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Promoter Region of the *nar* Operon of *Escherichia coli*: Nucleotide Sequence and Transcription Initiation Signals

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The *nar* operon, which encodes the three subunits of nitrate reductase in *Escherichia coli*, is fully induced under anaerobic conditions with nitrate. Two distinct regulatory domains have been delineated in the 5′ region of the operon which respond respectively to positive induction by the *fnr* gene product under anaerobic conditions and to positive induction by the *narL* gene product in the presence of nitrate (S. F. Li, T. Rabi, and J. A. DeMoss, J. Bacteriol. 164:25–32). To characterize these two regulatory regions, we determined the DNA sequence for a 500-base-pair (bp) region extending upstream from the first structural gene of the *nar* operon. Analysis of subsequent subclones of the operon established that the 5′ limit of the *nar* operon lies between 215 and 260 bp upstream from the translational start site of the first structural gene. The region required for induction by the *fnr* gene product is located within 160 bp from the translation start site, while the region responding to induction by nitrate extends an additional 100 bp upstream. Protein fusions of *lacZ* with the N-terminal sequence of the *narG* gene were constructed so that β-galactosidase formation was under the control of the *nar* promoter and one or both regulatory domains. Analysis of strains bearing these fusion plasmids indicated that the expression of the hybrid proteins paralleled that of nitrate reductase by the parent plasmids, demonstrating that the regulatory signals did not extend significantly into the first structural gene. The transcriptional start site and the level of the transcription were determined by the S1 mapping procedure. One major transcript was identified which was initiated −50 bp from the translational start site of the first structural gene. The synthesis of the transcript was repressed aerobically, was fully induced by nitrate anaerobically, and was greatly reduced in an Fnr⁻ mutant. Possible regulatory sequences were identified in the 200-bp regulatory region extending upstream from the transcription start site.

Escherichia coli can use nitrate as an effective electron acceptor for anaerobic respiration by reducing it to nitrite. The terminal enzyme in the electron acceptor pathway, nitrate reductase, is produced only under anaerobic conditions and is induced to its maximum level when nitrate is present in the medium (18, 20). The anaerobic formation of nitrate reductase is under the control of the *fnr* gene product (2, 22), a positive pleiotropic regulator which also affects the formation of several other enzymes involved in anaerobic metabolism (10, 19, 24). A second positive regulator, the product of the *narL* gene, is required for the further induction of the anaerobic production of nitrate reductase by nitrate. A mutation in the *narL* locus permits the normal anaerobic induction of nitrate reductase but makes the cells unresponsive to nitrate (22). The *narL* mutations map in the same region of the chromosome (22), but are located outside the *nar* operon (E. S. Edwards and J. A. DeMoss, unpublished data).

The *nar* operon, which encodes the three subunits of nitrate reductase, consists of three structural genes transcribed in the order *narG* (α subunit), *narH* (β subunit) and *narI* (γ subunit) (1, 4, 5, 23). The intact *nar* operon cloned into a multicopy plasmid overproduced the three subunits of nitrate reductase in the transformed strain under the control of the same factors which regulate the chromosomal operon (17).

Recently, a portion of the 5′ untranslated region of the *nar* operon was sequenced and the translation start site of *narG* was determined (11, 14). Studies with deletions into the 5′ end of the *nar* operon suggested that the 5′ untranslated region includes a promoter and two regulatory regions (11). A regulatory domain immediately adjacent to the *narG* gene apparently includes a transcriptional start site and the sequence recognized by the *fnr* gene product, which induces the anaerobic expression of *nar* operon. A second regulatory domain extending further upstream determines the interaction with the *narL* product, which is activated by nitrate and leads to an increase of the anaerobic expression of the operon.

In the studies reported here, we have sequenced the entire regulatory region, defined more precisely the 5′ limit of the operon, and determined the transcriptional start site under the several conditions of induction.

MATERIALS AND METHODS

Strains and growth conditions. All bacterial strains used in this study are listed in Table 1. Strains RK5285 and RK5288 were generously provided by Valley Stewart. All strains were grown and maintained in L broth or L broth supplemented with appropriate antibiotics.

Aerobic cultures were grown on a vigorously shaking water bath at 37°C and harvested when the turbidity reached approximately 100 Klett units (green filter). Anaerobic cultures were grown in filled bottles standing at 37°C and harvested at a turbidity of about 110 Klett units. Strains containing Tn*10* insertions or plasmids were grown on media supplemented with tetracycline (25 μg/ml) or ampicillin (50 μg/ml).

Plasmid construction. Plasmids pSL962, pSL964, pSR96, and pSR91 were constructed previously (11). Plasmid pZ178, kindly supplied by G. Weinstock, was constructed by R. Zagursky (personal communication). This plasmid is identi-

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TABLE 1. *E. coli* K-12 strains

Strain	Genotype	Reference
PK27	*thi* (Hfr)	5
SSR100	*rpsL nar* (F^-)	11
MC4100	F^- *araD139* Δ(*argF-lac*)*U169* *rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR*	21
RK5285	*fnr*$^+$ *zcg-622*::Tn*10 narG*::Mu d1 Φ(*nar-lac*)[a]	22
RK5288	*fnr-250 zcj-637*::Tn*10 narG*::Mu d1 Φ(*nar-lac*)[a]	22

[a] Derived from MC4100; for full genotype, see reference indicated.

cal to plasmid pMLB1034 (21), except that it contains a bacteriophage M13 intergenic region derived from plasmid PZ152 (25).

Plasmids pSL11, pSL12, and pSL13 were constructed as follows. Plasmid pSR96 was digested completely with *Sal*I and then partially with either *Nco*I or *Dra*I. After the ends were filled by incubation with T4 DNA polymerase, the fragments were religated with T4 DNA ligase. A Nar^- mutant, SSR100 (11), was transformed with the resulting mixture, and individual Amp^r transformants were selected and tested for the Nar phenotype by the overlay assay of Glaser and DeMoss (7). Minipreparations of the plasmids from the Nar^+ isolates were digested with either *Eco*RI or *Bal*I to establish that the appropriate regions had been deleted. The deleted plasmids resulting from partial digestion with *Nco*I were also checked to verify that the *Nco*I site in the *narG* gene was retained. Plasmids pSL11 and pSL12 were derived from the partial digest with *Nco*I, and pSL13 was derived from the partial digest with *Dra*I. The 5′ ends of the *nar* operon in plasmids pSL11 and pSL12 were verified by sequencing. Plasmid pSL12 was deleted at an unexpected position which did not contain an *Nco*I site (see Fig. 2).

Plasmid pSL17 was constructed by the following procedure. The *Pvu*II fragment containing the *nar* promoter and the first 176 nucleotides of the *narG* gene from pSL964 (Fig. 1) was isolated from an agarose gel and ligated with plasmid pZ178 which had been digested with *Eco*RI and then converted to a blunt-ended fragment with T4 DNA polymerase. Insertion of the *Pvu*II fragment regenerated two *Eco*RI sites. A plasmid with the correct orientation of the promoter region and in-frame fusion of *narG*::*lacZ* was isolated from Lac^+ transformants of strain MC4100 selected on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) plates. Plasmid pSL17 was derived from this plasmid by deletion of an *Eco*RI fragment containing the pBR322 sequences which were included in the original *Pvu*II fragment derived from pSL964. The structure of pSL17 (see Fig. 4) was confirmed by restriction endonuclease mapping with *Pst*I and *Eco*RI and nucleotide sequencing of the fusion region between *narG* and *lacZ*.

Construction of pSL19 involved extensive rearrangements because a procedure similar to that used to isolate pSL17 was unsuccessful. A *Bal*I fragment containing the *nar* promoter and the first 68 nucleotides of the *narG* gene from plasmid pSR9 (11, 17) was ligated with the same blunt-end derivative of pZ178 as that used to construct pSL17. No Lac^+ colonies were found among the transformants of MC4100, but plasmids were isolated which contained the inserted fragment with the promoter oriented in the incorrect direction. One of these plasmids was digested with *Bam*HI and *Eco*RI and then converted to blunt-ended fragments with T4 DNA polymerase, and the mixture was religated in an attempt to reorient and fuse the promoter and *narG* gene to *lacZ*. A plasmid was isolated from Lac^+ transformants of MC4100 which contained two copies of the *lacZ* gene sequence, one of which was fused to the *narG* gene and was under the control of the *nar* promoter. The promoterless *lacZ* gene sequence was deleted by digestion with *Nco*I and *Bal*I followed by religation. Plasmid pSL19 (see Fig. 4) was isolated from a Lac^+ transformant of MC4100, and its structure was confirmed by restriction endonuclease mapping and sequencing of the fusion joint.

Miniscale and large-scale plasmid preparations, transformations, digestion with restriction endonucleases, ligation, agarose and polyacrylamide gel analyses were carried out as described elsewhere (5, 17).

Nitrate reductase and β-galatosidase assays. Nitrate reductase activity was determined directly with cell suspensions (20). Protein concentrations in cell suspensions were determined by the method of Lowry et al. (12). Analyses on sodium dodecyl sulfate (SDS)-polyacrylamide gels of protein accumulated by plasmid-bearing strains were performed as described previously (17). β-Galatosidase activity was assayed as described by Miller (15).

DNA sequencing. The 1.5-kilobase *Eco*RI fragment containing the *nar* promoter from plasmid pSL962 was cloned in two orientations into M13mp10 and sequenced by using the commercial 17-mer primer and a synthetic 15-mer primer (positions −134 to −149; Fig. 2), respectively (11). The 5′ ends of *nar* operon fragments in pSL11, and pSL12 were determined by cloning the *Eco*RI fragments of both plasmids into M13mp11 and then sequencing with two primers: the 15-mer described above and a 16-mer which hybridizes with a sequence within the *narG* gene (11). To sequence the *nar* promoter fragment in plasmid pSL19, strain M103(pSL19) was infected and grown with helper M13 phage IRI1 (6). The mixture of helper phage and phages containing the single-stranded pSL19 were collected, and the purified DNAs were sequenced with the 15-mer primer described above.

Preparation of mRNA. Strains were grown aerobically or anaerobically in 100 ml of L broth with appropriate antibi-

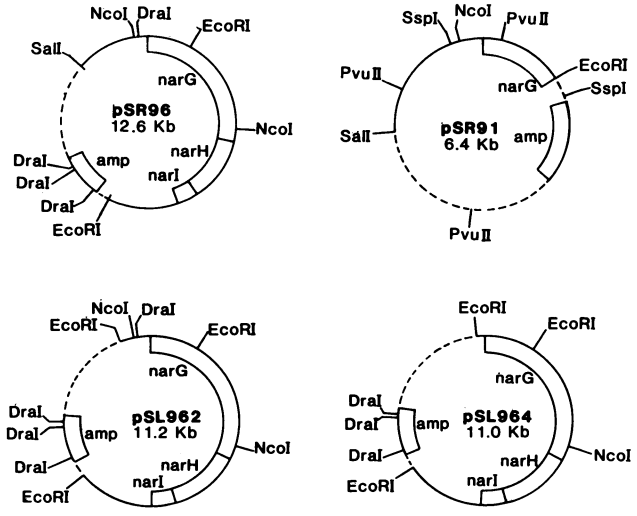

FIG. 1. Previously constructed plasmids (11) used in these studies. Symbols: ----, pBR322 sequences; ——, cloned *E. coli* sequences. The *nar* promoter and regulatory sequences are contained in the *E. coli* sequences adjacent to the *narG* gene.

otics and with or without nitrate. Rifampin was added to indicated cultures at a final concentration of 100 μg/ml. Cultures were poured into centrifuge tubes with ice and centrifuged, and pellets were suspended in 3 ml of 0.02 M sodium acetate (pH 5.5)–0.5% SDS–1 mM EDTA. After the addition to the suspension of 3 ml of phenol saturated with the same sodium acetate buffer, the mixture was incubated at 60°C for 5 min with gentle shaking. This phenol extraction procedure was repeated one more time, and the RNA was collected by ethanol precipitation (15).

S1 nuclease protection experiments. A phosphatase-treated *Ssp*I-*Pvu*II fragment from pSR91 was end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP, digested with *Nco*I, and denatured with sodium hydroxide. The labeled fragment (50 ng) was hybridized with 200 μg of extracted RNA as described by Maniatis et al. (13), and the mixture was digested with 800 U of S1 nuclease at 37°C for 2 h. The reactions were stopped by EDTA addition and phenol extraction. Samples were subjected to electrophoresis on a 7% polyacrylamide–urea sequencing gel. The sequence ladder was generated from the M13mp10 clone containing the 1.5-kilobase *Eco*RI fragment of pSL962 (Fig. 1). The sequence of the 17-mer primer used in generating the ladder is identical to the sequence of the 5′ end of the labeled probe at the *Pvu*II site (14). Thus the ladder had the same sequence as the protected DNA fragments.

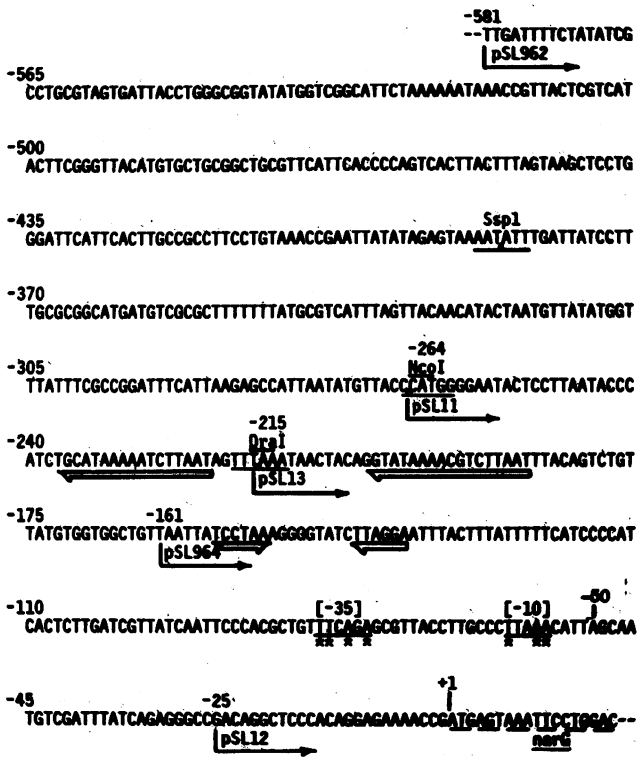

FIG. 2. Nucleotide sequence of the *nar* operon promoter and regulatory regions. The +1 position marks the translation start site for the *narG* gene (11, 14). The 5′ ends of the *nar* sequences contained in plasmids are marked with arrows labeled with plasmid designations, and the recognition sites for restriction enzymes *Nco*I and *Dra*I are underlined (their locations are shown above the sequence). The arrow at position −50 marks the transcription start site, and putative −10 and −35 promoter sequences are identified; asterisks identify bases in these sequences that are homologous to the corresponding consensus sequences for procaryotic promoters (8). Open arrows represent inverted and direct repeat sequences, which are discussed in the text.

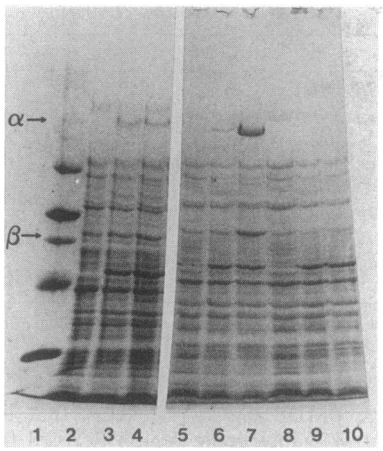

FIG. 3. Accumulation of α and β subunits of nitrate reductase by strains transformed with plasmids with subcloned derivatives of the *nar* operon. Strain SSR100 (*nar thi*) was previously shown to produce no detectable α or β subunit (11). Strain SSR100 transformed with pSL13 (lanes 2 to 4), pSL11 (lanes 5 to 7), or pSL12 (lanes 8 to 10) was grown aerobically (lanes 2, 5, and 8), anaerobically without nitrate (lanes 3, 6, and 9), or anaerobically with nitrate (lanes 4, 7, and 10). Cells were harvested, treated directly with SDS, and analyzed on SDS-polyacrylamide gels as described in Materials and Methods. Protein standards (lane 1) included β-galactosidase (116 kilodaltons [kDa]), phosphorylase A (97 kDa), bovine serum albumin (68 kDa), catalase (60 kDa), ovalbumin (43 kDa), and carbonic anhydrase (29 kDa).

RESULTS

Nucleotide sequence of the *nar* promoter region. Plasmid pSL962 (Fig. 1) was previously shown to carry the entire *nar* operon including the complete 5′ regulatory regions (11). The region (581 base pairs [bp]) extending upstream from the *narG* gene to the *Eco*RI site in plasmid pSL962 was sequenced as described in Materials and Methods (Fig. 2). This sequence verified the 161-bp sequence immediately adjacent to the *narG* gene which was previously determined with plasmid pSL964 (Fig. 1 and 2). This region was inferred to contain a transcriptional start site and the regulatory sequences which interact with the *fnr* gene product (11).

Delineation of the regulatory regions. To define more precisely the 5′ limit of the *nar* operon, the region containing the *nar* operon in plasmid pSR96 was subcloned by using two unique restriction sites, *Nco*I and *Dra*I, identified in the upstream sequence. Plasmids pSL11, pSL12, and pSL13 were constructed as described in Materials and Methods, with 5′ ends terminating as indicated in Fig. 2. The accumulation pattern of α and β subunits was examined directly in strains transformed with these derivative plasmids (Fig. 3). The Nar$^-$ mutant SSR100 did not produce detectable levels of either the α or β subunit of nitrate reductase. The accumulation pattern of SSR100(pSL11) was similar to that of SSR100(pSL962) (11), in which the production of the subunits was maximum under fully induced conditions (anaerobic conditions plus nitrate), was reduced under anaerobic conditions without nitrate, and was completely repressed under aerobic conditions. In contrast, expression in SSR 100(pSL13) was repressed aerobically and produced about the same amount of α and β subunits as the above strains

TABLE 2. Effect of *fnr* on expression of nitrate reductase by plasmids pSL962 and pSL964

Strain	Nitrate reductase activity[a] for:		
	Aerobic growth	Anaerobic growth	Anaerobic growth with nitrate
RK5285	0.00	0.00	0.00
RK5285(pSL962)	0.01	1.44	1.63
RK5285(pSL964)	0.03	1.31	1.61
RK5288	0.00	0.00	0.00
RK5288(pSL962)	0.01	0.20	0.19
RK5288(pSL964)	0.00	0.06	0.06

[a] Nitrate reductase activity was expressed as micromoles of nitrite per minute per milligram of protein.

anaerobically, but was not further induced by nitrate. Therefore this strain behaved similarly to SSR100(pSL964) (11). Strain SSR100(pSL12), which expressed a very low level of nitrate reductase in the overlay assay, did not produce detectable levels of subunits by the SDS-gel analysis under any of the growth conditions.

These results indicated that the subcloned *nar* operon in pSL11, which terminated at the *Nco*I site 264 bp upstream from the *narG* translational start site, contained intact regulatory and promoter sequences. The deletion which ended at the *Dra*I restriction site 215 bp upstream from the translational start site in pSL13 permitted the normal anaerobic expression of nitrate reductase but abolished the stimulation of the expression by nitrate. Therefore the 5′ limit of the *nar* operon must lie between the *Nco*I and *Dra*I restriction sites. In plasmid pSL12 the *nar* operon which terminated 25 bp 5′ to the translational start appeared to lose the regulatory and promoter sequences. The residual activity of nitrate reductase was presumed to result from readthrough transcription initiated from pBR322 vector sequences and extended into the *nar* structural genes.

Effects of the Fnr⁻ mutation. Fnr⁻ mutant strain RK5288 and its parent strain RK5285 (22) were transformed with pSL962 and pSL964. The levels of the nitrate reductase activity produced by these transformed strains are shown in Table 2. Although the wild-type strains carrying the plasmids, such as RK5285(pSL962) and RK5285(pSL964), overproduced the α, β, and γ subunits (11), the nitrate reductase activities did not reflect the level of subunit accumulation, since, as previously shown, much of the overproduced enzyme is inactive (17). For this reason the nitrate-induced activities of nitrate reductase in RK5285(pSL962) and RK5285(pSL964) reached the same maximum level. The levels of activity expressed by pSL962 and pSL964 were significantly reduced in the *fnr* mutant, RK5288, indicating that expression of the *nar* operon in both plasmids is dependent on the *fnr* gene product and that the 161-bp 5′ region present in plasmid pSL964 includes the sequences which are recognized and regulated by the *fnr* gene product. The difference in the level of expression of subunits in the *fnr* mutant and the wild-type strain is much greater than the activity levels shown in Table 2. The levels of subunits accumulated as visualized on gels by RK5285(pSL962) and RK5285(pSL964) were similar to those seen with SSR100 (pSL962) and SSR100(pSL964) (11), while the levels expressed by RK5288(pSL962) and RK5288(pSL964) were so low that subunits could not be easily visualized on SDS-gels (data not shown).

Regulation of expression of *nar*::*lacZ* fusion proteins. To establish a direct method for determining the level of expression of the *nar* operon in the various constructions, the promoter regions from plasmids pSL962 and pSL964 were fused with the *lacZ* gene, producing plasmids pSL19 and pSL17, respectively (Fig. 4). The hybrid proteins generated by pSL19 and pSL17 contain 22 and 58 amino acids, respectively, of the N-terminal sequence of the α subunits of nitrate reductase. Derivatives of Lac⁻ strain MC4100, transformed with plasmids pSL17 and pSL19, were grown aerobically, and portions of each were induced anaerobically with or without nitrate for 1.5 h. The specific activities of β-galactosidase produced from the plasmids and of nitrate reductase produced from the chromosomal copy of the *nar* operon were determined (Table 3). In both strains, nitrate reductase was expressed at a low level anaerobically and was fully induced by nitrate. The expression pattern of the β-galactosidase from pSL19, which contains an intact *nar* promoter, was similar to that of nitrate reductase produced from the chromosome. Similarly to the situation with the parent plasmid pSL964, from which the promoter was fused with the *lacZ* gene, the anaerobic production of β-galactosidase from pSL17 was not significantly stimulated by nitrate. The accumulation of the hybrid protein and the α subunit as visualized on SDS-polyacrylamide gels confirmed these observations (data not shown). These results show that expression from either the chromosome or plasmids does not interfere with expression from the other. They also demonstrate that the regulatory regions do not extend significantly into the structural genes of the *nar* operon and confirm the existence of the two regulatory domains.

Determination of the transcriptional start site. The location of the *nar* operon transcriptional start site was determined by S1 nuclease mapping of the 5′ end of mRNA which was induced when strains were grown either anaerobically or anaerobically with nitrate. In each culture used, the subunit accumulation patterns were confirmed by analysis on SDS-polyacrylamide gels. A radioactive probe was prepared as described in Materials and Methods by end labeling the 560-bp *Ssp*I-*Pvu*II fragment isolated from plasmid pSR91 (Fig. 1). The labeled probe was digested with *Nco*I to produce fragments of 120 and 440 bp (Fig. 5A), and the mixture was used for the S1 nuclease mapping experiments.

Small amounts of reannealed probe fragments were resis-

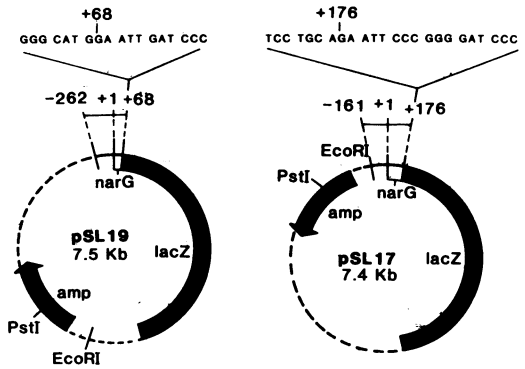

FIG. 4. Structure of plasmids containing *nar*::*lacZ* fusions. The plasmids were constructed as described in Materials and Methods, and the base sequences of the in-frame fusion joints between the *narG* and *lacZ* genes were established by nucleotide sequencing. -----, pBR322 sequences. The expanded regions represent fragments derived from the *nar* promoters region and the *narG* gene; +1 represents the translational start site for the *narG* gene, and the other numbers represent the number of nucleotides from the translation start site included in the subcloned fragment.

TABLE 3. Expression of nitrate reductase and β-galactosidase in strain MC4100 transformed with fusion plasmids pSL19 and pSL17

Strain	β-Galactosidase[a] activity for:			Nitrate reductase activity[b] for:		
	Aerobic growth	Anaerobic growth	Anaerobic growth with nitrate	Aerobic growth	Anaerobic growth	Anaerobic growth with nitrate
MC4100(pSL19)	0.00	0.61	4.42	0.00	0.11	0.82
MC4100(pSL17)	0.00	0.37	0.42	0.00	0.16	1.05

[a] β-Galactosidase specific activity is expressed as micromoles of *O*-nitrophenol per minute per milligram of protein.
[b] Nitrate reductase activity is expressed as micromoles of nitrite per minute per milligram of protein.

tant to S1 digestion and were visible on the gel at the top (440 bases) and near the bottom (120 bases). With mRNA prepared from induced cultures of PK27(pSL964) and PK27(pSL962), two major protected fragments of about 226 and 212 bases were produced (Fig. 5A, lanes 4 and 7, respectively). On the basis of the calibration of a specific sequence ladder (Fig. 5C), these protected fragments correspond to transcription start sites at −50 and −36 bp from the translation start site of the *narG* gene. The anaerobic production of the −50 transcript was stimulated by the presence of nitrate in the induction medium in PK27(pSL962) (Fig. 5A, lanes 6 and 7) but not in PK27(pSL964) (Fig. 5A, lanes 3 and 4).

Somewhat surprisingly, the 226-base protected fragment was also found with the aerobic cultures (Fig. 5A, lanes 2 and 5). To eliminate the possibility that anaerobically induced transcription of the *nar* operon occurred during the harvesting procedure, the experiment was repeated and rifampin was added to the cultures 5 min before the incubation was terminated. In this case no protected bands were detected in the aerobic cultures (Fig. 5B, lanes 4 ang 6). In other experiments (not shown) rifampin was added at the time of harvest, with similar results. Therefore the protected fragments observed with the aerobic cultures in Fig. 5A resulted from transcripts produced during the harvesting procedure. Furthermore, the fact that only the transcript that initiated at position −50 was detected in the aerobic cultures suggested that the −36 transcript detected in induced cultures was formed by degradation of the initial transcript during the longer induction period.

The kinetics of appearance and disappearance of the two transcripts in the following experiment were also in agreement with this suggestion. Aerobic cultures of PK27 (pSL962) were transferred to anaerobic conditions to induce expression of the *nar* operon, and after various periods rifampin was added, the cells were harvested, and mRNA

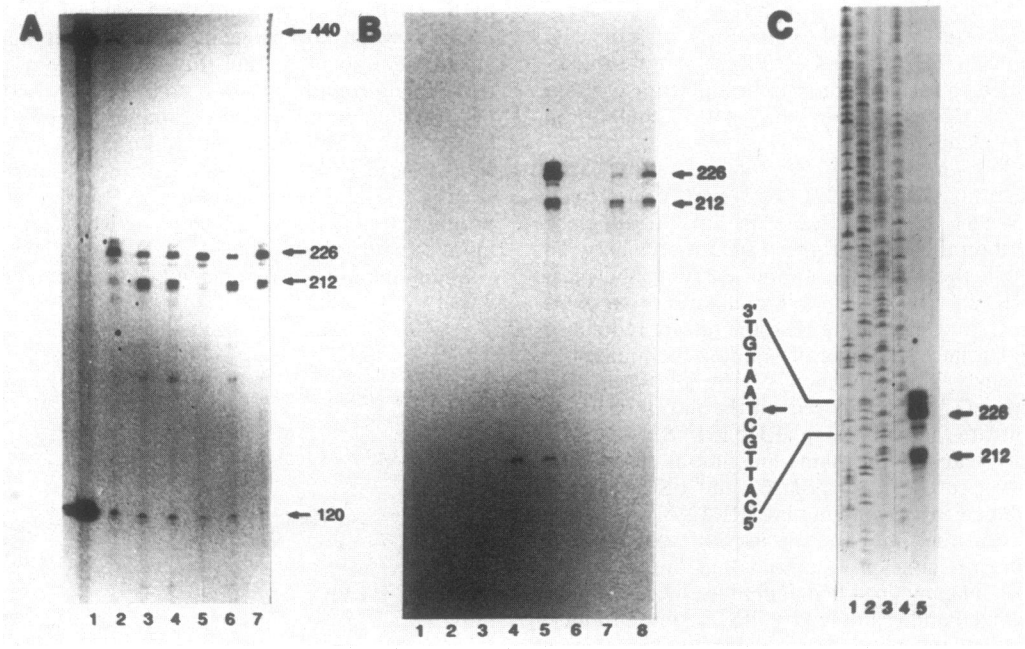

FIG. 5. S1 mapping of the transcriptional start site of *nar* operon. A 560-bp *Ssp*I-*Pvu*II fragment covering the entire promoter region and a segment of the first 176 bp of the *narG* gene was end labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP. The labeled DNA was digested with *Nco*I; resulting in a 120-bp fragment and a 440-bp fragment which includes a 264-bp sequence immediately adjacent to the structural gene and the 176-bp structural (*narG*) gene sequence. The DNA probe was subsequently denature and hybridized to RNA prepared from the strain grown under different conditions. The hybrids were digested with S1 nuclease and run on a sequencing gel with a DNA sequence ladder generated with the M13 system. The dry gels were subsequently autoradiographed with X-ray film. (A) *Nco*I-digested labeled probe (lane 1); PK27(pSL964) grown aerobically (lane 2), anaerobically (lane 3), or anaerobically with nitrate (lane 4); PK27(pSL962) incubated aerobically (lane 5), anaerobically (lane 6), or anaerobically with nitrate (lane 7). (B) Rifampin (100 μg/ml) was added to the cultures 5 min before harvest except for lane 5. RK5288(pSL962) grown aerobically (lane 1), anaerobically (lane 2), or anaerobically with nitrate (lane 3); PK27(pSL11) grown aerobically (lane 4) or anaerobically with nitrate (lane 5) (three times as much was loaded in lanes 4 and 5 as in the rest); PK27(pSL962) grown aerobically (lane 6), anaerobically (lane 7), or anaerobically with nitrate (lane 8). (C) Sequence ladder generated as described in Materials and Methods. Lanes: 1, C reaction; 2, T reaction; 3, A reaction; 4, G reaction; 5, same as panel B, lane 8, but overexposed.

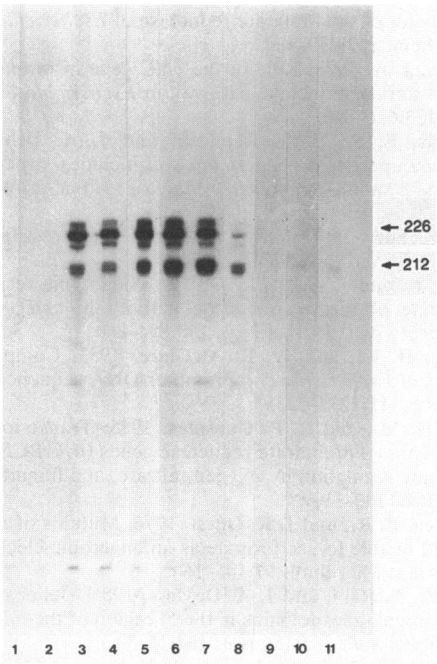

FIG. 6. Kinetic study of the anaerobic transcription of the *nar* operon by S1 mapping. The S1 mapping procedure was described in the legend to Fig. 5. The culture of PK27(pSL962) was grown aerobically to 100 Klett units. Part of the aerobic cultures was mixed with rifampin, and the incubation was continued aerobically for 5 min (lane 1). Another part of the aerobic cultures was transferred to anaerobic conditions for 0, 5, 10, 15, or 20 min (lanes 2 to 6, respectively), and rifampin was added at the time of harvesting. The remainder of the aerobic culture was shifted to anaerobic growth for 1.5 h, rifampin was added, and samples were removed after further anaerobic incubation for 0, 10, 15, 20, and 60 min (lanes 7 to 11, respectively).

preparations were analyzed as described above (Fig. 6). When rifampin was added 5 min before the transfer to anaerobic conditions, no protected fragments were detected. The appearance of the −50 transcript (226-base fragment) appeared to reach a maximum after 5 min, while the accumulation of the −36 transcript (212-base fragment) gradually increased up to 20 min (lanes 3 to 6). Cultures were also induced for 1.5 h, rifampin was added, and the cultures were incubated further for increasing periods before harvest. In this case an analysis of the mRNA by the same procedure revealed that the transcript corresponding to the 226-base fragment disappeared more rapidly than the shorter transcript did (lanes 7 to 10) and that both of them were almost not detectable after 20 min (lane 11).

These kinetic experiments, along with the observation that only the −50 transcript was produced in aerobic cultures during the short period of anaerobioses in the harvest procedure, favor the argument that transcription of the *nar* operon is initiated at position −50 relative to the translation start site and that the −36 position transcript is a degradation product.

Transcription of the *nar* operon as detected by this procedure was drastically reduced in an Fnr$^-$ mutant transformed with pSL962 (Fig. 5B, lanes 1 to 3). After prolonged exposure of the autoradiogram, a 226-base fragment could be detected in lanes 2 and 3 but not in the preparation from the aerobic culture in lane 1.

DISCUSSION

On the basis of the kinetics of formation and breakdown of the two major transcripts identified by the S1 nuclease protection procedure, we concluded that transcription of the *nar* operon is initiated at −50 bp from the translation start site of the *narG* gene. Putative promoter sequences can be identified (Fig. 2) which may correspond to the −10 and −35 consensus sequence of procaryotes (8), although the correspondence to the consensus sequence is rather poor (asterisks in Fig. 2 indicate homologies). A poor correspondence to the consensus sequence was also found in the *frd* (fumarate reductase) operon (3, 9), another operon which is regulated by the *fnr* gene product. The marked deviation of promoter sequences from the consensus sequence appears to occur generally in positively regulated promoters, and it has been suggested that positive regulators may be essential for binding RNA polymerase to otherwise weak promoter sequences (16).

Two additional bands were found on the autoradiographs in the S1 mapping experiments. One, corresponding to a 5′ end at −36 bp, most probably results from degradation of the −50 transcript. It is possible that degradation of the mRNA by cellular nucleases proceeds from the 5′ end and pauses upon encountering a ribosome bound to the ribosome-binding site (Fig. 2, positions −8 through −12), giving rise to the shorter transcript. However, we cannot exclude the less likely possibility that the shorter transcript is independently initiated after a delay and is more stable during growth under anaerobic conditions. Another protected band is visible on the autoradiographs immediately above the 226-base major band (Fig. 5A), representing a transcript several nucleotides longer than the major transcript. Possible overlapping −10 and −35 sequences can be identified in the nucleotide sequence in Fig. 2 which would correspond to this initiation site. However, it is not clear what role, if any, transcription initiation at this site plays in the expression of the *nar* operon.

In any case, the major transcription initiation site is located at about −50 bp from the *narG* translation start site, and the upstream regions involved in transcription regulation must include sequences involved in induction by the *fnr* gene product and in induction by the *narL* gene product. The subcloning experiments permitted us to define more precisely the 5′ limit of these sequences. Plasmids pSL11 and pSL19, in which the 5′ region is terminated at the *Nco*I site (−214 bp from the transcription start site), expressed their *nar* promoter-linked genes under the control of the same factors that regulate the chromosomal *nar* operon; i.e., they were induced by anaerobic conditions and nitrate. On the other hand, expression of the *nar* operon in plasmid pSL13, in which the 5′ region is terminated at the *Dra*I site (−165 bp from the transcription start site), was not stimulated by nitrate, although levels of expression were normal under anaerobic conditions. These results establish the 5′ limit of the *nar* operon promoter and regulatory regions between −165 and −214 bp from the transcription start site.

The 5′ limit of the region required for anaerobic induction of *nar* transcription by the *fnr* gene product was intact in plasmids pSL964 and pSL17, in which the *nar* regulatory region is terminated −111 bp from the transcription start site. Thus the sequences required for interactions with the *fnr* gene product are located within the first 111 bp upstream from the transcription start site, while the region required for the further induction by nitrate and the *narL* gene product extends 54 to 100 bp farther upstream. It is not possible from

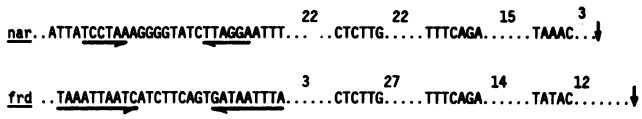

FIG. 7. Comparison of the *nar* and *frd* (3, 9) promoter sequences. The arrow represents the transcription initiation sites, and the numbers represent the number of base pairs between the homologous sequences or structures.

our results to conclude whether the region which determines interaction with the *narL* gene product overlaps physically with the region defined for reaction with the *fnr* gene product. However, the results with fusion plasmids pSL19 and pSL17 define the 3′ limit of the regulatory region. Sequences required for regulation by either of these factors do not extend significantly into or beyond the first structural gene.

Examination of the nucleotide sequence across the two regulatory regions delineated in these studies reveals some specific sequences which may be involved in the regulation by the *fnr* gene product and by the *narL* gene product. The sequence immediately upstream from the *nar* transcription start site was compared with the analogous region of the *frd* operon, which is also positively regulated by the *fnr* gene product (3, 9). The two operons contain three virtually identical short sequences within the first 60 to 70 bp upstream from transcription start sites followed by an inverted repeat upstream (Fig. 7). Furthermore, two nearly identical 15-base direct repeats separated by 17 bases are found near the *Dra*I site (Fig. 2).

The overall structural features of the nucleotide sequence of the *nar* promoter and regulatory regions suggest that the regulation of transcription involves the interaction of several regulatory proteins over a significant distance on the DNA. Transcription is initiated at the same site whether expression is partially induced by anaerobic conditions or fully induced by anaerobic conditions with nitrate. Interactions of at least two regulatory factors, involving sequences extending at least 160 bp upstream from the transcription start site, must cooperate to regulate the initiation of transcription from that site. A detailed definition of the binding proteins, their binding sites, or their interactions with RNA polymerase is required to establish the mechanisms involved in this complex regulation process.

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