with S1 nuclease. The reaction products were loaded on an 8% polyacrylamide–8 M urea gel along with the 1-kilobase ladder (Bethesda Research Laboratories) labeled by filling in the ends with DNA polymerase I large fragment. The intensity of each band was quantified and compared with those of serial dilutions of the probe so that the intensities of the bands could be converted to number of probe molecules protected by RNA, which is equivalent to the number of *mioC* transcripts. Data are corrected for 95% recovery, which is based on the amount of probe that was recovered when the reaction did not include S1 nuclease.

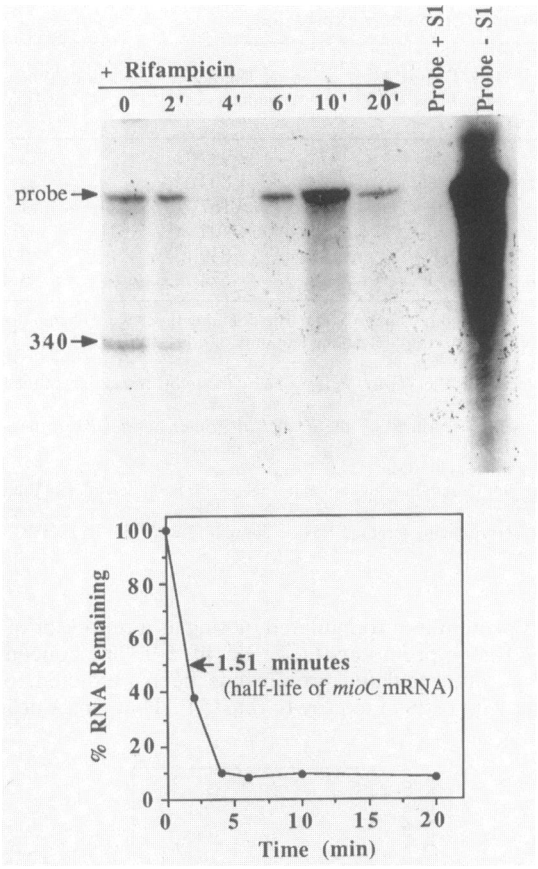

FIG. 7. Half-life of the *mioC* transcript. Total RNA from *E. coli* EMG2 grown with a doubling time of 30 min was isolated at the indicated time intervals after rifampin addition, and 150 μg of RNA was hybridized with the 5′-end-labeled *Xho*I-*Hin*cII probe (210 fmol, 17,390 cpm/fmol). The samples were treated with S1 nuclease and separated by polyacrylamide gel electrophoresis. The size of the *mioC* transcript is indicated on the left. The lane labeled probe + S1 represents the probe hybridized with 150 μg of yeast tRNA and digested with S1 nuclease, whereas the lane labeled probe − S1 contains the probe alone. Beneath the autoradiogram is a plot of percent of RNA remaining versus time after the addition of rifampin.

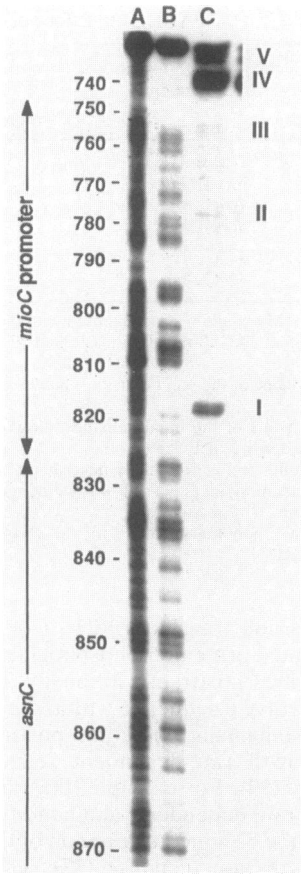

FIG. 8. S1 nuclease protection by transcripts originating from the *asnC* promoter. Lanes A and B contain the A+G and G (47) chemical reaction products of the probe, respectively. Lane C contains S1 nuclease digestion of the *Eco*RV-*Hpa*II probe labeled at the 3′ end of *Hpa*II (75 fmol, 1,733 cpm/fmol) and hybridized to 150 μg of RNA isolated from exponentially growing EC559 cells. Half of the reactions were run on 10% polyacrylamide–8 M urea gel until the xylene cyanole dye reached 45 cm. The gel was exposed to film for 3 days. Coordinates for *mioC* and *asnC* are shown to the left. Roman numerals indicate sites of termination in the *asnCt* terminator.

is growth rate regulated suggests that a control mechanism in addition to autorepression is affecting its expression. In the cell, DnaA protein exists in many forms, either bound to DNA at specific sequences (24); bound to other DnaA molecules (23); bound in a complex of 20 to 40 DnaA monomers with *oriC* (25); bound to ATP or ADP, both with high affinity (49); or bound to phospholipids in the membrane (50, 59). These different forms either are unavailable for repression or may have different binding affinities for the *dnaA* promoter, which may explain the seeming contradiction between autoregulation and growth rate regulation of *dnaA* gene expression.

The *mioC* promoter, the expression of which is required for efficient *oriC* activity (17, 34, 55), is shown here to be growth rate regulated (Fig. 6, Table 3). This was expected, because we have previously shown that this promoter is stringently controlled and inhibited by ppGpp (45, 46), and stringently controlled promoters are also growth rate regulated (13). In *Bacillus subtilis*, induction of the stringent response leads to an inhibition of chromosomal replication (51); indications are that this is also true for *E. coli* (26, 51), so a component of the initiation machinery appears to respond to changes in ppGpp concentrations in manner similar to that of rRNA operons.

Of interest is the differential inhibition by DnaA protein of the *mioC* promoter, depending on its location; DnaA repression and derepression were observed when the *mioC* promoter was located on a plasmid but not when it was in residence in the chromosome (Fig. 4, Table 2). There are at least two possible explanations for this difference in *mioC* promoter activity. The first is that the *mioC* promoter structure is different when in the chromosome than when in the plasmid, perhaps caused by differences in the superhelical density of plasmid DNA and the *mioC* chromosomal domain, and either the *mioC* promoter or binding by DnaA protein is sensitive to changes in supercoiling. Plasmid DNA may be more negatively supercoiled than chromosomal DNA (52). Also, DnaA protein inhibition of *mioC* promoter activity in a supercoiled template can be overcome by increasing the RNA polymerase concentration (40), which may be the result of local changes in the superhelical density of the template caused by increased transcription from other promoters on the template (58). The second explanation is that the promoter on the chromosome is sequestered in some

way, perhaps by insertion into the membrane. The origin may be associated with the membrane, especially during initiation (42, 59); *mioC* is contiguous with *oriC* (Fig. 1). Repression by DnaA protein may be cell cycle dependent, such that the *mioC* promoter is most sensitive to repression just after initiation, thereby contributing to an eclipse period proposed to result from hemimethylation (42).

In this study, both the amounts of the DnaA protein and the *mioC* transcript relative to total protein and total RNA, respectively, have been shown to be directly proportional to growth rate. The importance of these observations is that the mechanisms by which these concentrations are growth rate regulated may contribute to the coupling of initiation of DNA replication to growth rate and protein synthesis. A critical concentration in the cell of DnaA · ATP is probably required for the origin to acquire the correct number of DnaA molecules so that strand opening and loading of DnaB protein can occur (6). Because the concentration of ATP in the cell appears to be constant at different growth rates (21), this critical concentration of DnaA · ATP may be close to invariant with growth rate.

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