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AsnC: an Autogenously Regulated Activator of Asparagine Synthetase A Transcription in *Escherichia coli*

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The regulation of the asparagine synthetase A gene of *Escherichia coli* was studied in vitro with a coupled transcription-translation system. It was shown that the 17-kilodalton gene, which is transcribed divergently from the adjacent *asnA* gene, codes for an activator of *asnA* transcription. The synthesis of the 17-kilodalton protein, which we now call AsnC, is autogenously regulated. The stimulating effect of AsnC on *asnA* transcription is abolished by asparagine, while the autoregulation of *asnC* is not affected by asparagine. The N-terminal part of the *asnC* protein, inferred from the DNA sequence, is homologous to the DNA-binding domain of regulatory proteins like catabolite gene activator, cro, and cI. This homology and direct repeats found in the region of the two *asn* promoters suggest that the *asnC* protein regulates transcription by binding to DNA. The *asn* promoters were defined by mapping of the mRNA start sites of in vitro-generated transcripts.

Two different enzymes found in *Escherichia coli* convert aspartate to asparagine, thereby cleaving ATP to AMP and PP_i (12). These asparagine synthetases are coded for by two genes, i.e., the *asnA* gene next to the *E. coli* replication origin (*oriC*) at 84 min on the *E. coli* map and the *asnB* gene at 16 min on the *E. coli* map (8, 12, 25). AsnB differs from AsnA especially in that it can use glutamine in addition to ammonia as a nitrogen donor. As demonstrated by Cedar and Schwartz (3), the AsnA synthesis is repressed in vivo on addition of asparagine to the medium.

The *asnA* region (Fig. 1), present on *oriC*-containing minichromosomes (19), has been sequenced (2, 19). The sequence reveals an open reading frame adjacent to *asnA* coding for a 17-kilodalton (KDa) protein expressed in vivo (10). Stuitje and Meijer (23) suggested that this protein or its transcript interferes with *oriC* replication. Here we present evidence that this 17-KDa protein, which we now call AsnC, is an autogenously regulated activator of *asnA* transcription.

As a means to study the regulation of genes in vitro, we used the transcription-translation system (S 30 system) originally described by Zubay (27) and modified by Collins (4). To facilitate the measurement of gene activity, we employed promoter galactokinase fusions.

MATERIALS AND METHODS

Plasmids. Fragments of the minichromosome pCM959 (2, 25) were cloned forward of the *galk* gene of pFD51 (20). pLSK92-2 carries the *asnA* promoter on a 378-base-pair (bp) *AluI* fragment (position 1092 to 1470), pLSK92-11 carries the *asnC* promoter on a 579-bp *Hin*dII-*Rsa*I fragment (position 1495 to 916), and pLSK92-7 carries the 16-kDa gene promoter on a 359-bp *AluI* fragment (position 1017 to 658). pLSK1-1 contains the *lacUV5* promoter on a 205-bp *Eco*RI fragment of pKB252 (1) forward of *galk*. pLSK35-3 contains the *asnC* gene with a *Bal* 31 deletion in the *asnC* promoter region cloned forward of the *tac* promoter of plasmid pJF118u, kindly furnished by J. P. Fürste and E. Lanka. The *asnC* fragment extends to the *MluI* site of pCM959 (position 777). The minichromosome pOC77 is a deletion derivative of pCM959 (position 3966 to 2499). For cloning, standard techniques were used, i.e., essentially the methods of Maniatis et al. (16). Restriction enzymes, media, and chemicals were from commercial sources.

Transcription-translation system. In vitro synthesis of galactokinase was carried out as originally described by Zubay (27). The S 30 extract was prepared from strain CM1671 (20) *asnA*::Tn*10* *asnB32* Δ(*asnA-oriC-gid*)*1071 fuc lysA relA1 spoT1 thi-1* Hfr:: Φ(*rrnC-ilv*). To compensate for the loss of the chromosomal replication origin (*oriC*), CM1671 uses the origin of an integrated F plasmid for replication. Reaction mixtures contained in a 10-μl final volume 500 ng of supercoiled plasmid DNA, 2.5 μl of S 30 extract (52 μg of protein), 6 mM magnesium acetate, and 2.5 μl of low-molecular-weight mix as detailed by Collins (4), including all 20 amino acids. The reaction mixture was incubated for 45 min at 37°C, and then galactokinase activity was measured as described previously (14). For the synthesis of labeled proteins, methionine was omitted from the low-molecular-weight mix, and 6 to 10 μCi of [^{35}S]methionine (specific activity, 1,115 Ci/mmol) was added

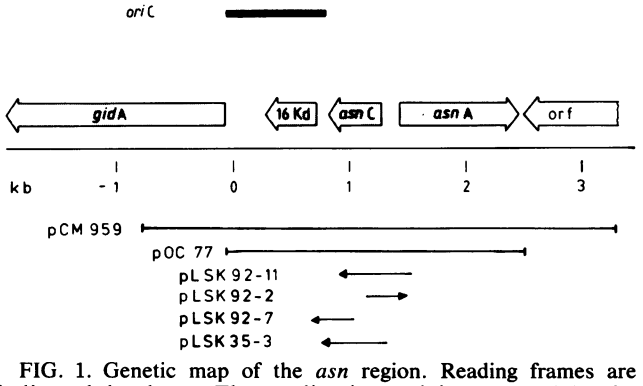

FIG. 1. Genetic map of the *asn* region. Reading frames are indicated by bars. The replication origin (*oriC*; 14), the minichromosomes pCM959 and pOC77, and the fragments subcloned from pCM959 are shown on the map. Arrows are oriented in the direction of transcription. kb, Kilobase; orf, open reading frame.

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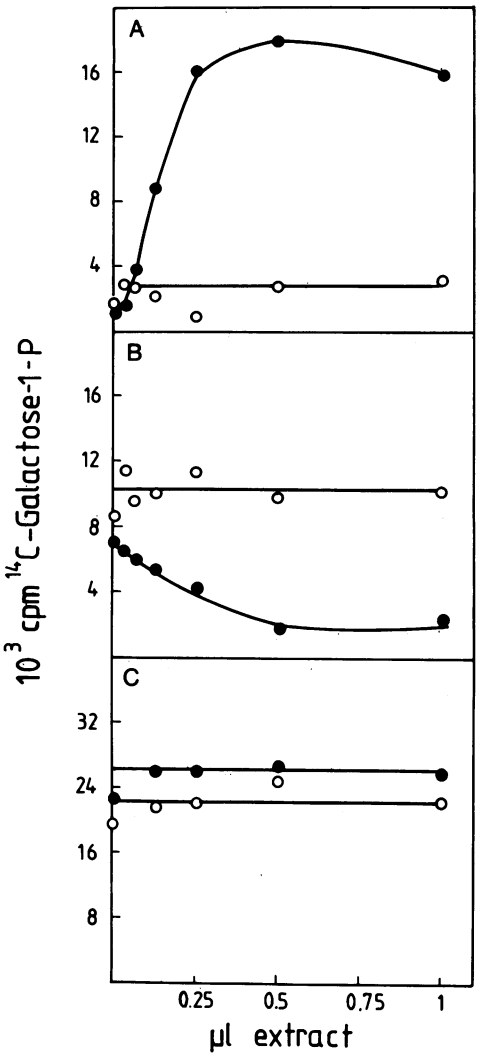

FIG. 2. Effect of *asnC* protein on the promoter activity of promoter-*galk* fusions in vitro. Galactokinase activity, expressed as counts per minute of [^{14}C]galactose phosphorylated, was measured after the transcription-translation system was incubated with different amounts of complementing extracts, i.e., AsnC$^+$ extract (CM1671 plus pLSK35-3, ●) and AsnC$^-$ extract (CM1671, ○). The protein contents of the two extracts were 6 and 5 mg/ml, respectively. The promoter fusions employed in each case are as indicated. (A) 92-2, *asnA* promoter; (B) 92-11, *asnC* promoter; (C) 92-7, 16-kDa promoter.

to the reaction mix. The proteins were separated on a 15% sodium dodecyl sulfate-polyacrylamide gel and examined by autoradiography.

Preparation of complementing extracts. Cells were grown at 30°C in TY medium to an A_{600} of 1.0, harvested, and washed once in buffer 2 (25 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 8], 100 mM KCl, 1 mM dithiothreitol, 1 mM *p*-aminobenzamidine). After being suspended in buffer 2 (1 ml/g of cells), the cells were lysed by a freeze-thaw procedure. After the first freezing and thawing step, lysozyme was added (1/50 volume, 15 mg/ml). Then the cells were frozen and thawed once again. Cell debris was removed by spinning the lysate in a 50 Ti rotor at 30,000 rpm. Ammonium sulfate was added to the supernatant (0.28 g/ml). The precipitate

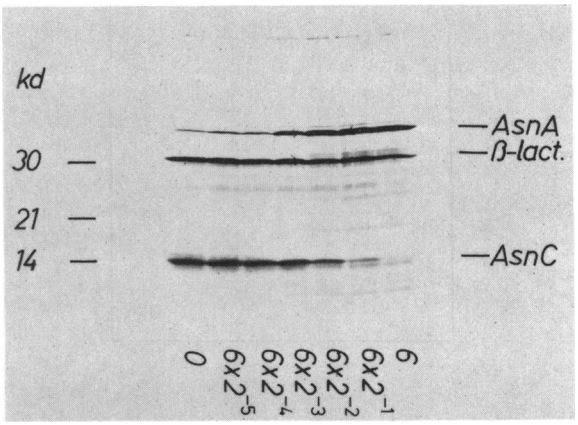

FIG. 3. Effect of *asnC* protein on the synthesis of proteins made in vitro from the *asnA-asnC* region. Proteins synthesized in the transcription-translation system were labeled with [^{35}S]methionine, resolved on a 15% sodium dodecyl sulfate-acrylamide gel, and examined by autoradiography. The template used was the minichromosome pOC77 containing reading frames for a 37-kDa protein (AsnA), a 17-kDa protein (AsnC), and the 16-kDa protein. pFD51 producing β-lactamase (31.5 kDa) was included as internal standard. From left to right, increasing amounts of AsnC$^+$ extract (CM1671 plus pLSK35-3, in micrograms of protein) were added as indicated. Positions of marker proteins are shown on the left.

was suspended in buffer 1 (1 ml/g of cells used; 25 mM Hepes, 100 mM KCl, 1 mM dithiothreitol) and dialyzed for 45 min against buffer 1. The cells containing pLSK35-3 were induced for about 5 h with 1 mM isopropyl-β-D-thiogalactopyranoside before being harvested.

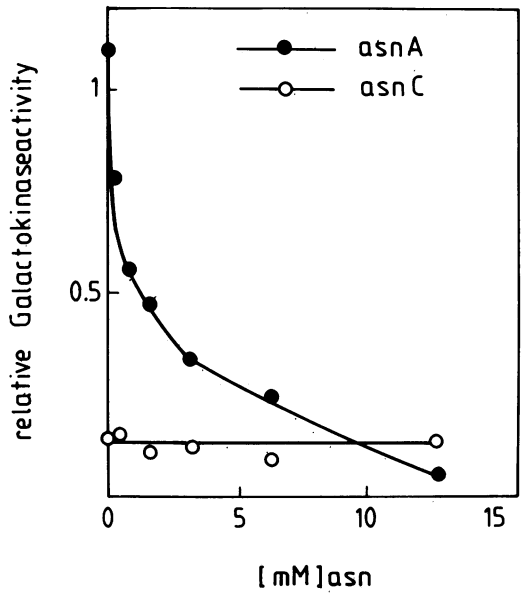

FIG. 4. Effect of asparagine on AsnC function in vitro. The promoter activity of the promoter-*galk* fusions was measured in the transcription-translation system as a function of the asparagine concentration added. A constant amount of 320 µM asparagine was always present to sustain protein synthesis. AsnC$^+$ extract (CM1671 plus pLSK35-3; 1 µl) was included. The galactokinase activities were normalized to the values obtained for the 16-kDa promoter (the control) because of unspecific stimulation of the system by asparagine. The promoters examined are as indicated.

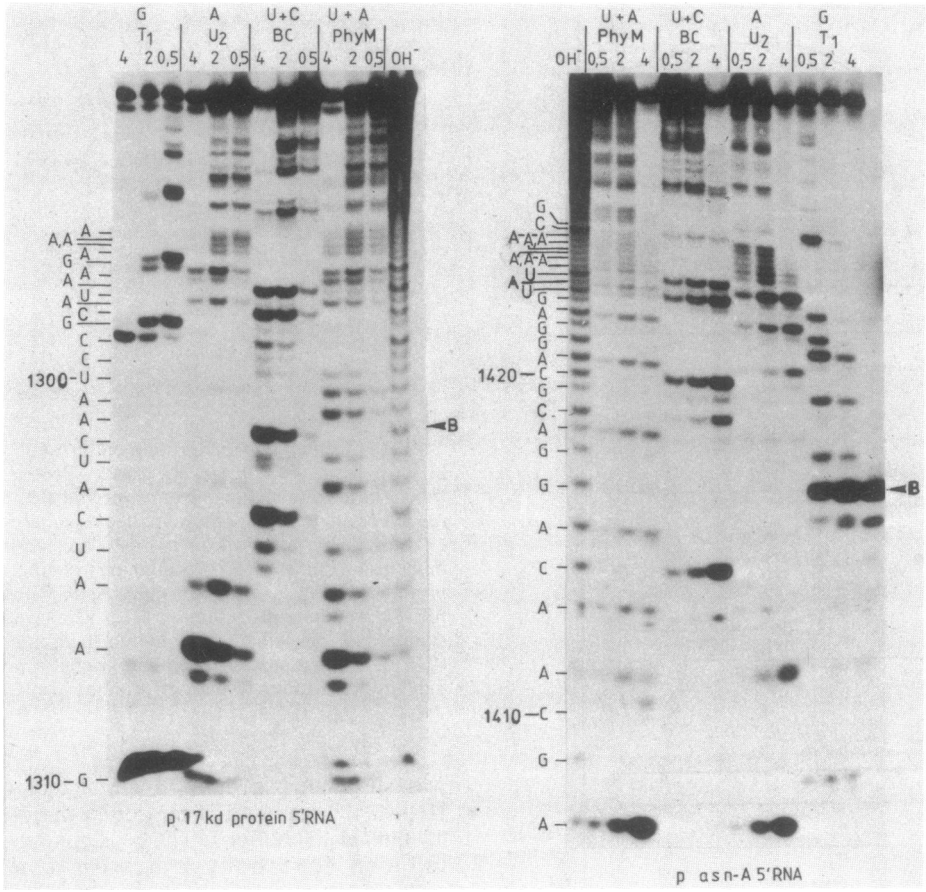

FIG. 5. Autoradiograms of sequencing gels of the 5′ ends of *asnAp* and promoter 17-kDa protein (AsnC) transcripts. Sequencing was performed with 0.5, 2, and 4 U of ribonuclease T1 (G specific), ribonuclease U2 (A specific), ribonuclease BC (U and C specific), and ribonuclease Phy M (A and U specific). Enzymes were obtained from P-L Biochemicals and assayed by the protocol of the company, based on the work of Donis-Keller et al. (7). 5′-end-labeled and alkali-hydrolyzed transcripts and bromphenol blue were run as size standards. Numbers next to sequences refer to pCM959 coordinates (2).

Sequencing of transcripts obtained in vitro. RNAs were synthesized in vitro basically as described elsewhere (15), with restriction fragments encompassing base pairs 1219 to 1533 of pCM959. Sequencing was done as detailed in the legend to Fig. 5. Sequence-specific RNases were obtained from P-L Biochemicals, Inc.

RESULTS

Promoter studies in vitro. To study the regulation of *asnA* and *asnC* transcription, various promoter-containing DNA fragments were fused to the galactokinase gene present on plasmid pFD51 (20). pFD51 is a derivative of pKO1 constructed by McKenney et al. (18). The plasmids were used as DNA templates in an in vitro transcription-translation system (S 30 system). Promoter activity was determined by measuring galactokinase produced under the control of the cloned promoters. The protein-synthesizing extract (21 mg/ml of protein) was prepared from strain CM1671 (24). This strain carries a chromosomal deletion including the *gidA*, *oriC*, *asnC*, and *asnA* genes. Therefore, no *asnC* protein was present to interfere with our measurements. The S 30 system was complemented with different amounts of extract (6 mg/ml of protein) prepared from strain CM1671 transformed with pLSK35-3. pLSK35-3 contains the *asnC* gene without its promoter, cloned forward of the inducible *tac* promoter of plasmid pJF118u. Sequences upstream from the *asnC* coding region were deleted by *Bal* 31 treatment, leaving the reading frame and the putative ribosome-binding site intact. As a control, we prepared a complementing extract (5 mg/ml of protein) from strain CM1671 without plasmid pLSK35-3.

The results of the complementation experiments are shown in Fig. 2. The activity of the *asnA* promoter (pLSK92-2) was raised by a factor of about 15 to 20 if *asnC* protein containing extract was added. The activity of the *asnC* promoter (pLSK92-11) was decreased by a factor of about 5. The basal level of *asnA* transcription without *asnC* protein was low. The promoter activities were not affected by the $AsnC^-$ extract. The 16-kDa promoter (pLSK92-7) as a control was affected by neither the $AsnC^+$ nor the $AsnC^-$ extract. This lack of effect was also true of the *lacUV5* promoter (data not shown).

Proteins made in the S 30 system. To see whether the results obtained with the promoter *galk* fusions also apply to the expression of the respective proteins from the intact region, we used the minichromosome pOC77 as a template. pOC77 includes the *asnA* and *asnC* genes as well as the 16-kDa protein reading frame (Fig. 1). The proteins produced in the S 30 system were labeled with [^{35}S]methionine, separated on a 15% sodium dodecyl sulfate-acrylamide gel, and examined by autoradiography. pFD51 producing β-lactamase was included as an internal standard. The S 30

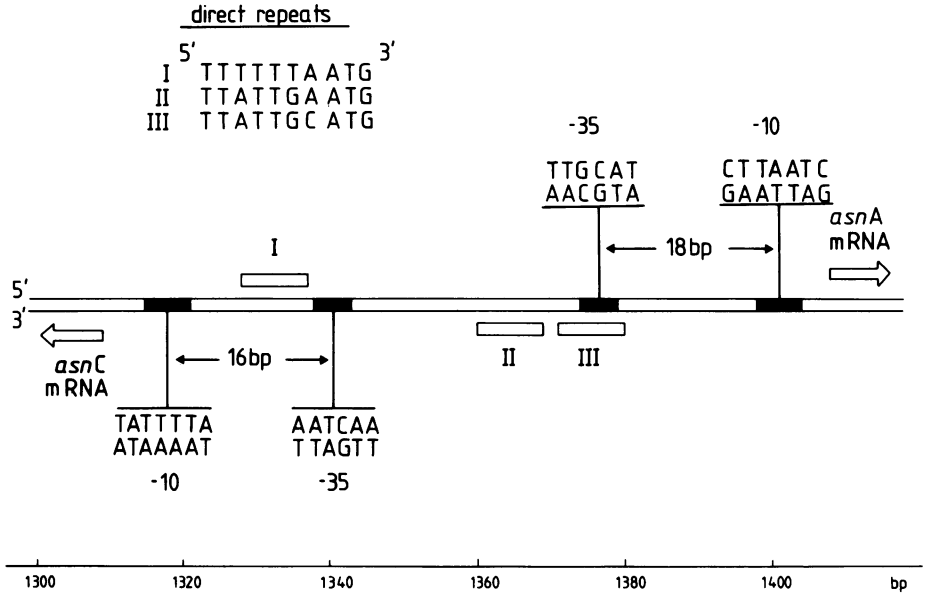

FIG. 6. Regulatory sites in the *asnA-asnC* promoter region. The presumptive *asnA* and *asnC* promotor sequences are shown. Indicated are the homologies to the −10 and −35 consensus sequences of a promoter. The mRNA start sites are marked by arrows. The three direct repeats found in this region are listed. Their positions in relation to the promoter sequences are demonstrated by open bars. As a scale we used pCM959 coordinates (2).

extract was the same as described above. The protein pattern obtained by adding increasing amounts of complementing extract (CM1671 plus pLSK35-3) confirmed the galactokinase measurements described above (Fig. 3). AsnA synthesis was stimulated, while the synthesis of AsnC was repressed. The β-lactamase band remained unchanged. The 16-kDa protein gave a faint band that runs just below the *asnC* protein band. To assign the proteins to the bands shown in Fig. 3, we selectively disrupted reading frames of the template DNA by cutting with appropriate restriction enzymes (data not shown).

Effect of asparagine on AsnC function. We then asked whether the amino acid asparagine has an effect on AsnC function, since it is common that metabolites related to operons act as cofactors for their repressors or activators. Again we measured the galactokinase activity produced under the control of the cloned promoters in the S 30 system. A constant amount of extract (CM1671 plus pLSK35-3) was added, giving optimal stimulation of the *asnA* promoter and optimal repression of the *asnC* promoter. With increasing concentration of asparagine, the stimulating effect of AsnC on *asnA* transcription disappeared, while the repression at the *asnC* promoter was not affected (Fig. 4). The amino acid glutamine had no effect on the promoters examined.

Regulatory sites in the promoter region. *asnA* and *asnC* are transcribed divergently from adjacent promoters (Fig. 1). To look for regulatory sites in this complex promoter region, we established the start points of the transcripts by RNA sequencing of in vitro-generated transcripts with purified RNA polymerase and various restriction fragments. A 120-base *asnA* transcript was obtained from a *Hha*I-*Hae*II fragment (position 1219 to 1532 of pCM959), and a 90-base *asnC* transcript was obtained from a *Hha*I-*Alu*I fragment (position 1219 to 1470 of pCM959). The transcripts synthesized in the presence of low amounts of [α-^{32}P]UTP were cut out of the acrylamide gel, subjected to 5′ end labeling with T4 kinase and [γ-^{32}P]ATP, and sequenced with sequence-specific RNases (7).

This analysis revealed that transcription started with an A at position 1408 for *asnA* and with a G at position 1310 for *asnC* (Fig. 5). At appropriate distances from these start sites, sequences were found that resemble the consensus −10 region of a promoter (21), i.e., CTTAAT for *asnA* and TAAAAT for *asnC*. Additional homologies to consensus promoter sequences were found in the -35 region (21), namely, TTGCAT for *asnA* and TTGATT for *asnC*. Futhermore, we found three direct repeats of 10 bp each in this region (Fig. 6). These repeats could be binding sites for the *asnC* protein.

Homology to DNA-binding proteins. It seems likely that *asnC* protein regulates transcription by binding to specific DNA sequences. Therefore we looked for homologies to other known DNA-binding proteins. The coding region for the *asnC* protein presumably starts with the first ATG of the 17-kDa protein reading frame. Upstream 6 bp from the first ATG there exists a reasonable homology to a Shine-Dalgarno sequence (22), i.e., AAGAA. The analysis of *Bal* 31 deletions in the *asnC* leader region suggests furthermore that the first ATG really marks the beginning of the coding region (data not shown). The N-terminal part of the protein, inferred from the DNA sequence, resembles the DNA-binding domain of established DNA-binding proteins like catabolite gene activator, cro, and cI (17; Fig. 7). This fact supports the notion that the *asnC* protein regulates transcription by binding to DNA.

DISCUSSION

Our in vitro results demonstrate that an activator protein is necessary for efficient *asnA* expression in *E. coli*. Without activator protein, the basal level of transcription is low. Asparagine, the product of asparagine synthetase A, turns off the stimulation of *asnA* transcription mediated by AsnC. This fact explains the finding of Cedar and Schwartz (3) that asparagine in the growth medium represses AsnA synthesis. The synthesis of the *asnC* protein itself is autogenously regulated. However, regulation of the promoter activity

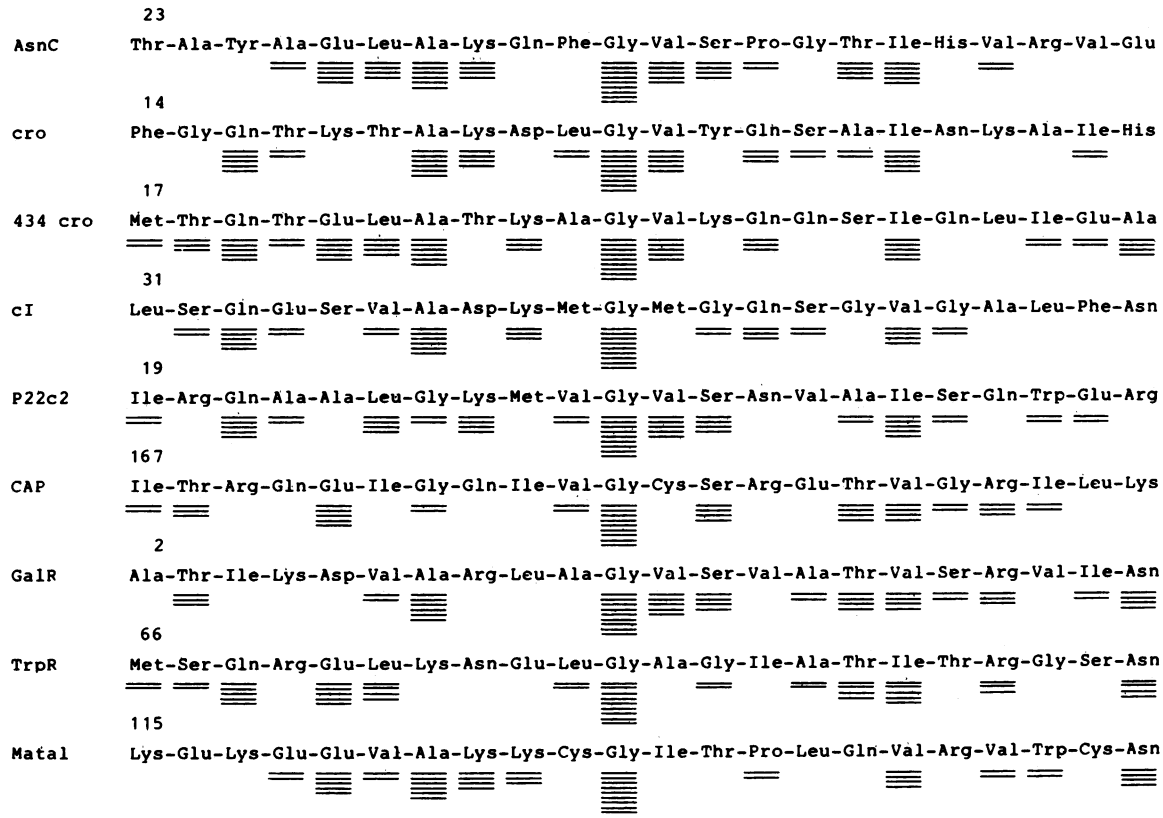

FIG. 7. Several regulatory proteins, including the *asnC* protein, which share sequence homologies with the DNA-binding domains of catabolite gene activator, cro, and cI. The number of lines below an amino acid residue indicates how often it is encountered at a certain position. Numbers above an amino acid refer to its position within the protein (redrawn from Matthews et al. [17]).

occurs only within a narrow range, similar to the regulation seen in the case of the *trp* repressor (13). In contrast to that of *trpR*, *asnC* autoregulation does not respond to an amino acid cofactor. Thus there seems to be a step, sensitive to a conformational change in AsnC protein, which is required for *asnA* activation but not for *asnC* repression. This step could be a protein-protein interaction between AsnC and RNA polymerase which is not needed for the simple repression of the *asnC* promoter.

Comparison of the protein structures of certain regulatory DNA-binding proteins reveals a common principle of protein-DNA interaction. For the proteins catabolite gene activator, cro, and cI, the DNA-binding domain has nearly the same configuration (17). Several other regulatory proteins have strong sequence homologies to the DNA-binding domain of catabolite gene activator, cro, and cI. The AsnC protein apparently belongs to this group of proteins, because it shows the same sequence homologies. Thus it seems likely that AsnC exerts its effects by binding to specific DNA sequences within the promoter region. It is tempting to speculate that the three direct repeats found in the *asn* promoter region are binding sites for the *asnC* protein. Their different locations with respect to the consensus promoter sequences could explain the opposite effect of *asnC* protein on the activity of the *asnA* and *asnC* promoters (Fig. 6). However, it is difficult to predict the effect of a protein from the location of the binding site within a promoter. *galR* acts as a repressor by binding to the classical activator site at the −60 region of a promoter (6), and *araC* reduces transcription at *araBAD* by binding to the −105 to −146 region (26).

So far we have studied only part of the *asn* regulation. One may expect the in vivo situation to be more complex. At present, we do not know to what extent the second asparagine synthetase in *E. coli*, AsnB, is involved in *asnA-asnC* regulation. Beyond that, there exist two asparaginases in *E. coli* which catalyze the hydrolysis of asparagine to aspartate (5). It is reasonable to assume that the breakdown of asparagine must be coupled somehow to the synthesis of asparagine, possibly via a common regulator. These problems remain to be clarified.

According to Stuitje and Meijer (23), the *asnC* region expresses incompatibility towards *oriC*-containing minichromosomes (*incC*). Although we cannot exclude the possibility that the *asnC* protein has another function besides regulating *asn* transcription, it seems unlikely that this protein is involved in DNA replication. The region defined as *incC* contains, in addition to *asnC*, a *dnaA* protein-binding site (9, 11). This sequence could serve as an incompatibility determinant by titrating *dnaA* protein, which is required for *oriC* replication.

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