Transcriptional Control of Genes Encoding CS1 Pili: Negative Regulation by a Silencer and Positive Regulation by Rns

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The adherence of enterotoxigenic Escherichia coli (ETEC) to the human small intestine is an important early event in infection. Attachment is thought to be mediated by proteinaceous structures called pili. We have investigated the regulation of expression of the genes encoding CS1 pili found on human ETEC strains and find that there are at least three promoters, P1 and P2, upstream of the coo genes, and P3, downstream of the start of cooB translation. We identified a silencer of transcription which extends over several hundred bases overlapping the cooB open reading frame. This silencer is dependent on the promoter and/or upstream region for its negative effect. The DNA binding protein H-NS is a repressor of coo transcription that acts in the same region as the silencer, so it is possible that H-NS is involved in this silencing, Rns, a member of the AraC family, positively regulates transcription of the coo operon and relieves the silencing of CS1 expression.

Human enterotoxigenic Escherichia coli (ETEC) strains cause diarrheal disease in travelers and infants and are considered to be a serious health problem in developing countries due to the number of children in which infection leads to mortality (1, 27). One of the earliest steps of infection is attachment of the ETEC pathogen to the intestinal mucosa. This is thought to be mediated by adherence factors called pili, which are proteinaceous appendages extending from the surface of the bacterial cell. Each pilus, which is composed of thousands of identical subunits of the major pilin protein with one or a few subunits of a different protein at the tip (40, 45), mediates specific interactions with target molecules exposed on the surface of the ileal cells. Among different ETEC strains isolated from humans, pilis with a limited number of distinct serotypes have been identified. These include coli surface antigen 1 (CS1), CS2, colonization factor antigen I (CFA/I), and CS4. For these pilis, the major pilin proteins are similar at the amino terminus (16). For CFA/I, CS1, and CS2, it has been shown that the other three genes required for production of pilis are highly homologous (13, 14, 22, 23).

The CS1 pilus structure is encoded by four linked genes, cooB, cooA (major pilin gene), cooC, and cooD (Fig. 1), that, if transcribed from a constitutive promoter on a multicopy plasmid, are sufficient in an E. coli K-12 background to produce functional pilis (13). When the coo genes are expressed from the wild-type promoter(s), the trans-acting protein Rns, which is a member of the AraC family, is required for pilin expression (4). In other members of this pilus family, the positive regulators are similar enough to Rns that Rns can functionally substitute for them (5, 8, 50, 51), suggesting that regulation of all of them may be similar. Some pilis of other human intestinal E. coli pathogens that are not related to the CS1 pilus family are also regulated by proteins with strong homology to Rns. These include AggR (32) of enteroaggregative E. coli, which regulates aggregative adherence fimbriae, and BfpT (47) of enteropathogenic E. coli, which is needed for expression of bundle-forming pili. Functional substitution has not been tested in these cases.

Rns homologs have also been identified in other gram-negative enteric bacteria, where these proteins have an important function as global regulators of virulence factors. These Rns-related regulators include VirF of Yersinia flexneri (39), VirF of Yersinia enterolitica (7), and ToxT/TcpN of Vibrio cholerae (33). This family of Rns-like proteins positively regulates transcriptional expression, although the mechanism of control has not been well defined in most of the systems.

In addition to specific activators like Rns and specific repressors, in E. coli there are small proteins which negatively regulate a large number of unrelated genes. One of these global regulators is the DNA binding protein H-NS (48). This small, relatively abundant protein binds to many sites on DNA. Among the genes that are regulated by H-NS are those in the pap (17) and type 1 pilin (26) operons. Also, the CFA/I pilus operon, which is very closely related to that of CS1, is negatively regulated by H-NS (20).

In this work, we studied the regulation of CS1 pili as a prototype of the family of pilis of human ETEC strains. In addition to the strong positive regulation by Rns, we found a negative regulatory region overlapping the first open reading frame that silences CS1 expression. We also found that the DNA binding protein H-NS inhibits transcription and that this inhibition involves the silencer region. It appears possible from our results that an additional negative regulator may also be involved.

MATERIALS AND METHODS

Media. Luria-Bertani broth (43) and MacConkey agar containing lactose (BBL) were used for bacterial growth. Antibiotics used were ampicillin (50 μg/ml), chloramphenicol (40 μg/ml), kanamycin (50 μg/ml), spectinomycin (100 μg/ml), and tetracycline (10 μg/ml).

Bacterial strains. E. coli K-12 strains MC4100 (6) and JM83 (29) were used for cloning. LMC10 is a lac deletion restriction-negative derivative of the O6:K15: H16 ETEC-derived strain C921b-2 (4, 36), which was cured of the plasmid encoding Rns and CS3. E. coli K-12 strain THK38 (25) and the isogenic hns-lacZ derivatives, THK40, were used in β-galactosidase assays. The mutant allele contains a lacZ gene inserted into a unique HindIII site 111 bp from the N terminus of H-NS. THK40 (24a) was constructed by transducing the hns-2 allele from THK30 (26) into ORN115 (34). Plasmids. The following plasmids were used for cloning: the high-copy-number vector pNEB193, which is a pUC derivative and carries ampicillin resistance (New England Biolabs); the low-copy-number pSC101-based vector pHSG576, which carries chloramphenicol resistance (46); and the single-copy vectors with an R100 origin of replication pEU720 and pEU730, which contain the pNEB193 polylinker cloned into the XhoI site of pEU720 (12, 15). Plasmids pEU730 and pEU710 carry spectinomycin resistance and a promoterless lacZ gene. In addition, they both have transcription terminators surrounding the

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5736
region containing the multiple cloning site and lacZ gene and an RNase III cleavage site between the multiple cloning site and the ribosomal binding site for lacZ. Plasmid pEU731 was constructed by cloning a 0.322-kb PvuII fragment from pUC19, which contains the lactose promoter, into the PmeI site of the pNEB193 polylinker in pEU730 (45a). Plasmid pEU756 (Fig. 2) was constructed by amplifying a 0.520-kb CS1 fragment from LMC10 with primers CS19 (5′ TTTGACAATTGTCAGGGATGGAGGG3′) and CS20 (5′ GTATGCTAG GTACCTGTGTTCTTTATAAGAAGACT3′), digesting with Asp718, and ligating into the Asp718 site of pEU731 between the lactose promoter and the RNase III site, which is upstream of the β-galactosidase gene. The synthesized Asp718 restriction enzymes sites of each primer are marked in the sequence in bold. Plasmid pEU753 (Fig. 2) was constructed by amplifying a 0.867-kb CS1 fragment from LMC10 with primers CS18 (5′ GTGGACTTGGTACCAGCGGATCC3′) and CS02A (5′ TTGAAGTTGGTACCAGCGGATCC3′), digesting with Asp718, and ligating into the Asp718 site of pEU731. The synthesized Asp718 sites of each primer are marked in the sequence in bold.

FIG. 1. Genetic organization of the coo genes of ETEC. The direction of transcription is shown by the arrow. The location of the omega-Km cassette (10) in cooB-1 (44) is indicated (it is not drawn to scale). The 1.3-kb region of DNA cloned into pEU720 to construct pEU745 is shown with an open bar.

FIG. 2. β-Galactosidase production by MC4100 carrying transcriptional fusion plasmids. The P1, P2, and P3 promoter sites are labeled. The extent of cooB and cooA is indicated by the lines. Only a part of cooA is shown on this map. The thick black bars indicate the regions of the coo genes cloned in the lac fusion vector pEU720, and the regions of CS1 cloned into pEU731 are shown by the white bars. The name of each plasmid is written beside each bar. The numbers above the bars designate the ends of the cloned fragments. pEU730 expresses 97 ± 7 U and pEU731 expresses 6,086 ± 373 U. Units are calculated as described by Miller (31). The results are the means ± standard percent error of at least three separate assays. –Rns, pEU2040 was absent; +Rns, pEU2040 was present; U, the strain segregates Lac– colonies; ND, not determined.
To construct pEU745 (Fig. 2), a 1.3-kb fragment from LMC10 was amplified with primers CS1 (5' CCGCGCAGGACCTCAACG3') and CS2 (5' CGGCCAGGACCTCAACG3'). This fragment (open bar, Fig. 1), consisting of 0.5 kb of the DNA upstream of the coo genes, the cooB open reading frame, and approximately 0.1 kb of the cooA gene, was ligated into the HindIII site of pNEB193 to construct pEU2060. In pEU2060 the cooB gene is transcribed from both the lactose promoter and the CS1 promoters. pEU745 was constructed by amplifying the 1.3-kb CS1 fragment from pEU2060, using primers PNEBPR2 (5' AAGACCTCGAGGTTAAACGAGGCGGCA3') and PNEBPR3 (5' TAGT AGTCTGACGCGGCCGCAAGCTT GCAATCGCCTT'). The synthesized XhoI restriction enzyme sites of each primer are marked in the sequence in bold, and the 5' end of the insert in pEU749 were destroyed. pEU742 and 5' I sites 3' of the CS1 insert in pEU745 were digested with XhoI and ligated into the XhoI site of the transcriptional fusion vector pEU720 (15) so that the CS1 promoters drive the promoterless lacZ gene. This construct contains the CS1 insert flanked on both sides by the pNEB193 polynucleotide kinase. 

Unidirectional deletion derivatives of pEU745 were constructed by digestion with exonuclease III, using the Erase-A-Base kit with a slight modification of the protocol described by Promega. Instead of S1 nuclease, mung bean nuclease was used, with exonuclease III, using the Erase-A-Base kit with a slight modification of the protocol. The synthesized restriction enzyme sites of the amplification primer were marked in the sequence in bold, and the 5' end of the insert in pEU749 were destroyed. Plasmid pEU2030 contains ms cloned into pUC18 (14), and pEU2040 contains ms cloned into pHSG576 (36).

**β-Galactosidase assays.** Cells were harvested from mid-log-phase cultures grown aerobically at 37°C in Luria-Bertani broth plus antibiotics to select for plasmid maintenance. All assays were performed in the lac deletion strain MC4100 as described previously or in the lac hns-2 mutant strain THK40 and the isogenic hns + strain, THK38 (15, 31).

**Primer extensions.** RNA was isolated from ETEC strain LMC10/pEU2030 (which carries the ms gene). Reverse transcriptase was used to extend a complementary DNA strand from a primer (CS13) located 66 bases downstream of the translational start of cooB. Two predominant products that mapped upstream of the cooB gene were identified (Fig. 3). These primer extension products were separated by 1 bp and were of equal intensity, suggesting that transcriptional initiation occurred at both G residues (S1 and S2 in Fig. 3) with similar frequency. The 5' end of S1 is defined as position +1, and it is 15 bases from the translational start of cooB. Sequence analysis of the DNA directly upstream of these transcriptional start sites revealed a match to the consensus prokaryotic −10 and −35 promoter elements in four of six bases, and the distance between the two regions (17 bases) is

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**RESULTS**

**Location of transcriptional initiation by primer extension.** The four genes needed to produce CS1 pili are organized as shown in Fig. 1. The gene that encodes the major pilin protein, cooA, is preceded by cooB. Previous studies with a cooB mutant (cooB-1) of the ETEC strain LMC10 (strain JEF100) showed that insertiondisruption of cooB by an omega-Km element encoding transcriptional and translational stop signals reduced expression of the CooA pilin protein (44). This indicates that CooB and CooA are encoded on one transcript, although there may be an additional weak promoter for cooA downstream from the insertion in JEF100.

To define the initiation site of coo transcription, RNA was isolated from ETEC strain LMC10/pEU2030 (which carries the ms gene). Reverse transcriptase was used to extend a complementary DNA strand from a primer (CS13) located 66 bases downstream of the translational start of cooB. Two predominant products that mapped upstream of the cooB gene were identified (Fig. 3). These primer extension products were separated by 1 bp and were of equal intensity, suggesting that transcriptional initiation occurred at both G residues (S1 and S2 in Fig. 3) with similar frequency. The 5' end of S1 is defined as position +1, and it is 15 bases from the translational start of cooB. Sequence analysis of the DNA directly upstream of these transcriptional start sites revealed a match to the consensus prokaryotic −10 and −35 promoter elements in four of six bases, and the distance between the two regions (17 bases) is
optimal for sigma-70 holoenzyme recognition (Fig. 3) (19, 37). We named this promoter P2 (see below for promoter P1).

Promoter location by lac fusion analysis. In wild-type ETEC strains, the coo genes are carried on a large low-copy-number plasmid (2). To study CS1 promoter activity and to localize regions of regulation, we maintained a low gene dosage by using an R100-based vector system. The single-copy transcriptional fusion vector pEU720 (15) was used to place the report gene, lacZ, under the control of cloned coo fragments. Plasmid pEU720 has transcriptional terminators, which reduce exogenous transcription, surrounding the cloning site (XhoI) and the lacZ gene. An RNase III cleavage site is located between the XhoI site and the ribosome binding site of lacZ in pEU720, thus ensuring that the 5’ end of the lac transcript is the same in each construct. Therefore, any regulation observed by β-galactosidase analysis should be at the level of transcription and not due to RNA processing. Since all of the clones containing CS1 fragments fused to the lacZ gene also have the pNEB193 polylinker inserted in the XhoI site (see Materials and Methods), we used pEU730 (12) as a control for the basal level of β-galactosidase activity expressed by the vector during our assays.

To investigate the possibility of another promoter upstream of P2, we used pEU742, which does not have P2 but contains bases −411 to −36 of coo fused to lacZ (Fig. 2). MC4100/pEU742 exhibited strong transcriptional activity (Fig. 2). Although it is possible that the promoter defined by pEU742, P1, was created at the cloning junction and may contain vector sequences, analysis of the DNA sequence showed no sigma-70 recognition site at the junction.

We were unable to identify the P1 start site by primer extension of RNA isolated from LMC10/pEU2030 with several primers, even when the reaction mixture contained 100 times more RNA than is needed to identify P2, because the bands observed were very weak and not consistently present. It seems possible, therefore, that the apparent strength of P1 in pEU742 (Fig. 2) may be due to deletion of downstream sequences which repress transcription from P1. Experiments described below (see Fig. 4) confirm this and define the location of P1.

Identification of P3 by using β-galactosidase transcriptional fusions. Western blot analysis with anti-CS1 antisera indicated that although pilin expression was greatly reduced in the cooB-1 omega-Km insertion mutant JEF100 (Fig. 1), detectable levels of CooA pilin were still present (44). This suggests that there might be a weak promoter downstream of the omega element in JEF100. To investigate this, we used pEU749, which contains the region of DNA downstream of the omega element (bases +93 to +929) fused to lacZ. MC4100/pEU749 expressed 358 U of β-galactosidase (Fig. 2), which is 3.7 times higher than that expressed by the strain with the vector alone (Fig. 2). This indicates that transcriptional initiation occurs in the region downstream of the cooB translational start.

To further localize the downstream promoter defined by pEU749, we tested pEU751 (containing bases +517 to +929 fused to lacZ). Strain MC4100/pEU751 expressed approximately 6.8-fold more β-galactosidase activity than the strain with the vector alone (Fig. 2), suggesting that promoter P3 is located between bases +517 and +929. Because MC4100/pEU751 exhibited only about one-ninth as much β-galactosidase activity as did MC4100/pEU743, P3 seems to be significantly less active than the upstream promoters.

To determine the exact site of promoter P3 initiation, RNA from LCM10/pEU2030 was used in primer extension with primer CS02 (bases 930 to 912). The 5’ end of the transcript was located at base 751 in the intergenic region between cooB and cooA and is 25 bases downstream from the end of cooB (data not shown). Sequence analysis of the DNA upstream of this start site revealed a match to a consensus sigma-70 promoter. The −10 region extends from base 734 to 739 and matches the consensus in four of six bases. The −35 region, which is at the end of the cooB open reading frame, extends from base 712 to 717 and matches the consensus in only three of six bases, and the spacing between the elements is 16 bp (19, 37).

Regulation by Rns. In ETEC strains, including LMC10, no products are detectable from the coo operon in the absence of Rns (4, 40a). Therefore, we asked whether any or all of the promoters we identified were dependent on Rns for activity.

For promoter P1, MC4100/pEU742 produced about the same amount of β-galactosidase in the absence of the Rns-encoding plasmid as in its presence (Fig. 2). Because of the lack of Rns activation and the weakness of P1 in its normal context (i.e., in LMC10/pEU2030, which has the entire coo operon; see above), it seems unlikely that this promoter is used significantly in the natural situation.

From P3 on the other hand, Rns increased transcriptional activity of pEU749 approximately 2.9-fold (Fig. 2) and of pEU751 about 2.2-fold. RNA isolated from LMC10 in the absence of Rns showed no transcription starting from P3 in primer extension with the same primer (CS02) used to locate P3 (data not shown), suggesting that significant transcription from P3 in the natural situation (i.e., with upstream coo sequences present) requires Rns.

For P2, no RNA was detected in primer extension analysis using RNA from LMC10 in the absence of pEU2030 (Fig. 3). This suggests that the two bands observed with RNA isolated from LMC10/pEU2030 represent Rns-dependent transcription initiation sites and that promoter P2 is regulated by Rns.

To localize the site at which Rns acts to increase transcription initiating from P2 and to quantitate the magnitude of the increased expression, we examined pEU743, which contains a fragment of the coo region consisting of bases −411 to +7 fused to the lacZ gene (Fig. 2). Although plasmid pEU743 also contains promoter P1, any effect of Rns on pEU743 transcription should be at promoter P2 since our results show that P1 is not Rns regulated (Fig. 2; pEU742). Strain MC4100/pEU743 exhibited a high level of β-galactosidase activity. This level increased at least sixfold (results not shown) when pEU2040 containing Rns was added to the strain, but the amount of β-galactosidase varied widely with each assay performed on MC4100/pEU743/pEU2040. Analysis of this strain on MacConkey indicator medium showed Lac+ and Lac− segregants. When plasmid DNA from several segregants was transformed into MC4100/pEU2040, the DNA isolated from the Lac− segregants yielded a mixture of Lac+ and Lac− transformants, while the plasmids isolated from several Lac− segregants yielded only Lac− transformants. This suggests that the presence of Rns induced such a high level of β-galactosidase in cells containing pEU743 that mutants were selected which no longer express lac. Thus, although we could not accurately determine the magnitude of Rns-mediated activation of transcription from pEU743 because the Lac phenotype was unstable, these results strongly suggest that a region of DNA between −411 and +7 is necessary to detect Rns-regulated activation of P2.

Negative regulation within the region encoding CooB and CooA. Although the E. coli K-12 strain MC4100/pEU743 showed very strong transcriptional activity in the absence of Rns (Fig. 2), no transcriptional initiation was detectable by primer extension in the absence of Rns with the ETEC-derived strain LMC10 containing the complete coo operon (Fig. 3). This may be due to differences between the wild-type ETEC
strain and *E. coli* K-12 or to negative regulation of transcription that is dependent on the *coo* region downstream of the end of pEU743 (base −411 to +7). To test this, we examined pEU745, which includes all of the region in pEU743 and an additional 922 bases downstream. In the absence of Rns, there was 30-fold-less β-galactosidase activity in MC4100/pEU745 than in MC4100/pEU743 (Fig. 2), showing that the additional 922 bp of *cooB-cooA* in pEU745 exerts a negative effect on transcription.

To localize this negative regulatory region further, plasmid pEU748, a smaller clone that does not include the *cooB-cooA* intergenic region or the 5′ terminus of *cooA*, was tested. MC4100/pEU748 expressed 3.3-fold-more β-galactosidase activity than did MC4100/pEU745 (Fig. 2). Further progressive deletions into *cooB* showed additional increases in the level of β-galactosidase activity (e.g., pEU747 and pEU746; Fig. 2), indicating the presence of more than one negative regulatory site between bases +7 and +929.

This negative regulatory region appears to act on transcription that originates at P3 as well as that originating at P1 and P2. Comparing two plasmids that have neither P1 nor P2, but only P3, we found that pEU751 showed a 1.8-fold-higher constitutive level of transcription than did the larger plasmid pEU749, which includes the negative regulatory region, was used for primer extension, neither the P1 nor the P2 start was visible (Fig. 4). The finding that transcription from P3 in pEU749 is detectable (Fig. 2), while that from P3 in LMC10 is not, suggests that the part of the negative regulatory region present in pEU749 is incomplete.

**Localization of P1 in pEU746 by primer extension.** Since pEU746 is partially deleted for the negative regulatory region (see above), RNA from MC4100 containing this plasmid was used in primer extension with primer CS13 (bases 83 to 63) to try to localize P1. In addition to the two 5′ transcript ends generated from P2 (S1 and S2), we saw a start upstream of P2 (S) at base −34 (Fig. 4). On the other hand, when RNA isolated from MC4100 containing pEU745, which includes the negative regulatory region, was used for primer extension, neither the P1 nor the P2 start was visible (Fig. 4). Sequence analysis of the DNA upstream of this potential P1 start showed two matches to a consensus sigma-70 promoter (Fig. 4). The furthest downstream (P1A in Fig. 4) matches the consensus at four of six bases at both the −10 and −35 regions, with a spacing of 16 bases between the elements. The second predicted promoter (P1B) matches the consensus at three of six bases at both the −10 and −35 regions, with a spacing between the elements of 17 bases. Both of these promoters are probably close enough to the consensus to be functional (19, 37).

Plasmid pEU742 (Fig. 2), which contains *coo* DNA from base −411 to −36, includes both predicted P1 promoters, although it lacks the transcriptional start site (which is at base −34). Therefore, the high constitutive level of β-galactosidase activity observed for pEU742 (Fig. 2) is probably due to the presence of the P1 promoters and the absence of the downstream silencing region in this plasmid.

**Effect of Rns on negative regulation.** When pEU2040 (encoding Rns) was added to MC4100/pEU745, the transcriptional activity varied greatly and, like MC4100/pEU743/pEU2040, this strain exhibited a mixture of Lac′ and Lac− segregants when streaked on MacConkey indicator medium. This type of unstable phenotype was observed when pEU2040 was added to MC4100 containing any of the transcriptional fusions described above (pEU746, pEU747, and pEU748). PCR analysis of the plasmid DNA isolated from Lac− segregants of MC4100/pEU745/pEU2040, MC4100/pEU747/pEU2040, and MC4100/pEU748/pEU2040 demonstrated that deletions had occurred in the CS1 inserts cloned in each of the plasmids (data not shown), suggesting that the high level of β-galactosidase activity induced by Rns was toxic to the cells. Toxicity of high-level β-galactosidase synthesis has been observed previously (9, 49). Although the instability of these strains made it impossible to measure β-galactosidase activity, the data suggest that Rns increases transcription to more than 15,000 U in all these strains (data not shown).

**Negative regulation is dependent on sequences upstream of the start of transcription.** To determine whether the negative regulation exerted between bases −7 and +929 of *coo* requires P2 and/or the upstream region, two overlapping fragments of the *cooB-cooA*-negative regulatory region were inserted in turn between the lac promoter and the *lacZ* gene of pEU731 (which is a derivative of pEU730) to yield pEU756 and pEU753 (Fig. 2). The control strain with the lac promoter driving *lacZ*, MC4100/pEU731, exhibited 6,086 U of β-galactosidase activity, which is very similar to the amount in MC4100/pEU745 (5,916 U) in the absence of Rns (Fig. 2). This suggests that the lac promoter is similar in strength to P1 plus P2 of *coo*, and therefore the degree of negative regulation by the inserted *coo* DNA might be anticipated to be similar. However, the insertion of a DNA fragment from *coo* with bases −5 to +518 (pEU756) or +63 to +930 (pEU753) between the lac promoter and the *lacZ* gene had no detectable effect on transcription initiated from the lac promoter (Fig. 2). Therefore, the negative effect observed previously in the *cooB-cooA* coding region is dependent on the DNA upstream of base −5 of the *coo* genes. These results rule out the possibility that the neg-
The results demonstrate that a mutation in hns used for promoter P3. No products were seen for any of the fusion plasmid pEU745 (which contains the silencer repressor) in the mutant strain. The fact that Rns does increase transcription in the absence of Rns is caused by the extra transcription resulting from the difference in stability of pEU743 and pEU745 in the presence of Rns. H-NS exerts most of its inhibitory effect in this deleted region. The small DNA binding protein H-NS regulates the genes encoding CS1 pili. In vivo, Rns is needed for expression of the coo genes. We found by primer extension analysis and by lac fusion studies that the major Rns-regulated promoter of this operon is P2, located upstream of these genes (Fig. 3). The sequence of P2 suggests that it should be a strong sigma-70-dependent promoter. In addition to the homology in the −10 and the −35 regions (four of six bases for each) and optimal spacing between the hexamers (17 bases) (30), there is an A+T-rich region upstream of the −35 element, which, in other systems, has been shown to increase promoter strength by about 30-fold (38). However, primer extension analysis indicated that transcription initiating from P2 requires Rns in LMC10. It is possible that transcriptional initiation from P2 is inhibited by a negative regulator and that Rns activation is necessary to relieve this inhibition.

**Promoter P1.** The sequences of the overlapping P1 putative promoters, which are located upstream of P2, suggest that they should be fairly strong sigma-70-dependent promoters. In addition to the homologies in the −10 and −35 regions (four of six bases for each) and three of six bases for each element of P1B) they both have close to optimal spacing between the hexamers (16 and 17 bases for P1A and P1B, respectively) (Fig. 4). However, primer extension analysis demonstrated that transcription from P1 can be detected only upon deletion of the downstream negative regulatory region (Fig. 4). The high constitutive level of transcription from pEU742 is due to the absence of this silencer (Fig. 2). Unlike the situation with P2, transcription from P1 does not appear to be regulated by Rns in pEU742 (Fig. 2). These results demonstrate that in its normal context (in the presence of the silencer), under the conditions we tested, P1 is too weak to be important in transcription of the coo operon.

**Promoter P3.** By lac fusion analysis and primer extension, we also identified P3, which is at the 3' end of the cooB reading frame and extends into the intergenic region between cooB and cooA (Fig. 2, pEU751). Transcriptional activity from P3 is regulated by Rns, but in the presence of the coo DNA upstream of it, P3 activity appears to be weak compared to the combined activity of P1 and P2 (Fig. 2, pEU751 compared to pEU743). The great reduction in amount of CooA protein (44) both in whole-cell extracts of JEF100, which has an insertion mutation in cooB upstream from P3, is consistent with P3 being a weak promoter.

**Negative regulation of coo transcripts.** We identified a region that negatively regulates transcription of promoters P1, P2, and P3 between bases +7 and +929 (Fig. 2). This is downstream of P2 and partially upstream of P3. Deletions show that there are at least four sites within this region at which negative regulation occurs (Fig. 2) or that the regulation does not occur at a discrete site. This negative control could be mediated by inhibition of transcriptional initiation and/or elongation from P1, P2, and P3.

The negative regulatory systems most similar to coo appear to be those of transcriptional silencers exemplified by proU and hly. In these cases, the negative regulatory region cannot be localized to one or several discrete sites but is composed of an extended region of DNA, including a long region downstream of the promoter (11, 24, 35). As in the case we have described for coo, deletions into the silencer region result in increased
transcription. The silencing effect in all three cases is dependent on the presence of the cognate promoter and/or upstream regions. In both proU and hly, the silencer has a maximal size of about 200 bp, while for coo, the silencer is at least 900 bp long. However, the maximal silencing effect observed is similar for all three: 29-fold, 20-fold, and 30-fold for proU, hly, and coo, respectively.

For proU, a mutation in hns greatly reduces negative regulation (11), and for hly, hha plays a similar role (24). For coo we found that, like proU, H-NS decreases transcription from promoters P1, P2, and P3. Our results suggest that the H-NS-dependent repression largely occurs in the downstream region responsible for silencing (+7 to +929), since in the hns mutant, deletion of the silencer resulted in only a 1.2-fold increase in transcription (Table 1, compare pEU743 to pEU745, which contains the silencer region). However, in the apparent absence of the silencer (pEU743), the hns mutation still has a small effect. This suggests that either the silencer was not completely deleted in pEU743 or that H-NS can act upstream of the silencer to repress transcription.

H-NS binds to specific sites that usually contain runs of five or more A·T base pairs. Many but not all of these sites are in curved DNA (28). Since both the promoter region and the silencer of coo contain numerous A·T runs, it seems likely that H-NS would bind to both of these regions in coo. Therefore, we predict that H-NS will be found to act directly at the coo locus.

Because H-NS inhibition of transcription requires both the cognate promoter and/or upstream sequences and the silencer region that overlaps the first open reading frame, H-NS appears to act over a long distance. Lucht et al. (28) identified several H-NS binding sites spanning the silencer region of proU and extending into the promoter region. These sites had differing affinities for H-NS. It was proposed that the strong affinity sites in the silencer serve as nucleation centers for H-NS binding. Additional H-NS molecules could then bind cooperatively at the lower-affinity sites, leading to a DNA-protein complex that extends into the promoter region, where it inhibits transcription. It is possible that H-NS acts by a similar mechanism in coo.

In the coo operon, there may be an additional negative regulatory factor that acts on the coo silencer region, since, in the hns mutant, deletion of the silencer led to a small increase in transcription (Table 1, compare pEU743 with pEU745). Negative regulation by this putative factor may be facilitated by alteration of the conformation of the coo DNA by H-NS. A more detailed understanding of regulation of coo requires determination of the H-NS binding sites in the coo region and isolation of mutations in the other potential negative regulator.

Positive regulation of coo. Activators of sigma-70 promoters in E. coli usually bind within or upstream of the promoter and appear to act by making direct contact with the RNA polymerase (3, 18). The Rns homolog in the CS1-related CFA/I operon may act directly as an activator since it requires a region upstream of the start of transcription for induction (21). We have demonstrated that in the coo operon, Rns induces promoter P2 by acting upstream of base +7. Because of this similarity in sites of action and because Rns also induces expression of CS2 (4), a conserved binding site near the promoters in all three was sought. We did not find an obvious conserved sequence in the region of the P2 promoter of CS1 and the DNA encoding CS2 and CFA/I.

Although deletion of the silencer region downstream of the P2 promoter resulted in elevated transcriptional activity, Rns activated each of the deletion derivatives to an even higher level (Fig. 2, pEU743, pEU746, pEU747, and pEU748). Furthermore, Rns increased transcriptional activity as much as 153-fold from the CS1 promoters in the presence of the silencer region (not shown), demonstrating that Rns can overcome the silencing by H-NS. In the absence of the silencer, Rns increased transcriptional activity at least sixfold, and in an hns mutant, Rns increased transcription at least 1.4-fold. This suggests that Rns does not act solely on H-NS silencing to induce transcription. Similarly, for CFA/I, it was found that the Rns homolog, CfaR, does not act solely to diminish the H-NS-dependent silencing (20). We propose instead that Rns overcomes the silencing effect indirectly by activating transcription of the coo genes from P2.

Virulence factors in many enteric pathogens are positively regulated by a protein in the Rns family. Many of these same factors show negative regulation by H-NS, Hha, and related small DNA-binding proteins. Because of these similarities, it will be interesting to discover whether the presence of a silencer region within the first open reading frame is also a common feature among virulence operons in these organisms and whether they share a common mechanism of action of their positive regulators.

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