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Regulation of the Operon Encoding Ribonucleotide Reductase: Role of the Negative Sites in *nrd* Repression

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Expression of the *nrd* genes was previously shown to be controlled by both positive and negative regulation (C. K. Tuggle and J. A. Fuchs, EMBO J. 5:1077–1085, 1986). Two regions, one located 5′ and one located 3′ of the *nrd* promoter (*nrdP*), were identified as negative regulatory sites since deletion of these sequences increased *nrd* expression. These regions of DNA have sequence similarities, and a looping mechanism was proposed to explain the requirement for two distant sites in *nrd* repression. To investigate the role of these sequences in regulating *nrd*, a gel electrophoresis assay was used to detect the proteins that bind to the *nrd* regulatory sites. A protein that bound to restriction fragments containing the negative regulatory sites but not to other DNA fragments was identified in cell extracts and was partially purified. DNase I footprinting experiments showed that the binding protein protects the 5′ negative site previously identified in vivo. The 3′ negative site also identified in vivo was not required in vitro for high-affinity protein binding to the 5′ site, but lower-affinity binding to this site could be detected. Specific binding to the 5′ site was found to be elevated approximately 10-fold in crude extracts from thymine-starved cells as compared with that in extracts from unstarved cells. This higher activity was also evident in purified preparations, suggesting that thymine starvation increases the expression of the negative regulatory protein. The finding that a purified protein preparation binds both negative regulatory sites indicates that this preparation contains the *nrd* repressor protein or proteins. Insertion of 37 base pairs (3.5 helix turns) of DNA at a *Hpa*II site or 35 base pairs (3.3 turns) at a *Mnl*I site between the 5′ regulatory sites and *nrdP* abolished the increase in *nrd* expression resulting from thymine starvation in vivo, but negative regulation appeared to be less affected than when either negative site was deleted. Insertion of DNA in these constructs was shown not to affect repressor binding in vitro, indicating either that a simple model of DNA looping to bring equivalent operator sites into physical proximity does not explain repression at *nrd* or that the distance between sites is sufficient that helical turns are of little importance.

Ribonucleotide reductase is an essential enzyme that catalyzes the first unique step in DNA synthesis, the conversion of ribonucleotides to deoxyribonucleotides (21). The enzyme present in *Escherichia coli* has been well characterized. The genes encoding the two subunits of ribonucleotide reductase (*nrdA*, *nrdB*) appear to be regulated in parallel with DNA initiation (6). Inhibition of DNA synthesis (i.e., by thymine starvation, shift of *dna* mutants to a nonpermissive temperature, or treatment with nalidixic acid or bleomycin) causes an increase in ribonucleotide reductase activity (6, 7) because of an increased rate of transcription of *nrdAB* (13). This regulation of *nrd* has been proposed to reflect the ability of the cell to sense the need for DNA initiation in inhibited cells; attempts to increase DNA initiation would lead to an increase of *nrd* expression for synthesis of DNA replication precursors (6, 7).

The *nrdAB* genes have been cloned and sequenced (1, 2, 5, 18). The start of *nrd* transcription has been mapped 110 base pairs (bp) 5′ of the ATG start codon of the *nrdA* gene (22). Galactokinase activity from plasmids with the *nrd* promoter regions fused to the galactokinase (*galK*) gene is induced by thymine starvation in parallel with induction of the chromosomal *nrd* gene, indicating that regulation of *nrd* is not affected by the addition of multiple copies of the regulatory region (22). This result indicates either that the pool of regulatory molecules is large or that the regulatory circuitry has feedback control. Deletion analysis of these fusions has indicated that the *nrd* operon is controlled in both a negative and a positive manner (22). Deletion of sequences at about −135 bp (relative to the start of *nrd* transcription at position +1) results in the loss of the ability to respond to thymine starvation. This result was interpreted as a deletion of a positive regulatory site. Deletion of sequences at −158 bp or at +80 bp increased GalK activity 5- to 10-fold throughout the period of thymine starvation, indicating that a negative regulatory site(s) had been removed from the DNA (Fig. 1). The effects of deletion of both proposed negative sites on GalK activity were further shown not to be additive when they were combined in the same construct. The two sites had approximately 70% sequence similarity, and these sites were proposed to interact to inhibit *nrd* transcription.

A popular model that is used to explain the phenomenon of two regions involved in repression invokes looping of the DNA to bring the distant sites into physical proximity (for reviews, see Ptashne [19] and Gralla [11]). The interaction of proteins bound at both sites may promote and/or stabilize such a conformation. The best evidence for the looping model has been obtained for the lambda repressor (16) and the *araC* protein (4, 12), in which insertion of nonintegral helical-turn lengths of DNA affects the ability of the repressors to function. In addition, recent studies on the *lac* operon (8) indicate interaction between the high-affinity operator near the *lacZ* initiation site and the lower-affinity, intragenic operator. Flasher and Gralla showed that the lower-affinity, intragenic operator has a dual function: strengthening of binding of the *lac* repressor to the high-affinity operator and

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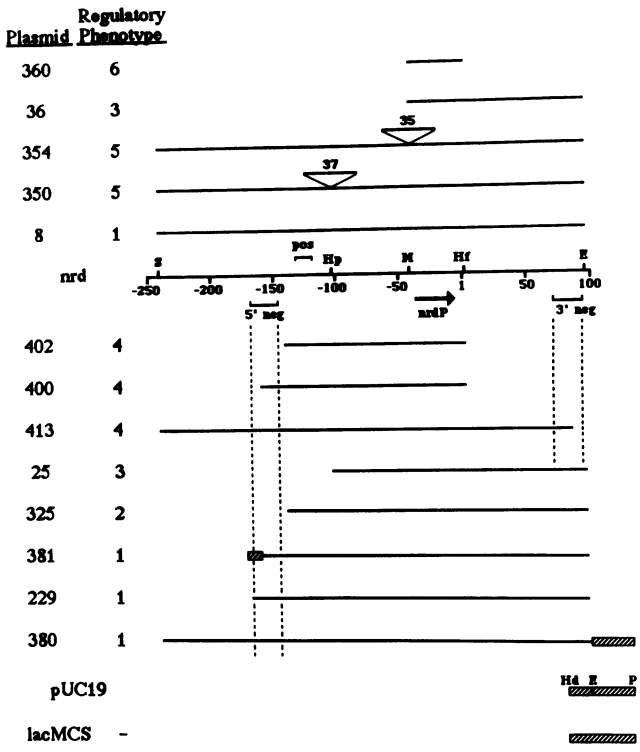

FIG. 1. Restriction map of the *nrd* regulatory region and DNA fragments used in binding assays. Regulatory phenotype classes 1 through 4 have been discussed previously (22), while data defining classes 5 and 6 are shown in Table 2. Briefly, class 1 is wild-type, low-level expression that is inducible upon thymine starvation to an approximately 10-fold higher level of expression. Class 2 is high-level (five times that of the wild type because of deletion of 5′ sequences) expression that is inducible to a very high level upon thymine starvation. Class 3 is low-level, uninducible expression because of deletion of additional 5′ regulatory sequences. Class 4 expression is high level (5 to 10 times that of the wild type because of deletion of 3′ sequences) and thymine inducible. Class 5 is slightly elevated expression (1.5- to 2-fold) which cannot be induced. Class 6 is high-level expression (sixfold over that of the wild type) which is uninducible by thymine starvation. The slashed box indicates vector sequences on the fragment. Abbreviations: E, *Eco*RI; Hd, *Hin*dIII; Hf, *Hin*fI; Hp, *Hpa*II; M, *Mnl*I; P, *Pvu*II; S, *Sau*3A; *nrdP*, *nrd* promoter elements; *lacMCS*, polylinker and *lacZ* sequences from pUC19.

functioning as a transcriptional block when the *lac* repressor is bound. To investigate whether the two sites in the *nrd* operon may, in fact, regulate transcription in a manner analogous to those of the systems described above, DNA was inserted between the negative regulatory sites and the effect of this insertion on regulation of *nrd* was determined.

The role of these negative regulatory sites was further examined by assaying for the *trans*-acting factors that bind at these sites and, presumably, that regulate the rate of *nrd* transcription. DNA restriction fragments containing the *nrd* promoter and various amounts of flanking regulatory sequences were used to identify specific DNA-binding proteins in cell extracts. We identified and partially purified protein activity that bound the *nrd* regulatory region with a high affinity only if the 5′ negative site was present, although binding to the lower-affinity 3′ site was observed. This DNA-binding activity was further shown to be much higher in extracts from thymine-starved cells compared with that in extracts from nonstarved cells. The implications of these findings on the proposed model for regulation of *nrd* expression are discussed.

MATERIALS AND METHODS

Bacterial strains. Strain N110 (*galK thyA recA* [22]) was used both as a host for *nrd-galK* fusions and as a source of protein for repressor studies.

Plasmid constructions and *GalK* assays. To insert a nonintegral length of DNA at the *Hpa*II site (at position −104 relative to the *nrd* transcriptional start site at position +1) of pCKT8 (22), the *Hin*dIII-*Eco*RI fragment of pCKT122 (22) was isolated by electroelution, digested with *Msp*I, and ligated into pUC19 via the *Hin*dIII and *Acc*I sites to create pCKT123. pCKT123 was digested with *Hin*dIII and *Sma*I and ligated to *Hin*dIII-digested pCKT25. After sticky-end ligation, the remaining *Hin*dIII ends were filled in with Klenow enzyme and the plasmid blunt ends were closed with ligase to create pCKT35, in which pCKT8 was regenerated but in which 37 bp (3.5 helix turns) of restriction site sequences was added at the *Hpa*II site. For insertion of nucleotides at the *Mnl*I site (at position −33), the *Hin*dIII-*Eco*RI fragment from pCKT122 was inserted into pCKT36 as described above for pCKT25. The resulting plasmid (pCKT354) regenerated pCKT8 with 35 bp (3.33 helix turns) inserted at the *Mnl*I site at position −33. pCKT360 was constructed from pCKT36 by deletion of the *Hin*fI-*Eco*RI fragment as described previously (22). The *Sal*I-*Eco*RI restriction fragment from pCKT8 (22) was cloned into pUC19 via the *Sal*I and *Eco*RI sites to create pCKT380. The *Bam*HI-*Eco*RI restriction fragment from pCKT300 (22) was cloned into pUC19 via the *Bam*HI and *Eco*RI sites to create pCKT381. This cloning was done to allow for easier identification of potential protein binding to either fragment near the *Eco*RI site or near the *Bam*HI linker used to construct pCKT300. GalK assays were performed on triplicate cultures as described previously (22).

DNA-protein binding assays. DNA restriction fragments (Fig. 1) were prepared as *Hin*dIII-*Pvu*II (pCKT380 and pCKT381; see above), *Hin*dIII-*Eco*RI (pCKT25, pCKT325, pCKT400, pCKT402, and pCKT360), or *Bam*HI-*Eco*RI (pCKT229 and pCKT234) fragments from CsCl-purified plasmid preparations. Fragments were radiolabeled either before or after gel isolation with [^{32}P]dATP or [^{32}P]dGTP and the Klenow enzyme. Binding assays (9, 10) contained the following: TMD buffer (20 mM Tris hydrochloride [pH 7.5], 10 mM $MgCl_2$ 5 mM dithiothreitol), 0.1 mM EDTA, 0.05 mg of bovine serum albumin per ml, approximately 50 mM KCl, 1 to 5% glycerol, 50 to 2,000 ng of sonicated salmon sperm DNA, 1 to 4 ng of labeled DNA fragment, and various amounts of protein extracts in a final volume of 0.02 ml. Constituents were mixed gently (15) and incubated at 37°C for 20 to 25 min. One-fifth volume TMD with 80% glycerol and bromphenol blue was added, and the reaction was immediately loaded onto a 6% (acrylamide-bisacrylamide, 29:1) polyacrylamide gel. Electrophoresis was carried out for 1 to 2 h. Gels were dried and exposed to X-ray film for 2 to 18 h. Densitometry, which was performed to quantitate the level of protein binding, required several exposures of the dried gel to ensure that scanning was in the linear response range of the film.

DNase I protection study. DNase I (supplied at 2 mg/ml; RQ1; Promega Biotec) was diluted 1,000- to 20,000-fold in the following buffer: 20 mM Tris hydrochloride (pH 8.0), 10 mM $MgCl_2$, 10 mM $CaCl_2$, 0.1 mM EDTA, 0.1 mg of bovine serum albumin per ml, 50% glycerol, and 0.001 mg of

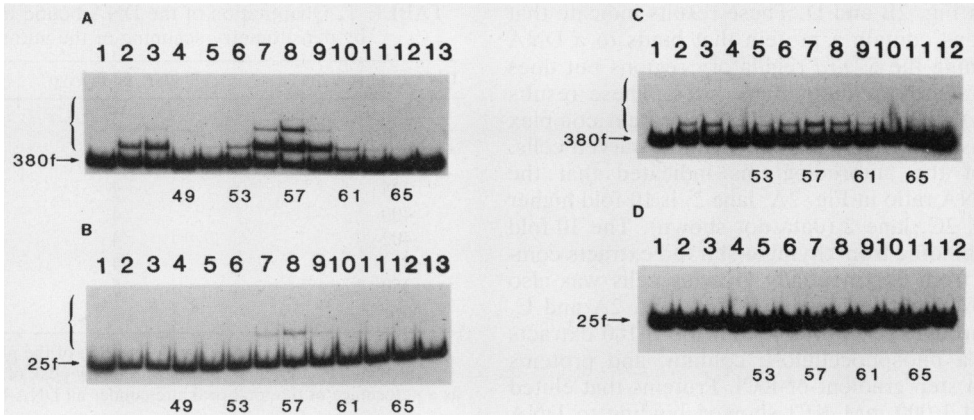

FIG. 2. DNA-protein binding assays of crude extracts and phosphocellulose column fractions from induced and noninduced cell extracts. Assays were performed as described in the text. Nonspecific DNA was used at 1,000 ng per reaction. (A and B) Starved cell extracts; (C and D) nonstarved cell extracts. (A through D) Lanes 1, no protein added; lanes 2, 7 μg of protein from nucleic acid-free S-100; lanes 3, 1.2 μg of protein from 600 to 1,000 mM KCl phosphocellulose batch fraction; lanes 4 through 13 (A and B) or lanes 4 through 12 (C and D), phosphocellulose salt gradient fraction (2 μl; approximately 300 ng of protein for fraction 57, starved cell extract). Arrows indicate free DNA; the braces indicate bound DNA.

sonicated salmon sperm DNA per ml. We found RQ1 activity to be unstable at these concentrations if the carrier DNA was omitted from the dilution buffer. To perform a DNase I protection experiment, the binding assay described above was quadrupled in size. A total of 0.02 ml of appropriately diluted DNase I was added to the binding reaction (0.08 ml) after the 37°C incubation period. DNase I digestion was allowed to proceed for 10 min at 37°C. Digestion was stopped by the addition of an equal volume of 4 M ammonium acetate and 50 mM EDTA containing 0.4 mg of yeast tRNA per ml as the carrier. The samples were extracted once with 1:1 phenol-chloroform, ethanol precipitated, washed twice with 1 ml of 70% ethanol, and electrophoresed next to a dideoxy sequencing reaction (20) that was used as a molecular weight standard.

Protein purification. Strain N110 was grown in 12-liter batches in Davis-Mingioli minimal medium with 0.05% casein hydrolysate, 0.004 mg of vitamin B_1 per ml, 0.2% glucose, Lwoff salts, 0.04 mg of thymine per ml, and 0.2 mg of deoxyadenosine per ml. Strain N110 was then thymine starved as described previously (22). Cell paste was stored at −70°C until sonication. A total of 20 g of wet cell paste of thymine-starved or nonstarved cells was thawed, suspended in DEAE-cellulose buffer (17), and sonicated; and cell debris was removed by centrifugation. A nucleic acid-free S-100 extract was prepared as described by Ishii et al. (17), except that the S-100 was applied to the column at 100 mM KCl, since preliminary results indicated that a DNase activity is removable by washing the column-bound protein with 100 mM KCl. Two column volumes were allowed to pass through, and then the majority of proteins were eluted with 300 mM KCl. A portion of this fraction was run through a small phosphocellulose column (P-11; Whatman, Inc., Clifton, N.J.) to determine which salt fraction contained specific binding activity. Proteins that were retained at 0 mM KCl were eluted in batches with 100, 300, 600, and 1,000 mM KCl. Activity was found only in the last (600 to 1,000 mM salt) batch. A 15-ml phosphocellulose column was then run with a 0 to 1 M KCl gradient with the remainder of the DEAE-cellulose extract. Binding activities in 2 μl of every other fraction were measured as outlined above.

RESULTS

Characterization of a protein that binds specifically to *nrd* regulatory DNA. To identify proteins that bind specifically to the regulatory sites of *nrd*, an assay was developed based on the gel electrophoresis DNA-protein binding assay (9, 10). DNA-protein complexes are apparently stabilized by the polyacrylamide matrix and migrate more slowly during electrophoresis than free DNA does, resulting in the separation of free DNA from DNA-protein complexes. After electrophoresis, the extent of binding was visualized by autoradiography of the dried gel and quantitated by densitometric scanning of the autoradiogram. Binding was expressed as the amount of DNA bound relative to the total amount of DNA present.

Strain N110 was grown to the exponential phase and starved for thymine for 3 h. Cells from time zero of starvation were used as a control. An S-100 extract was prepared from sonicated cells, and nucleic acids were removed by chromatography on DEAE-cellulose (17). Preliminary assays indicated that a DNase activity present in this extract could be removed by first binding proteins to DEAE-cellulose at 100 mM KCl. DNA-binding activity was then eluted (along with the majority of cellular proteins) from the column with 300 mM KCl. This modified nucleic acid-free S-100 extract was used for further studies. Figure 2 presents the results of protein-DNA binding studies under various conditions. Figure 2A and B presents assay results with proteins that were extracted from a thymine-starved culture, while Fig. 2C and D presents results from extracts of an exponentially growing, nonstarved culture. In Fig. 2A and C, a DNA fragment from pCKT380 (designated 380f; see Fig. 1 for schematic of all fragments used in this study) containing all known *cis*-acting *nrd* regulatory sequences was used, while in Fig. 2B and D a DNA fragment from pCKT25 (designated 25f), in which both the 5′ negative and the positive regulatory sites were deleted, was used. In lanes 1 of Fig. 2A through D, no protein was added and only a band of free DNA was detected. In lanes 2 of Fig. 2A through D, 7 μg of protein from a nucleic acid S-100 fraction was added. A slower-migrating band was observed in lanes 2 of Fig. 2A

and C but not in Fig. 2B and D. These results indicate that the S-100 fractions contain a protein that binds to a DNA fragment containing the 5′ *nrd* regulatory regions but does not bind to a fragment without these sites. These results further indicate that the amount of DNA-protein complex formed is increased in extracts from thymine-starved cells. Densitometry of the autoradiograms indicated that the bound to free DNA ratio in Fig. 2A, lane 2, is 10-fold higher than that in Fig. 2C, lane 2 (data not shown). The 10-fold increased DNA binding from thymine-starved extracts compared with that from exponentially growing cells was also observed in subsequent purification steps (Fig. 2A and C, lanes 3 and 5 through 11). Protein from the S-100 extracts were bound to a phosphocellulose column, and proteins were eluted by a step gradient of KCl. Proteins that eluted between 600 and 1,000 mM KCl showed binding to DNA fragment 380f that mimicked the binding seen with the crude extract (Fig. 2A through D, lanes 3). A low level of binding to DNA fragment 25f could also be seen in this protein preparation. To obtain a greater purification of this binding activity, protein from the S-100 fraction was bound to phosphocellulose and eluted with a 0 to 1 M KCl gradient. The DNA binding of every other fraction around the peak of activity is presented in Fig. 2A through D, lanes 4 through 12 or 13. In the extract from a thymine-starved culture, binding to 380f was highest in fraction 57 (Fig. 2A, lane 8). We interpret the multiple bands observed in reactions with high levels of binding activity to be additional protein interactions with 380f. These multiple interactions could be the same protein or different, copurifying binding proteins. Binding to 25f appeared to parallel binding to 380f, but binding of the protein was significantly reduced (Fig. 2B, lanes 7 through 9). In extracts from an exponentially growing culture, similar patterns of elution of the binding activity were obtained, but the level of binding was much decreased with 380f (Fig. 2C) and undetectable with 25f (Fig. 2D). Comparison of the specific activity of binding indicated that the protein present in fraction 57 was purified approximately 200- to 300-fold compared with the specific activity of the S-100 extract.

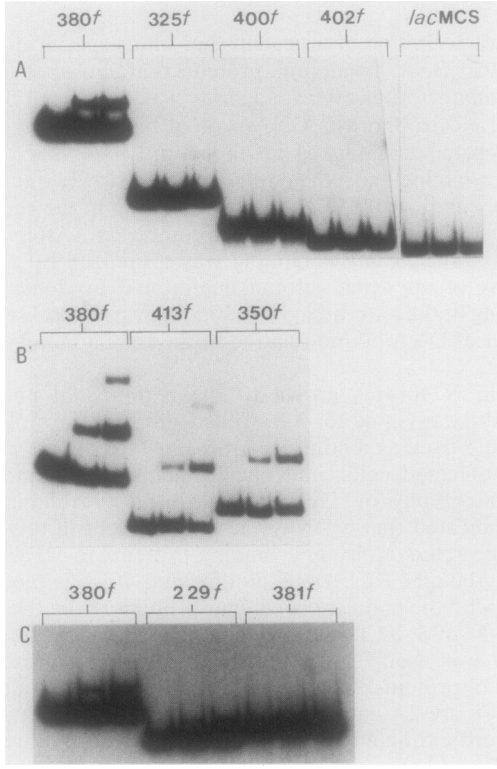

FIG. 3. Negative sites in the *nrd* regulatory region are required for binding of fraction 57 in vitro. Assays were performed as described in the Fig. 2 legend. Each fragment was assayed in three lanes: lanes 1, control with no added extract; lanes 2, 25 ng of fraction 57 extract; lanes 3, 50 ng of fraction 57 extract (from left to right, respectively, under each fragment). (A) The fragments test for a requirement of the various regulatory regions for binding. (B) The fragments test for a requirement of correct spacing between the 5′ and 3′ negative sites (350f) as well as for the presence of the 3′ negative site for binding (413f). (C) The fragments test for differences in binding because of the alterations in the sequence at the negative site between 229f and 381f.

TABLE 1. Quantitation of the DNA bound to protein in Fig. 3 by densitometry scanning of the autoradiogram

DNA fragment	Phenotype in vivo[a]	% Bound[b]
380	1	48
229	1	45
381	1	7
325	2	7
400	4	<1
402	4	1
413	4	36
350	5	25
lac		<1

[a] See Fig. 1 legend and text for description of the phenotypes.
[b] Calculated as area under the densitometry peak of the bound DNA band as a percentage of the total peak area under all DNA bands.

Since 25f is missing both 5′ negative and positive regulatory regions (22), the DNA-binding protein in peak fraction 57, which showed a strong preference for 380f, could be either a repressor protein or a protein that is involved in the response to thymine starvation. To differentiate between these two possibilities, the activity of the peak fraction from thymine-starved cells (peak fraction 57) was studied further.

Binding correlates with the presence of the negative regulatory sites. A series of restriction fragments containing various segments of the *nrd* promoter-operator region (Fig. 1) were used in binding assays to characterize the activity in thymine-starved cell extracts (peak fraction 57). *nrd* DNA fragments were chosen in an attempt to correlate in vivo regulation as previously determined by deletion analysis (22) with the binding activity observed in vitro. Strong binding of protein to DNA fragment 380f, weaker binding to 325f (5′ negative site deleted), and very low or undetectable binding to 400f (the 3′ negative site was deleted and the 5′ negative site was altered) and 402f (both negative sites were deleted)

TABLE 2. Expression of various *nrdP-galK* fusion operons

Plasmid	GalK activity at the following times (min) after thymine starvation[a]:			
	0	60	120	180
pCKT8	0.3	0.41	1.2	3.24
pCKT350	0.67	0.31	0.5	0.73
pCKT354	0.52	0.29	0.46	0.69
pCKT36	0.22			
pCKT360	1.25			

[a] Activity is in nanomoles of galactose phosphorylated per minute per femtomole of plasmid.

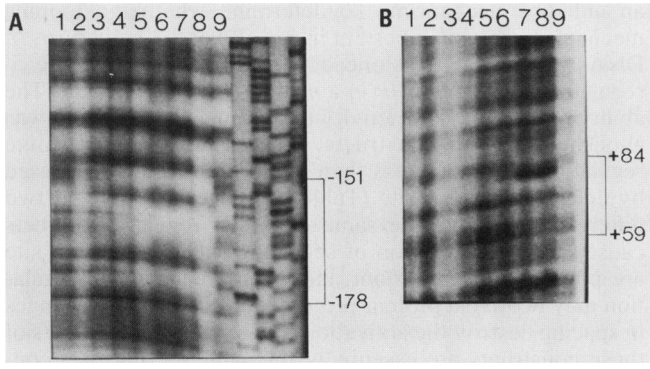

FIG. 4. Observed binding activity protects the 5′ and 3′ operator sites from DNase I digestion. A binding assay with fraction 57 and DNA fragment 380f with subsequent DNase I digestion was performed as described in the text. (A) Footprint pattern near the *nrd* 5′ negative regulatory site; (B) footprint pattern near the 3′ negative site. Nonspecific DNA was used at 1,000 ng per reaction. Lanes 1 and 9, control lanes with no protein added; lanes 2 through 8, twofold increase in fraction 57 protein added in each successive lane: lane 2, 0.156 μl (about 23 ng), to lane 8, 10 μl (about 1,500 ng). Unmarked lanes in panel A are a GATC series of sequencing reactions. Brackets indicate protected regions. The lengths noted in the figure are relative to the *nrd* transcription start site (position +1; Fig. 1).

was observed (Fig. 3A and Table 1). Thus, high-affinity binding is seen only if the 5′ negative site is present on the DNA being tested (380f), although binding at a reduced level is still detected (325f) if the 3′ negative site is present, as was also seen for 25f in Fig. 2B. Furthermore, failure to detect binding of the protein to DNA fragments 400f and 402f, which contain sites of binding of the proposed positive regulatory protein, indicates that the protein that was purified is not the positive regulatory protein. Figure 3B shows that insertion of a nonintegral number of turns between negative sites (350f; see below) or deletion of the 3′ negative site (413f) alone does not affect high-affinity binding, indicating that the 5′ negative site is capable of binding protein(s) in fraction 57 independently of the 3′ site. To map this proposed binding site further, two deletion fragments with endpoints near the 5′ negative site were tested for binding activity. Both 229f and 381f are repressed in vivo (22), but Fig. 3C shows that while 229f is strongly bound by fraction 57, 381f is bound poorly. DNA fragment 229f has an additional 9 bp (from positions −166 to −157) of *nrd* sequences relative to that in 381f, and these binding results indicate that these additional base pairs are necessary for stable in vitro binding. The fact that 381f was repressed in vivo may be due to the fact that several (five) nucleotides in a *Bam*HI linker that was used to construct the deletion series are in register with the *nrd* sequences in this 9-bp region (see Fig. 5 and Discussion). Densitometry of the autoradiograms in Fig. 3 was performed to quantitate the DNA bound to protein in Fig. 3 (Table 2) and indicates that the affinity of the fraction 57 protein(s) to the 5′ site (380f, 229f, 413f) is approximately six- to 7-fold greater than that to the 3′ site (325f, 381f).

The observed binding activity protects the *nrd* negative regulatory sites during DNase I Digestion. To determine the specific protein-binding site in DNA fragment 380f, a DNase I protection experiment was performed. Protection from limited digestion by DNase I has been successfully used to identify regions which are recognized and bound by various proteins (16). DNA-protein complexes were formed and subjected to DNase I digestion. DNA fragment lengths which were underrepresented relative to those obtained from the digestion of naked DNA indicate those regions that were protected from digestion by a DNA-binding protein. Figure 4 shows the results obtained with 380f DNA and fraction 57 from starved extracts. Two regions, one of about 27 bp centered at position −164 and the other of about 25 bp centered at position +71, were protected from DNase I digestion by added fraction 57 (Fig. 4A and B; the sequences of the indicated sites are shown in brackets in Fig. 5). These two regions correlate well with the in vivo deletion studies, which indicated negative regulatory sites that were at or that overlapped these locations of the *nrd* operon (22). The affinity of the protein(s) in fraction 57 was much higher (approximately four- to eightfold) for the 5′ site than it was for the 3′ site, as can be directly observed as the four- to eightfold higher amount of fraction 57 required to observe protection at the 3′ site in the DNase I footprinting assay (Fig. 4).

Insertion of DNA between the 5′ regulatory sites and *nrdP* alters regulation of the *nrd* operon. A DNA fragment of 37 bp (3.5 helix turns of B-DNA) was inserted into a *Hpa*II site at position −104 from *nrdP* to create pCKT350, and 35 bp (3.33 helix turns of B-DNA) was inserted into a *Mnl*I site at position −33 from *nrdP* to create pCKT354 (Fig. 1). Cells containing these plasmids were tested for their response to thymine starvation and were found not to increase GalK

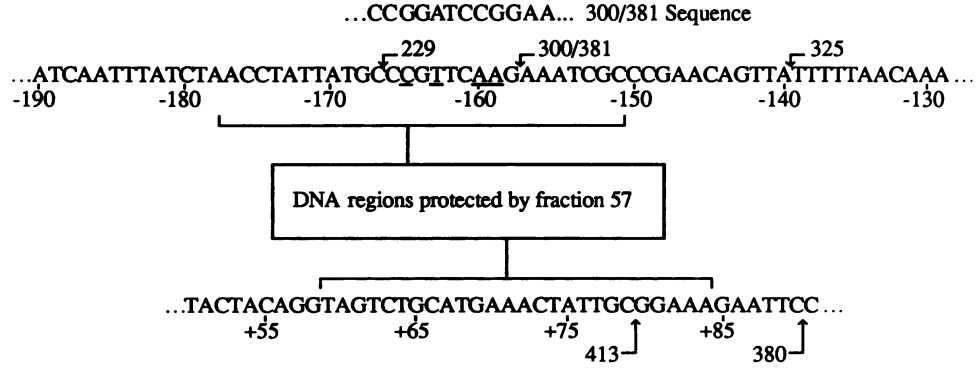

FIG. 5. Sequence of protected bases at the repressor-binding sites and relationship to in vivo deletion analysis. Brackets indicate the extent of sites protected by fraction 57 in the DNase I experiment. The underlined nucleotides are bases in this region that differ between pCKT229 and pCKT381. The pCKT381 sequence in the region of interest is shown above the *nrd* chromosomal sequence. Arrows show the deletion endpoints of the indicated constructs.

activity in vivo, in contrast to the 10- to 12-fold induction observed for the *nrd-galK* fusion (pCKT8) without insertions (Table 2). Spacing between the positive regulatory site and *nrdP* thus appears to be important for correct regulation, since the same loss of positive regulation was observed upon insertion of DNA at either of two locations between *nrdP* and the DNA region that is critical for the response to thymine starvation. This result, especially for the insertion at the *Mnl*I site, since it is about 100 bp 3′ to the positive regulatory site, may indicate that the response to thymine starvation involves a protein-protein interaction between RNA polymerase and a protein that binds at the positive regulatory site. The data in Table 2 indicate that there was also a slight increase in GalK activity at time zero (exponentially growing cells) in these constructions relative to the activity in pCKT8 (50 to 100% elevated), indicating that negative regulation is affected by these insertions; however, this effect was 10-fold lower than that seen when either site is deleted, in which transcription increases 500 to 1,000% (22). This regulatory phenotype of a slightly elevated basal level with no ability to increase transcription upon thymine starvation is termed class 5.

Previous results (22) indicated that deletion of the 5′ negative site increases *nrdP* expression fivefold, while deletion of the 3′ negative site or both sites results in 10-fold increased GalK activity. To determine whether the 3′ negative site could affect *nrdP* expression in the absence of other *nrd* regulatory sequences, a *Hin*fI-*Eco*RI fragment containing the 3′ negative site was deleted from pCKT36 to generate pCKT360, which contained only 38 bp of *nrd* sequences. GalK activity in exponentially growing cells harboring these two plasmids was measured, and the results indicated that pCKT360 has about sixfold increased GalK activity compared with that of pCKT36 (this phenotype is termed class 6 and reflects the strength of the *nrdP* in the absence of flanking *nrd* sequences [Table 2]). The increased promoter activity of the unregulated *nrdP* gene observed for pCKT360 relative to that observed for pCKT36 indicates that the 3′ site can negatively regulate *nrdP* in the absence of sequences 5′ to *nrdP*.

DISCUSSION

The mechanism of repression in several operons has been found to be more complicated than originally proposed. Early ideas on the lambda repressor and operator system and the *lac*, *ara*, and *gal* operons focused primarily on the fact that the repressor in these systems binds to an operator site close to the promoter. Steric hindrance on RNA polymerase was believed to fully explain the action of many repressor proteins. However, regions too far from the promoter to invoke direct competition for overlapping sites between RNA polymerase and a repressor protein have been reported as operator sites (for reviews, see Ptashne [19] and Gralla [11]). A model that was used to explain these results used looping of the DNA so that repressor proteins can contact more than one site on the DNA simultaneously. Insertion of DNA to change the integral number of helical turns between operators has been shown to alter repression in the lambda repressor and *ara* operons (4, 12, 16), indicating that the looping model has validity.

Recent investigations on the regulation of the *nrd* operon showed that two sites of negative regulation border the *nrd* promoter (22). Since neither site is close to *nrdP* (at least 75 bp pairs between either site and the RNA polymerase-binding site), steric hindrance of RNA polymerase seemed an unlikely mechanism. To determine whether a looping mechanism may be important in *nrd*, 3.5 or 3.3 helix turns of DNA were inserted at one of two places between the 5′ regulatory sites and *nrdP* in a *nrd-galK* fusion plasmid. The ability to induce GalK activity upon thymine starvation was abolished in both constructs, but the effect on negative regulation was much less than that which would be caused by deletion of either site (Table 2). Since insertions at two different sites have the same effect on regulation, effects caused by the disruption of sequences at the insertion site are probably not important, indicating that positive regulation may involve a protein-protein interaction since changes in spacing destroy the activation of *nrdP*. The phenotypes of these constructs are essentially the same as that of a construct in which sequences 5′ of *nrdP* are deleted (22), indicating that the movement of the 5′ sites even farther 5′ has the same effect as deletion of these regions does. If the two negative regulatory sites directly interact to repress *nrd* transcription, the mechanism may be independent of either spacing or topographical constraints.

To characterize the protein(s) responsible for regulation of *nrd* transcription, an in vitro DNA-protein binding assay was developed, based on a gel electrophoresis assay method (9, 10). Specific binding to *nrd* restriction fragments was observed in crude extracts eluted from a DEAE-cellulose column to remove nucleic acids. Binding activity was higher (Fig. 2) with a DNA fragment that contained both negative sites and the positive site (380f) than it was with a DNA fragment that contained only the 3′ negative site (25f). Stronger binding to both fragments was observed with extracts from cells that were starved for thymine, indicating that this DNA-binding activity may be regulated in parallel with the *nrd* operon. These properties are consistent with those expected for both the negative and positive regulatory proteins which presumably bind at *nrd* (22). Synthesis of the positive regulatory protein would be expected to increase during thymine starvation, since in a previous study (14) it was indicated that continuous protein synthesis is required during thymine starvation for induction of the *nrd* operon. An increase in repressor activity late in thymine starvation could also be expected if *nrd* expression is autoregulated, which is one of the models suggested to explain the fact that multiple copies of the operator and promoter region do not affect *nrd* regulation on either the chromosome or the plasmid (22). In addition, *nrd* expression is thought to be cell cycle regulated (J. Carlson and J. Fuchs, unpublished data), and such strict temporal control might require a system to shut off expression soon after induction. This would give a pulse of ribonucleotide reductase synthesis, which is needed for DNA replication. It is conceivable, then, that repression would also be turned on by the same mechanism that induces *nrd* expression. The repressor concentration would increase and eventually compete for binding at the *nrd* locus with positive regulatory proteins to shut off *nrd* synthesis. Such competition between the positive and negative regulatory proteins is possible at *nrd*, since the two regulatory sites have been mapped close together (22).

To determine whether the observed activity was that of the positive or the negative regulator, the binding activity was then partially purified by separation over a phosphocellulose column. Properties of the peak activity fraction were consistent with those found for the nucleic acid-free crude extracts discussed above. Further characterization of the binding activity in the peak fraction from starved cells (fraction 57) was accomplished by testing the binding activity of fraction 57 to several DNA fragments of the *nrd*

operon. Each phenotypic regulatory class identified by deletion analysis (22) was tested, as was an additional class that was constructed by insertion of DNA between the 5′ regulatory sites and *nrdP* (Table 1). DNA fragment 380f, a class 1 or wild-type class fragment, and DNA fragment 413f, a class 4 construct in which the 3′ negative regulatory site has been deleted, are bound strongly by fraction 57. Class 2 (5′ negative site deleted) and class 3 (both positive and negative 5′ sites deleted) showed little or no binding by fraction 57 (Fig. 2 and 3). If a positive regulatory protein that is responsible for induction of *nrd* during thymine starvation was present in this fraction, strong binding to high-activity, thymine-inducible DNA fragments which contain the positive regulatory site (classes 2 and 4) would be expected. However, strong binding was observed only when the 5′ negative regulatory site was present on the DNA fragment. The results are consistent with the hypothesis that the *nrd* repressor is present in fraction 57 and is responsible for the binding activity that we observed.

A DNase I footprinting experiment (Fig. 5) showed that a region from positions −178 to −151, as well as a region from positions +59 to +84, was protected by fraction 57 from DNase digestion. These results agree very well with those for the postulated negative regulatory site found by deletion analysis (22) (Fig. 5), further indicating that the *nrd* negative regulatory protein(s) is present in fraction 57.

The correlation between the presence of the 5′ negative regulatory site and both in vivo repression of *nrd* promoter activity and in vitro DNA-binding activity of fraction 57 is strong, but fails in the case of 381f. pCKT300, the parent plasmid of 381f, appears to be fully repressed in vivo (22), but 381f is not bound by fraction 57, as was observed for all other fragments that showed repression in vivo. Upon inspection of the joint between plasmid and *nrd* sequences, it was observed that 5 bases of the *Bam*HI linker that was used to construct pCKT300 are in common with 5 of the 9 bases of the *nrd* sequence that were absent on pCKT300 and pCKT381 but that were present in pCKT229, a construct which was bound strongly by fraction 57 (Fig. 5). It is tempting to speculate that these 5 bases allow recognition of the operator site in vivo, perhaps in the context of additional factors or conditions that are not duplicated when DNA binding of fraction 57 to this region was measured. In any event, it is possible to state that one or more of the 4 bases that changed from positions 229f to 381f in this region (nucleotides boxed in Figure 5) must be critical for stable in vitro complex formation of the *nrd* operator by the activity in fraction 57.

How does a repressor exert its function upon binding so far from the RNA polymerase-binding site? One possible answer is competition with a positively acting protein that binds at the nearby sequences that are required for response to thymine starvation. In accordance with this idea, we found that a protein bound with high affinity and protected from DNase I digestion the 5′ negative regulatory region, which is approximately 15 to 20 bp from the sequences that are required for induction of *nrd* transcription (22). A second possibility may involve transduction of a signal through the DNA molecule to alter the structure of the *nrdP* region and thus allow binding by RNA polymerase, since insertion of a short length of DNA between the operator and the promoter only slightly affected in vivo repression and did not affect in vitro protein binding (Table 2 and Fig. 3B). A third alternative is that one function of the 5′ repression site is to increase the local concentration of repressor molecules so that the repressor can bind at the low-affinity 3′ regulatory site to block RNA chain elongation. This situation appears to be the case in the *lac* operon, in which binding of the *lac* repressor to the high-affinity operator at the RNA start site both blocks transcription and stabilizes binding to the intragenic, lower-affinity operator to block RNA elongation (3, 8). This mechanism may be active at *nrd*, since although only the 5′ binding site is required for high-affinity in vitro binding by fraction 57, repression of *nrd* transcription in vivo occurs only if the 3′ site is also present (22). Furthermore, the finding that deletion of the 3′ negative regulatory site increases expression of GalK activity in the absence of 5′ sequences (Table 2) indicates that at least some of the function of this site is independent of 5′ sequences. The 3′ site may function independently of 5′ sequences by blocking transcription of *nrdP* upon binding of a protein at this site, and low-affinity, noncooperative binding to block transcription through this site may explain the repression seen in pCKT36. If the *nrd* repressor binds to the 5′ site as a multimeric protein, looping of the DNA to allow binding of the repressor to both the 5′ and 3′ sites simultaneously could block transcription and account for the increased local concentration of repressor. The failure to observe significantly decreased regulation when nonintegral lengths of DNA were inserted between the two negative sites may result from the flexibility of the DNA as a result of the fact it had over 280 bp between the negative sites.

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