Escherichia coli Fis and DnaA Proteins Bind Specifically to the *nrd* Promoter Region and Affect Expression of an *nrd-lac* Fusion

LANCE B. AUGUSTIN, BLAKE A. JACOBSON, AND JAMES A. FUCHS*

Department of Biochemistry, University of Minnesota, St. Paul, Minnesota 55108

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The *Escherichia coli* *nrd* operon contains the genes encoding the two subunits of ribonucleoside diphosphate reductase. The regulation of the *nrd* operon has been observed to be very complex. The specific binding of two proteins to the *nrd* regulatory region and expression of mutant *nrd-lac* fusions that do not bind these proteins are described. A partially purified protein from an *E. coli* cell extract was previously shown to bind to the promoter region and to regulate transcription of the *nrd* operon (C. K. Tuggle and J. A. Fuchs, J. Bacteriol. 172:1711–1718, 1990). We have purified this protein to homogeneity by affinity chromatography and identified it as the *E. coli* factor for inversion stimulation (Fis). Cu-phenanthroline footprinting experiments showed that Fis binds to a site centered 156 bp upstream of the start of *nrd* transcription. Mutants with deletion and site-directed mutations that do not bind Fis at this site have two- to threefold-lower expression of an *nrd-lac* fusion. The previously reported negative regulatory nature of this site (C. K. Tuggle and J. A. Fuchs, J. Bacteriol. 172:1711–1718, 1990) was found to be due to a change in polarity in the vectors used to construct promoter fusions. Two nine-base sequences with homology to the DnaA consensus binding sequence are located immediately upstream of the *nrd* putative –35 RNA polymerase binding site. Binding of DnaA to these sequences on DNA fragments containing the *nrd* promoter region was confirmed by in vitro Cu-phenanthroline footprinting. Footprinting experiments on fragments with each as well as both of the mutated 9-mers suggests cooperativity between the two sites in binding DnaA. Assay of in vivo expression from wild-type and DnaA box-mutated *nrd* promoter fragments fused to *lacZ* on single-copy plasmids indicates a positive effect of DnaA binding on expression of *nrd*.

The ribonucleoside diphosphate reductase (RDP reductase) (EC 1.17.4.1) of *Escherichia coli* catalyzes the reduction of ribonucleoside diphosphates to the corresponding deoxyribonucleoside diphosphates in the first reaction of the pathway specific to DNA synthesis in *E. coli* (43). Ribonucleoside diphosphate reductase consists of two nonidentical subunits, R1 and R2 (9), encoded by the *nrdA* and *nrdB* genes, respectively. These two genes are transcribed as a polycistronic message from the *nrd* operon (20), located at 48 min on the *E. coli* chromosome (15).

Growth conditions that result in a decreased DNA/mass ratio also result in increased levels of ribonucleoside diphosphate reductase (13). Ribonucleoside diphosphate reductase expression is regulated at the level of transcription (19). Protein synthesis is necessary for both the initial induction and the continued increase of *nrd* mRNA synthesis during DNA inhibition. These results suggest that a protein involved in positive regulation of *nrd* transcription may accumulate during DNA inhibition (21). Recently, *nrd* transcription was shown to correlate with the cyclic initiations of DNA replication in phosphate-synchronized *E. coli* cultures (41).

The *nrd* operon has been sequenced, including approximately 3 kb of upstream flanking sequence (10). The *nrd* promoter region was analyzed by Tuggle and Fuchs (46). A partially purified protein fraction isolated from thymine-starved cells showed increased binding to *nrd* promoter region DNA compared with the same fraction isolated from non-

starved cells. The protein extract from a thymine-starved culture protected several sites in the promoter region from DNase I digestion (47). Here, we report the identity of this protein as the *E. coli* factor for inversion stimulation (Fis).

Fis is an 11.2-kDa protein initially identified by its ability to stimulate Hin-, Gin-, and Cin-mediated DNA inversion (18, 23, 25). The involvement of Fis in other site-specific recombinations, including excision and integration recombination reactions of bacteriophage λ DNA, was also demonstrated (3, 4, 44). Fis has also been shown to be a transcriptional activator at rRNA and tRNA promoters in *E. coli* (6, 38). In vitro footprinting experiments show protection of specific sites in the *E. coli* *oriC* sequence by Fis (17), and examination of DNA synthesis in *fis* *gyrB* double mutants indicates a synergistic involvement of Fis and DNA gyrase in the initiation of DNA replication at *oriC* (14).

The *fis* gene has been cloned, sequenced, and mapped to 72 min on the *E. coli* chromosome (23, 26). The gene encodes an 11,239-Da, 98-amino-acid polypeptide. The *fis* operon promoter has been located 1 kb upstream of the *fis* start codon (36), and Fis synthesis is autoregulated. Transcription of *fis* is controlled as part of the stringent response. A degenerate consensus binding site for Fis has been proposed: (G/T)nnYRnn(A/T)nnYRnn(C/A) (where Y is a pyrimidine, R is a purine, and n is any nucleotide) (22). However, local DNA topology and the ability of the DNA duplex to bend, accommodating the helix-turn-helix DNA-binding domains of the Fis dimer, appear to be more important in determining Fis binding at any given site (51) than the sequence per se. In activating transcription of stable RNA operons in *E. coli*, binding of Fis to upstream activating sequences lowers the dissociation constant of the RNA polymerase-promoter complex (34). This

* Corresponding author. Mailing address: Department of Biochemistry, University of Minnesota, 140 Gortner Laboratory, 1479 Gortner Avenue, St. Paul, MN 55108-1022. Phone: (612) 624-1215. Fax: (612) 625-5780. Electronic mail: james-f@molobiol.cbs.umn.edu.

phenomenon is due at least in part to the effect of a Fis-induced DNA bend on the local geometry of the promoter region (49). Western blot (immunoblot) analysis has revealed a correlation between in vivo Fis concentration and increased growth rate (35). The concentration of Fis peaks in early log phase, declines through mid- to late log phase, and is undetectable in stationary-phase cells. A burst of Fis-dependent transcription of stable RNA operons upon reinitiated growth of stationary-phase cultures and nutritional shift-up to a faster growth rate was shown to correlate with a burst of Fis expression and led Bosch et al. (6) to speculate that de novo synthesis of Fis is a basic element of growth control in *E. coli*. Examination of Fis expression in nutrient upshift experiments demonstrated rapid transduction of growth medium quality to the control of Fis synthesis, leading Ball et al. (5) to speculate that Fis may communicate nutritional information to various cellular processes, including coupling of the cell cycle to the growth rate. Here, we present results indicating that Fis enhances the expression of an *nrd-lac* fusion through a specific binding site upstream of the *nrd* promoter.

Tuggle and Fuchs (48) noted that, immediately upstream of the -35 region in the *nrd* promoter, there are two 9-bp sequences with significant homology to a 9-bp sequence that occurs in the origin of DNA replication in a number of bacterial species. This sequence has since been shown to be the consensus binding sequence for the bacterial DNA replication initiation protein DnaA (16). We report here that DnaA binds to these two sites in the *nrd* promoter region and that mutations in these sites reduce the expression of an *nrd-lac* fusion gene.

The protein product of the bacterial *dnaA* gene is required for in vivo initiation of DNA replication at *oriC* in *E. coli* (45). A number of experimental observations have led to speculation that DnaA is involved in coupling DNA initiation to the *oriC*/mass ratio of a cell (2). However, this view of DnaA as the cell cycle oscillator is the subject of an ongoing debate in the literature (8). Here, we report that DnaA binds cooperatively to two sites adjacent to the *nrd* promoter. Cultures containing an *nrd-lac* fusion in which these sites are mutated so that DnaA no longer binds have decreased *lac* expression.

MATERIALS AND METHODS

Strains. Strains RZ211 [$\Delta(lac-pro)$ *ara recA56 srl str* (24)], RJ1561 (RZ211 *fis* [23]), N110 (*galK thyA recA* [46]), and BK99T (a thymine auxotroph generated in this study from BK99 *galK his ile* [$\Delta[pro lac]$] [27] by screening trimethoprim-resistant colonies for thymine auxotrophy) are derivatives of *E. coli* K-12 and were used in this study.

Plasmid constructions. pLBA3 and pLBA4 are pUC118 and pUC119 (50) derivatives, respectively, containing the *Bam*HI-*Eco*RI *nrd* promoter region fragment from -165 to $+85$ relative to the start of transcription (see Fig. 1) from pCKT229 (46). pLBANKE contains the mung bean nuclease blunt-ended *Kpn*I-*Eco*RI 1.6-kb *nrd* promoter region fragment from pPS2 (37) inserted into pUC118 (50) at the Klenow fragment-filled-in *Bam*HI and *Eco*RI sites. pLBAR229 was made by replacing the sequence in pJEL170 (31) from the unique *Eco*RI site to the *Cl*aI site at codon 280 of *lacZ* with three restriction fragments as follows: (i) the Klenow enzyme-filled-in *Bam*HI end of the -165 to $+85$ *Bam*HI-*Eco*RI *nrd* promoter fragment from pCKT229 was ligated to the mung bean nuclease blunt-ended *Eco*RI end of pJEL170; (ii) the *Eco*RI end of the $+85$ to $+518$ *Eco*RI-*Cl*aI *nrd* operon fragment from pPS2 was joined to the *Eco*RI end of the pCKT229 fragment; and (iii) the mung bean nuclease blunt-

ended *Bam*HI end of the 814-bp *Bam*HI-*Cl*aI DNA that encodes the N-terminal *lacZ* fragment from pMC1403 (11) was ligated to the Klenow enzyme-filled-in *Cl*aI end of the pPS2 fragment. The construct was closed by ligating the *Cl*aI overhang of the pMC1403 fragment to the *Cl*aI overhang of pJEL170. pLBAR2 was constructed in the same manner as pLBAR229 except that the 1.6-kbp *Bam*HI-*Eco*RI *nrd* promoter fragment from pLBANKE was used in place of the *nrd* promoter fragment from pCKT229.

pLBAR229T, pLBAR300T, pLBAR325T, and pLBAR307T were constructed by replacing the 770-bp fragment in pLBAR229 extending from the *Sca*I site in the *bla* gene upstream of the *nrd* promoter to the *Eco*RI site with the corresponding *bla* through *nrd Sca*I-*Eco*RI fragments from pCKT229, pCKT300, pCKT325, and pCKT307 (46), respectively. pKOTW8 was constructed by inserting the *Sal*I-*Eco*RI *nrd* promoter region fragment from pCKT8 (46) into *Sal*I- and *Eco*RI-digested pKOTWI (46). pKOTWI was constructed by partial *Eco*RI digestion of pKO-11 (1) and fill-in of the pKO-1 (33)-derived *Eco*RI site. pLEJ229, pLEJ325, and pLEJ307 were constructed by insertion of the *Eco*RI-*Nru*I *lacZ* fragment of the transcriptional *nrd-lac* fusion from pLBARET into *Eco*RI- and *Nru*I-digested pCKT229, pCKT325, and pCKT307, respectively. pLBARET contains an *nrd-lac* transcriptional fusion constructed by insertion of the *Bam*HI-*Eco*RI *nrd* promoter fragment from pCKT229 into *Bam*HI- and *Eco*RI-digested pLBA5. pLBA5 was constructed by replacing sequence in pJEL170 from the mung bean nuclease blunt-ended *Eco*RI site into the *lacZ* coding sequence at *Cl*aI with a 955-bp fragment from pRS550 (40) extending from the *Bam*HI site, filled in with Klenow enzyme, to the *Cl*aI site in the *lacZ* coding sequence. pBJ229 was constructed by replacing the *Bam*HI-*Eco*RI *nrd* promoter fragment in pCKT325 with the *Bam*HI-*Eco*RI *nrd* promoter fragment from pCKT229. pBJ325 was constructed by replacing the *Bam*HI-*Eco*RI *nrd* promoter fragment in pCKT229 with the *Bam*HI-*Eco*RI *nrd* promoter fragment from pCKT325.

Site-directed mutagenesis. Mutagenesis of specific nucleotides in the *nrd* promoter region was accomplished by the oligonucleotide-directed in vitro mutagenesis method of Kunkel et al. (28). Reactions were performed with the MutaGene in vitro mutagenesis kit (Bio-Rad) by the manufacturer's protocol. Cytidines were inserted at -165 and -158 by oligonucleotide-directed in vitro mutagenesis with the 21-mer oligonucleotide GCCCGTTCAACGAAATCGCCG. Plasmids pLBAR2UD, pLBAR2DD, and pLBAR2D2 are derivatives of pLBAR2 with mutations in the DnaA binding box consensus sequences located between -52 and -32 relative to the start of *nrd* transcription. The 22-mer oligonucleotide CTCCTGAGTTAACCACAAAGT was used to mutate the -50 upstream DnaA box (UD) by changing the T at -49 to A. The 20-mer oligonucleotide ATCCACAAACATATGCACCTT was used to mutate the downstream DnaA box (DD) by changing the G at -41 to C and the T at -40 to A. The 33-mer oligonucleotide CTCCTGAGTTAACGTCAAACAAATGCACTTGC was used to mutate both DnaA boxes (D2) by changing the T at -49 , C at -47 , A at -46 , G at -41 , T at -40 , and T at -39 to A, G, T, C, A, and A, respectively. After confirmation of the correct mutations in the plasmids by DNA sequence analysis, the *Bam*HI-*Eco*RI *nrd* promoter region fragments from the mutated plasmids were electroeluted from excised slices of agarose electrophoresis gels and used to replace the wild-type *Bam*HI-*Eco*RI *nrd* fragment in pLBAR2 or pLBAR229.

Affinity purification of an *nrd* promoter region-binding protein. A biotin end-labeled *nrd* promoter region DNA

FIG. 1. *nrd* promoter region. The sequence is numbered relative to the start of transcription. Deletion endpoints in various constructs used in this study are indicated by numbered arrows. The +85 *EcoRI* cleavage site used to construct promoter fusions is indicated by an arrow. The position of inserted cytidines resulting in elimination of the upstream Fis footprint are shown below the sequence at -158 and -164. The putative -35 and Pribnow RNA polymerase-binding sites and a 9-bp consensus DnaA-binding sequence centered at -48 are indicated by dashed boxes. Putative ribosome-binding sites, based on homology to the Shine-Dalgarno rRNA sequence, are overlined and labeled R.B.S. The start and stop codons for a 15-codon open reading frame located immediately upstream of the *nrdA* start codon are also indicated.

fragment was prepared as follows: 75 μ g of plasmid pLBA4 was digested with *Hinc*II and *Eco*RI; the *Eco*RI ends were filled in with dTTP and biotin-14-dATP by using Klenow enzyme; the resulting fragments were separated by agarose gel electrophoresis; and the 269-bp biotinylated *Hinc*II-*Eco*RI fragment containing the 254-bp *nrd* promoter region fragment was recovered by electroelution. A streptavidin-agarose column was prepared in a 1-ml pipette tip as follows. The tip was plugged with glass wool and wetted in binding buffer (20 mM Tris-Cl [pH 7.6], 50 mM KCl, 1 mM EDTA, 5 mM dithiothreitol, 12% glycerol). Then, 200 μ l of a 1:1 homogenous slurry of streptavidin-agarose beads and binding buffer was packed in the column. The column was washed sequentially twice with 300 μ l of binding buffer, then with 300 μ l of binding buffer containing bovine serum albumin (BSA, 0.5 mg/ml) and double-stranded poly(dI · dC) (0.2 mg/ml), then twice with 300 μ l of elution buffer (20 mM Tris-Cl [pH 7.0], 1 M KCl, 5 mM MgCl₂, 1 mM EDTA, 5 mM dithiothreitol, 12% glycerol), and finally twice with 300 μ l of binding buffer. The column was then loaded with four 162- μ l binding reaction mixes at a flow rate of approximately 10 μ l/min. Binding reaction mixes, containing 3 μ g of biotinylated DNA fragment, 15 mg of fractionated protein from a thymine-starved cell extract (fraction 57 [47]), and 30 μ g of double-stranded poly(dI · dC) per ml, were incubated in binding buffer at 37°C for 15 min. After the last binding reaction mix was loaded, the column was washed with 350 μ l of binding buffer. Protein was eluted from the column with elution buffer. One-drop fractions were collected and stored at -80°C.

N-terminal sequence determination. The N-terminal sequence of the 11-kDa polypeptide in fraction 57, identified by affinity purification on *nrd* promoter region DNA (see above), was determined by the method of Matsudaira (32) with the following modifications. Fraction 56 (50 μ g of protein) from thymine-starved cells (47) was precipitated with trichloroacetic acid, resuspended, and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on an 18% polyacrylamide gel. Proteins were transferred to an Immobilon polyvinylidene difluoride membrane (Millipore) with a Bio-Rad Trans-Blot SD semidry electrophoretic transfer cell by the manufacturer's protocol. The 11-kDa band was cut from the Coomassie blue-stained membrane, air dried, and stored at -20°C. The protein was sequenced on an automated sequencer by Ben Madden at the Protein Core Facility of the Mayo Clinic in Rochester, Minn.

Gel retardation. DNA fragments were prepared by restriction digestion of various plasmids, followed by agarose gel electrophoresis and electroelution of fragments from excised gel slices. Fragments were end labeled by being filled in with Klenow enzyme with appropriate α^{32} P-labeled and unlabeled deoxynucleotides. Binding reactions were performed by preincubating proteins for 15 min at 37°C in binding buffer (see individual experiments in the Results section). [32 P]DNA fragments or a deionized H₂O blank was added, and the reaction mixes were incubated at 37°C for 20 min. At the end of the 20-min incubation period, a 0.2 reaction volume of 5 $\times$ loading buffer (80% glycerol, 1 $\times$ binding buffer, 0.25% bromophenol blue) was added, and the reaction mixes were loaded on prerunning 6% acrylamide-bisacrylamide (29:1) gels buffered with TBE (5.4 g of Tris and 2.75 g of boric acid per liter with 5 mM EDTA). Gels were dried on Whatman 3MM paper and exposed to X-ray film for various amounts of time, depending on signal strength.

Cu-phenanthroline footprinting. Footprints of protein-DNA complexes, both in solution and on gel-retarded frag-

ments in situ, were obtained by the methods of Kuwabara and Sigman (29).

Preparation of nucleic acid-free S100 *fis*⁺ and *fis* mutant cell extracts. RZ211 and RJ1561 cells were grown in TB medium (42) at 37°C to an optical density at 595 nm (OD₅₉₅) of 0.7. Protein was fractionated from cells pelleted from 100 ml of culture by the procedure of Tuggle and Fuchs (47) through the 300 mM KCl elution from a DEAE column. Column fractions across the protein peak, with the OD₂₈₀ determined spectrophotometrically, were stored at -80°C.

Growth of cells. Unless otherwise indicated, cultures used for quantitation of β -galactosidase were started by inoculating TB medium with a single colony and incubating at 30°C. At an OD₅₉₅ of approximately 0.6, an aliquot of the culture was diluted to an OD₅₉₅ of 0.05 in TB medium supplemented with 10 mM NaHCO₃. At an OD₅₉₅ of 0.6, the cultures were again diluted to an OD₅₉₅ of 0.05 and harvested at an OD₅₉₅ of 0.6. Cultures containing pLEJ plasmids were incubated at 37°C. Cultures used for quantitation of galactokinase were grown as described above except that Davis-Mingioli minimal medium (12) supplemented with casein hydrolysate (0.05%), thymine (40 μ g/ml), deoxyadenosine (200 μ g/ml), vitamin B₁ (4 μ g/ml), glucose (0.2%), and ampicillin (35 μ g/ml) was used and incubations were done at 37°C.

Assay of β -galactosidase and galactokinase activity. For β -galactosidase assays, the production of 2-nitrophenol (ONP) from 2-nitrophenyl- β -D-galactopyranoside (ONPG) by cellular extracts was determined by measuring the change in A_{412} of reaction mixes in a kinetic microplate reader (Thermomax Microplate Reader; Molecular Devices). The extinction coefficient for a 1 M solution at 412 nm for 300 μ l of ONP in a 96-well flat-bottomed microtiter plate (Linbro Titertek; Flow Laboratory catalog no. 76-381-04) was determined to be 562 M⁻¹. This value was used to calculate the β -galactosidase activity in cellular extracts as nanomoles of ONP produced per minute. Culture aliquots were thawed on ice, sonicated for 10 s, and clarified by centrifugation at 14,000 $\times$ g for 5 min. Reactions were started by addition of 90 μ l of Z buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, 50 mM β -mercaptoethanol, 900 μ g of ONPG per ml) to 10 μ l of cell extract. The Δ OD₄₁₂ per minute was linear for at least 2 min in all reactions. The total protein concentration in each extract was determined by the method of Bradford (7) with a protein assay kit (Bio-Rad) by the manufacturer's microassay procedure. Plasmid copy numbers were determined by the method of Adams and Hatfield (1). Galactokinase assays were performed as described previously (46).

In thymine starvation experiments, cultures of *E. coli* BK99T containing the *nrd* promoter fusion constructs were grown at 30°C in supplemented Davis-Mingioli minimal medium (12) to an OD₅₉₅ of 0.5. The cultures were then swirled in an ice-water slurry to chill them quickly, pelleted at 4°C, washed twice in ice-cold unsupplemented Davis-Mingioli minimal medium, resuspended in supplemented Davis-Mingioli medium minus thymine, and grown at 30°C. Samples of the cultures were harvested at various time points, quickly chilled, and stored at -20°C.

RESULTS

Previous work on *nrd* regulation (46) was done with transcriptional fusions to the *galK* gene in the pKO vector system of McKenney et al. (33). The pKO plasmids are maintained in multiple copies, and the copy number of the pKO vector has been shown to be influenced by the strength of inserted promoters (1). A system based on a single-copy plasmid vector,

TABLE 1. Relative exponential-phase expression from upstream-deleted *nrd* promoter fusions

Plasmid	Relative activity ^a	Sp act ^b (SE)
pLBAR229	1.0	2,210 (91)
pLBAR229F	0.35	779 (57)
pLBAR229T	1.0 (1.0)	2,620 (224)
pLBAR300T	0.50 (1.0)	1,320 (61)
pLBAR325T	0.36 (5.0)	943 (28)
pLBAR307T	0.07 (1.0)	183 (14)
pLEJ229	1.0	57.9 (4.5)
pLEJ325	0.39	22.4 (0.9)
pLEJ307	0.06	3.7 (0.03)
pCKT229	1.0	2.25 (0.20)
pBJ325	0.65	1.50 (0.24)
pBJ229	1.0	17.4 (3.6)
pCKT325	0.53	9.3 (1.4)

^a Values are normalized to the activity of the 229 construct in each group. Values in parentheses are relative activities reported by Tuggle and Fuchs (46) for the corresponding pCKT constructs.

^b Values for pLBAR plasmids were determined in *E. coli* RZ211 and are reported as nanomoles of ONP produced per minute per milligram of protein. Values for pLEJ plasmids were determined in *E. coli* N110 and are reported as nanomoles of ONP produced per minute per femtomole of plasmid. Values for pCKT and pBJ plasmids were determined in *E. coli* N110 and are reported as nanomoles of galactose phosphorylated per minute per femtomole of plasmid. Values in parentheses represent the standard error of triplicate assays of triplicate cultures.

pJEL170 (31), was used for additional studies of the regulation of the *nrd* promoter to eliminate the possibility of titration of regulatory protein by the high copy number of the vector. The pJEL170 vector contains a promoterless *lac* operon that can be fused to a promoter to express β -galactosidase.

Some of the upstream deletions generated by Tuggle and Fuchs (46) were reconstructed in the pJEL170 system to express an *nrd-lac* translation or transcription fusion. Three classes of deletions upstream of the *nrd* promoter were reported by Tuggle and Fuchs (sites are numbered from start of transcription): (i) deletion up to -156 gave no change from the parental phenotype; (ii) deletions ending from -151 to -139 resulted in fivefold-increased *nrd* expression; and (iii) deletions from -122 to -104 resulted in parental-level expression in log-phase cultures but noninducibility by thymine starvation. Upstream *nrd* promoter region deletions from pCKT229 and pCKT300 (class 1), pCKT325 (class 2), and pCKT307 (class 3) were transferred into pJEL170-based translational fusions of codon 136 of *nrdA* to codon 8 of *lacZ*, creating the constructs pLBAR229T, pLBAR300T, pLBAR325T, and pLBAR307T, respectively. β -Galactosidase expression in log-phase RZ211 cultures harboring these constructs indicated results different from those reported by Tuggle and Fuchs (46) (Table 1). The DNA deleted in pLBAR300T reduced activity by 2-fold and the deletion in pLBAR325T reduced activity 2.4-fold compared with activity in pLBAR229T. This contradicts the previous report (46) that the deletion in pCKT300 had no effect and that the deletion in pCKT325 increases expression fivefold (Table 1). We obtained similar results using transcriptional fusion vectors (data not shown).

The deletion analysis of Tuggle and Fuchs (46) was performed with two different pKO-based vectors to simplify analysis of deletions. The vector used for all class 1 deletions

TABLE 2. Exponential-phase expression from downstream-deleted *nrd* promoter fusions

Plasmid	Relative activity	Sp act ^a (nmol/min/fmol)
pCKT8	1.0	2.13 (0.18)
pKOTW8	13.0	27.7 (1.19)
pCKT419	3.9	8.25 (0.33)

^a Values were determined in *E. coli* N110 and are reported as nanomoles of galactose phosphorylated per minute per femtomole of plasmid. Values in parentheses represent the standard error of triplicate assays of triplicate cultures.

and pCKT300 was pKO-9, whereas pKOTWI was used for class 2 and 3 deletions. Since the complete nucleotide sequence of pKO-9 and pKOTWI could not be ascertained from the literature, the unknown DNA sequence was determined. Stop codons in each reading frame were found to be dispersed over 100 nucleotides of the leader sequence. We have observed in vivo translation originating from the AUG start codon of a 15-codon open reading frame located at +67 to +111 in the mRNA leader sequence immediately upstream from the +110 *nrdA* start codon (2a). Translation of this open reading frame to different translation stops could lead to a polar effect on expression of downstream open reading frames.

To verify that the differences seen in the pKO system versus the pJEL system were due to polarity, the sequence downstream of the *EcoRI* site in pCKT deletion constructs representing each of the three upstream deletion classes was replaced with a DNA fragment containing a *lacZ* coding sequence that includes a single translational stop codon in the reading frame encoding *lacZ*. Relative expression from the *nrd* promoter in each of the three constructs (pLEJ229, pLEJ325 and pLEJ307) was very similar to that found in the pJEL-based plasmid and differed from the *galK* expression reported previously (46) (Table 1). Galactokinase assays of extracts of exponentially growing cultures of N110 gave results (Table 1, pCKT229 versus pBJ325 and pBJ229 versus pCKT325) very similar to those found for the pJEL system (pLBAR constructs) as well as the multicopy pKO vector containing an insert of the promoterless *lac* operon and having a constant region between the insert and the reporter gene (pLEJ constructs). These results indicate that a region deleted upstream of -139 has a two- to threefold effect on *nrd* expression and that a second region upstream of -122 has a further five- to sixfold positive effect.

Tuggle and Fuchs (46) reported a fourth class of *nrd* promoter region deletions revealing a putative negative regulatory site located approximately 80 bp downstream from the start of *nrd* transcription. However, in this experiment, a pKO-9 vector was compared with a pKOTW1 vector containing the deletion. When the downstream deletion, pCKTR419, is compared with the appropriate vector, pKOTW8, expression of galactokinase was more than threefold lower rather than increased, as found previously (Table 2). Although this result equivocates the assignment of a negative regulatory site downstream from the start of *nrd* transcription, it does suggest a regulatory role for sequence downstream from the start of *nrd* transcription.

Identification of a protein that binds to the *nrd* promoter as *Fig*. While the region deleted between -139 and -122 seems to play a major role in *nrd* regulation, it may be necessary to characterize all proteins that bind to the *nrd* regulatory region to understand its complex regulation. The protein previously reported to bind the *nrd* regulatory region (47) was purified to homogeneity by affinity chromatography. The starting material

FIG. 2. Gel retardation of *nrd* promoter region fragment 380f. Binding reaction mixes contained 21 mM Tris-Cl (pH 7.8), 205 mM KCl, 6 mM EDTA, 7.5 mM MgCl₂, 2 mM dithiothreitol, 100 μ g of bovine serum albumin per ml, 50 μ g of double-stranded poly(dI · dC) per ml, the protein to be assayed, and 0.15 μ g of ³²P-end-labeled 380f DNA per ml. First lane, no protein; second lane, 2.5 μ g of fraction 57 from thymine-starved *E. coli* N110 cell extracts (41) per ml; third lane, 0.33 μ g of pure Fis per ml. The fourth and fifth lanes contain 2 μ g of nucleic acid-free S100 extract from *E. coli* RZ211 (*fis*⁺) and RJ1561 (*fis* mutant) per ml, respectively.

was fraction 57 from a phosphocellulose column, which Tuggle and Fuchs (47) had found to be the most active fraction in DNA binding. The *Eco*RI overhang at the downstream end of a 254-bp *nrd* promoter region fragment (–165 to +89 relative to the start of transcription) was filled in with biotinylated dATP and mixed with the partially purified protein under the binding conditions developed by Tuggle and Fuchs (47). The binding reaction mix was passed through a streptavidin-agarose column. The column was washed extensively with binding buffer and eluted with high salt. Eluted fractions were analyzed by SDS-PAGE. The silver-stained gel revealed a single 11-kDa band in fractions 4 and 5, corresponding to an 11-kDa band present in a sample of the partially purified protein (data not shown). Samples of streptavidin-agarose column fractions 2 through 6 were assayed for their ability to retard the electrophoretic mobility of fragment 229f in a polyacrylamide gel. Mobility shifts identical to those seen across the previously purified activity peak suggested that the 11-kDa polypeptide is responsible for the binding activity observed in fraction 57 (data not shown).

To obtain a sufficient quantity of the protein for N-terminal sequence determination, a sample of fraction 57 was separated on SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and stained with Coomassie blue. The 11-kDa band was cut from the membrane and sequenced. A search of the GenBank protein sequence data base with the resulting 18-amino-acid N-terminal sequence MFEQVNSDVLTVSTVNS revealed exact homology to the N terminus of the *E. coli* protein Fis.

Identification of Fis as the binding activity observed by Tuggle and Fuchs (47) was further substantiated by comparison of the binding of fraction 57 (Fig. 2, lane 2), a homogenous sample of Fis purified in a way that did not depend on binding

to the *nrd* promoter and obtained as a kind gift from R. C. Johnson (University of California at Los Angeles) (Fig. 2, lane 3), and partially purified extracts from a *fis* mutant strain (Fig. 2, lane 5) and its parent (Fig. 2, lane 4) to the *nrd* promoter region. The electrophoretic mobility of the fragment of DNA containing the *nrd* promoter region sequence from –234 to +85 (fragment 380f [47]) gave identical gel retardation patterns whether bound by fraction 57, pure Fis, or the partially purified parental extract, while the extract from the *fis* mutant did not retard the fragment, indicating that Fis is the protein partially purified by Tuggle and Fuchs (47).

Comparison of the site-specific binding activities of fraction 57 and pure Fis on fragment 380f was done by gel retardation and Cu-phenanthroline footprinting analyses. After electrophoresis of binding reaction mixes containing either fraction 57 or pure Fis, gels were treated with Cu-phenanthroline, DNA fragments were eluted from excised bands, and footprints were obtained from autoradiograms of a denaturing urea-acrylamide electrophoresis gel. The binding conditions in these experiments permitted the excision and analysis of six retarded bands, with additional bands not being sufficiently abundant for analysis (data not shown). The binding of Fis and fraction 57 to fragment 380f was identical. The only site protected in all six retarded bands was at –142 to –160. This site corresponds to the site previously identified by deletion analysis as an upstream negative regulatory site bound by fraction 57 (47). Footprinting on the opposite strand protected –152 to –169 (data not shown). The center of the Fis consensus sequence is –156. A downstream footprint between +59 and +84 observed by Tuggle and Fuchs (47) with the partially purified protein fraction after DNase footprinting was not observed with pure Fis after Cu-phenanthroline footprinting in the current study; however, both fraction 57 and pure Fis protected a site from –10 to –28 on the second through sixth retarded bands (data not shown).

Mutated *nrd-lac* fusions with altered binding at the –156 site have decreased *nrd-lac* expression. To specifically determine the effect of Fis binding at the –156 site, the wild-type *nrd* sequence (–169)TATGCCGTTCAAGAAATCGCCG (–142) was mutated to TATGCCGTTCAAcGAAATCGCCG. The choice of bases to mutate was based on the consensus sequence for Fis binding, (G/T)nnYRnn(A/T)nnYRnn(C/A), where n is any nucleotide, Y is a pyrimidine, and R is a purine (22). Cu-phenanthroline footprinting of Fis bound to the 2-kbp *nrd* wild-type and mutant fragments (–1552 to +518 relative to the start of transcription [Fig. 1]) indicated that Fis binding is eliminated or at least greatly decreased at the mutated site (Fig. 3, compare lanes 2 to 5 with lanes 8 to 11). There were additional binding sites at –187 to –204, –268 to –285, and –310 to –328 that do not appear to be relevant to *nrd* regulation, since deletion of these sites did not affect *nrd-galK* expression (46).

To verify that regions upstream of the mutated site were not required for Fis activation of *nrd* expression, as indicated by the deletion study, the mutated DNA was transferred into a translational fusion pJEL vector and analyzed. *E. coli* DNA from 167 bp upstream of the start of *nrd* transcription to 518 bp downstream in the *nrdA* coding sequence was translationally fused to *lacZ* in a pJEL170-based vector to generate pLBAR229. Plasmid pLBAR229F, a similar construct containing the mutated Fis binding site centered at –156 with inserted cytidines at –165 and –158, was also made. *E. coli* RZ211 containing the resulting construct, pLBAR229F, grown exponentially in rich medium had a 2.4-fold decrease in *nrd* expression compared with pLBAR229 carrying the nonmutated *nrd* promoter grown under similar conditions (Table 1).

FIG. 3. Footprint of Fis on wild-type and -156 Fis binding site-mutated *nrd* promoter region fragment 2f. Solution Cu-phenanthroline footprinting reactions were performed as detailed in Materials and Methods. A 6- μ l amount of each reaction mix was electrophoretically separated on a denaturing 6% acrylamide-urea gel along with an M13mp18 dideoxy sequencing ladder. Lanes 1, 6, 7, and 12 contain no Fis. Four sites of protection, seen on the wild-type 2f fragment at the lowest Fis concentration tested (lane 2), are indicated by brackets, numbered relative to the start of *nrd* transcription. Specific binding of Fis at the -142 to -160 site appears to be eliminated on the mutated fragment (lanes 8 to 10). Less-specific binding, outside the four bracketed sites, is apparent at the higher Fis concentrations in lanes 4, 5, 10, and 11.

The difference in expression between pLBAR229 and pLBAR229F is the same as the difference in expression observed between pLBAR229T and pLBAR325T, indicating that the difference is due to the single Fis binding site. An in vitro transcription assay with supercoiled template DNA confirmed that this site-directed mutation eliminated the positive effect of Fis binding on transcription (41a).

DnaA protein binds near the -35 region in the *nrd* promoter. The *nrd* promoter region sequence (-52)TTATCACA(-44) is exactly homologous to two of the four 9-bp DnaA-binding sequences in the *E. coli* minimal origin of DNA replication, *oriC* (16). Schaefer and Messer (39) showed this to be one of the stronger binding permutations of the DnaA consensus binding sequence (T/C)(T/C)(A/T/C)T(A/C)C(A/G)(A/C/T)(A/C). The *nrd* promoter region sequence (-40)TTATgCACT(-32) contains two nonconsensus bases (lowercase letters), and binding by a strain carrying this sequence was too low to measure in the Schaefer and Messer study (39). To determine whether DnaA protein bound to these sequences in the *nrd* promoter, a 32 P-end-labeled *nrd* promoter region fragment (fragment 229f, -165 to +85 relative to the start of *nrd* transcription) was mixed with increasing amounts of DnaA and subjected to Cu-phenanthroline footprinting. At a DnaA concentration of 10 μ g/ml, protection of bases -55 to -35 was observed (wild-type footprint, Fig. 4), demonstrating in vitro binding of DnaA immediately upstream of the *nrd* promoter.

Three different mutated *nrd* promoter region fragments, D2, UD, and DD, were created by site-directed mutagenesis of the consensus DnaA-binding boxes immediately upstream of the -35 RNA polymerase binding site. In D2, both DnaA boxes were mutated by six transversions (shown in lowercase letters), (-52)TTAaCgtCAAaCAAATgCACT(-32); in UD, the upstream DnaA box was mutated by a single T-to-A transversion at position -49, (-52)TTAaCCACA(-44); and in DD, the downstream DnaA box was mutated by a T-to-A transversion at position -40 and a G-to-C transversion at position -41, (-41)caTATgCACT(-32). To determine the effect of the mutations on DnaA binding, the 261-bp *Bam*HI-*Eco*RI *nrd* promoter fragment 229f, containing the DnaA box mutations, was mixed with increasing amounts of DnaA and subjected to Cu-phenanthroline footprinting. The resulting protection patterns demonstrate cooperative binding of DnaA to the two boxes (Fig. 4). The double DnaA box mutation resulted in no detectable DnaA binding at the highest DnaA concentration tested (20 μ g/ml); the downstream box mutation resulted in no detectable binding at the downstream box and diminished binding at the upstream box; and the upstream box mutation resulted in diminished binding at both boxes, suggesting that binding of DnaA to the downstream box occurs only after binding to the upstream box.

Mutations in the DnaA boxes of the *nrd* promoter reduce *nrd-lac* expression. The effect of DnaA binding on *nrd* expression in vivo was examined by assaying β -galactosidase expression from *nrd-lacZ* translational fusions in constructs with either *nrd* promoter region wild-type or mutated DnaA-binding boxes. All three mutated promoter regions, translationally fused to *lacZ*, resulted in decreased β -galactosidase expression compared with expression from the wild-type pLBAR2 construct in log-phase RZ211 cells. The largest effect resulted from the single T-to-A transversion at -49 in the upstream DnaA box in pLBAR2UD (Table 3). RZ211 containing pLBAR2 contained 2.5 times as much β -galactosidase as RZ211 containing pLBAR2UD. These results suggest that DnaA binds cooperatively to two sites immediately upstream of the -35 RNA polymerase-binding site and that this binding results in increased *nrd* transcription. At present, we cannot eliminate the possibility that the T-to-A transversion happens to independently alter both DnaA binding and *nrd* expression.

Surprisingly, when DNA synthesis was inhibited by thymine starvation, strains carrying a plasmid mutated in either the upstream or downstream or both DnaA-binding boxes increased *nrd* expression in parallel with a strain carrying the

FIG. 4. Solution Cu-phenanthroline footprinting of DnaA protein on wild-type and DnaA box-mutated *nrd* promoter region fragment 229f. Reactions were performed as detailed in Materials and Methods. A 4- μ l amount of each footprinting reaction was electrophoretically separated on a denaturing 6% polyacrylamide-urea gel along with an M13mp18 dideoxy sequencing ladder. The concentration of DnaA in each lane is indicated at the bottom. In the far right panel, reactions without DnaA exhibit hypersensitive bands in the DnaA box region of the downstream (lane D) and double (lane Dbl) DnaA box mutant fragments, indicating a possible structural difference from the wild-type (lane WT) fragment. This difference is not observed in the upstream DnaA box mutant fragment (lane U).

nonmutated plasmid (Fig. 5). Since both *nrd* expression and DNA initiation increase in parallel in synchronized exponentially growing cells, and DnaA appears to be involved in the regulation of both, it is surprising that the DnaA-binding boxes do not appear to be required for increased *nrd* expression during DNA inhibition.

DISCUSSION

Reevaluation of *nrd* promoter region deletions in constant vector backgrounds has demonstrated that the two *nrd* promoter region "negative" regulatory sites reported by Tuggle and Fuchs (46) are artifacts resulting from polarity differences introduced during their deletion analysis. Mutations or deletions of the previously reported negative regulatory site centered at -156 from the start of transcription, shown in this present report to bind Fis, result in a 2- to 2.4-fold reduction in expression from the *nrd* promoter in several gene fusion systems. The most important site of regulation appears to be a site located between -122 and -139; deletions here decrease

expression of an *nrd-lac* fusion gene five- to sixfold. This site was shown (46) to be required for increased expression of the *nrd* promoter during thymine starvation. Because of the polarity difference in the vectors used in the previous study (46), the "parental" construct used for comparison had 10-fold lower activity than the appropriate parental construct. Thus, the apparent fivefold negative effect found for deletion of the -156 site should have been reported as a twofold positive effect. In the previous study (46), constructs containing deletions of sequence upstream of -122 were reported to show five- to sixfold lower expression than a construct with only the -156 site deleted but the same level of expression as the nondeleted "parental" construct. In this study, we find that deletion of the -156 site results in a 2- to 2.4-fold decrease in expression and deletion of the -122 to -139 site results in a further five- to sixfold decrease in expression.

Since transcription from the *nrd* promoter either on the chromosome or on a single-copy plasmid has been found to increase in a cell cycle-dependent manner when DNA synthesis is initiated and DnaA is a good candidate for the key component in DNA initiation (41), a site for the binding of DnaA adjacent to the -35 site of the promoter appeared to be a good candidate for regulation of *nrd*. DnaA was found to bind at both boxes in a cooperative manner. Site-directed mutations that alter binding at each as well as both of the DnaA boxes were found to have a twofold effect on expression of *nrd-lac* fusions in exponentially growing cultures as well as in thymine-starved cultures. These results are in agreement with the previous finding (13) that indicated that shifting a *dna*(Ts) mutant to nonpermissive conditions and thus inhibiting DNA replication was sufficient to induce *nrd* expression. However, one cannot exclude the possibility that the *dna*(Ts) alleles used in that study retained the ability to bind the *nrd* regulatory

TABLE 3. Exponential-phase expression from wild-type and DnaA box-mutated *nrd* promoter fusion constructs

Plasmid	Relative activity	Sp act ^a (nmol/min/mg of protein)
pLBAR2	1.00	1,760 (25)
pLBAR2UD	0.40	702 (15)
pLBAR2DD	0.72	1,270 (50)
pLBAR2D2	0.65	1,150 (24)

^a Values were determined in *E. coli* RZ211 and are reported as nanomoles of ONP produced per minute per milligram of protein. Values in parentheses represent the standard error of triplicate assays of triplicate cultures.

FIG. 5. Expression from wild-type and DnaA box-mutated *nrd* promoter fusions during thymine starvation. *E. coli* BK99T carrying the *nrd-lac* fusion constructs pLBAR2 (wild type), pLBAR2UD (upstream DnaA box mutated), pLBAR2DD (downstream DnaA box mutated), and pLBAR2D2 (both DnaA boxes mutated) was grown to early log phase in defined medium, washed, and resuspended in medium without thymine at time zero as described in Materials and Methods. Aliquots of the cultures were harvested at the times indicated and assayed for β -galactosidase activity.

region. Although the *in vivo* expression and *in vitro* DnaA-binding results reported here demonstrate that DnaA increases *nrd* expression, the correlation between diminished binding and decreased expression was not exact. A single base change in the upstream DnaA box, resulting in diminished *in vitro* DnaA binding, had a greater effect on expression than did the total elimination of DnaA binding resulting from six base changes in the double DnaA box mutant promoters. A difference in the Cu-phenanthroline cleavage patterns of DNA observed on the downstream DnaA box and double DnaA box mutant fragments relative to that on the upstream DnaA box mutant and wild-type fragments in the absence of added DnaA protein (see Fig. 4, lanes WT, D, U, and Dbl) suggests that mutations in the downstream DnaA box affect DNA structure.

Although both Fis and DnaA appear to be positive regulators of *nrd* expression, mutated *nrd* promoter regions unable to bind one or the other of these proteins show decreased expression in both exponentially growing cultures and thymine-starved cultures. One explanation for this observation is that an effector that controls the timing of *nrd* expression acts at the site located between -122 and -139 (a site that is also required for induction by DNA inhibition) and that Fis and DnaA, and perhaps other proteins, regulate the degree of expression. Since an exponentially growing culture contains cells at all stages of the cell cycle, a mutated promoter region lacking the ability to respond to an effector that controls timing would have low-level *nrd* expression in all parts of the cell cycle. In contrast, mutated promoters unable to bind proteins that affect the degree of expression would have decreased expression in both the presence and absence of thymine starvation, assuming that the increased expression of *nrd*

during DNA inhibition is due to a change in the timing effector.

Would there be a need to control *nrd* expression other than by controlling timing? This may depend on how the timing mechanism works. If the timing mechanism increases expression for a given period of time, the amount of transcription and translation may have to be adjusted to ensure that sufficient ribonucleotide reductase is synthesized. All experiments conducted in this study were done with cultures that had been growing in the exponential phase for five or more generations. Since Fis expression varies greatly with growth conditions, this study may not have detected major effects of Fis on the regulation of *nrd* that may exist under changing growth conditions. A nutritional shift-up increases Fis synthesis (5) as well as both *nrd* expression and DNA initiation (13). This effect could be either direct or indirect. Since the amount of ribonucleotide reductase required by a cell is proportional to the number of replication forks and *nrd* expression parallels DNA initiation, proteins that alter DNA initiation may also need to alter *nrd* expression to keep a balanced supply of precursors.

Identification of Fis and DnaA as regulatory factors at the *nrd* promoter, along with the observation that transcription of the *nrd* operon is cell cycle dependent (41), suggests that understanding the regulation of ribonucleotide reductase expression in *E. coli* may provide insights into the mechanism regulating the initiation of DNA replication at *oriC*.

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