Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 4134–4138, July 1984
Genetics

DNA sequence of the *carA* gene and the control region of *carAB*: Tandem promoters, respectively controlled by arginine and the pyrimidines, regulate the synthesis of carbamoyl-phosphate synthetase in *Escherichia coli* K-12

(carbamoyl phosphate/cumulative repression/arginine operator/unusual translation start)

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Communicated by Sarah Ratner, December 16, 1983

ABSTRACT **The *carAB* operon of *Escherichia coli* K-12, which encodes the two subunits of carbamoyl-phosphate synthetase (glutamine hydrolyzing) [carbon-dioxide: L-glutamine amido-ligase (ADP-forming, carbamate-phosphorylating); EC 6.3.5.5], is cumulatively repressed by arginine and the pyrimidines. We describe the structure of the control region of *carAB* and the sequence of the *carA* gene. Nuclease S1 mapping experiments show that two adjacent tandem promoters within the *carAB* control region serve as initiation sites. The upstream promoter P1 is controlled by pyrimidines; the downstream promoter P2 is regulated by arginine. Attenuation control does not appear to be involved in the expression of *carAB*. A possible mechanism by which control at these promoters concurs to produce a cumulative pattern of repression is discussed. The translational start of *carA* is atypical; it consists of a UUG or AUU codon.**

Carbamoyl phosphate, a common precursor of the arginine and pyrimidine pathways, is synthesized in *Escherichia coli* and related organisms from glutamine, ATP, and CO_2 by a single enzyme (1, 2), carbamoyl-phosphate synthetase (glutamine-hydrolyzing) [carbon-dioxide: L-glutamine amido-ligase (ADP-forming, carbamate-phosphorylating); EC 6.3.5.5], which is controlled by antagonistic effectors: an inhibitor, uridine 5′-monophosphate, and two activators, ornithine and inosine 5′-monophosphate (2–4). The enzyme consists of two subunits (5), the products of the adjacent genes *carA* and *carB* (6), which constitute an operon oriented from A to B (7, 8). *carA* encodes a small subunit of M_r 42,000 that carries the glutamine-binding site; *carB* encodes a subunit of M_r 130,000 that catalyzes the synthesis of carbamoyl phosphate from NH_3.

The synthesis of carbamoyl-phosphate synthetase is subject to a unique type of regulation that is of obvious physiological significance, cumulative repression by arginine and the pyrimidines (1, 2): partial repression occurs in the presence of either arginine or a pyrimidine base and a nearly complete repression occurs in the presence of both (Table 1). The arginine repressor, which controls the transcription of the genes of the arginine pathway by recognizing specific operator sequences (10–12), participates in cumulative repression as well (9, 13); arginine—and not arginyl-tRNA—was shown to be involved in repression (9, 13). Cumulative repression appears to be exerted strictly at the transcriptional level, because variations in the rate of synthesis of carbamoyl-phosphate synthetase and *carAB* messenger RNA correlate closely with one another (14).

Table 1. Cumulative repression of carbamoyl-phosphate synthetase by arginine and pyrimidines*

Strain	Relevant genotype	Addition (100 μg/ml) to minimal medium† None	Arginine	Uracil	Arginine and uracil
P4X	*argR*$^+$	1.04	0.52	0.38	0.05
P4XB2	*argR*$^-$	1.93	1.71	1.65	1.10

*Adapted from ref. 9.
†Values represent specific activity, expressed as μmol of carbamoyl-phosphate formed per hr per mg of protein.

To further investigate the mechanism of this regulation, we have cloned the *car* operon (ref. 8; this paper) and analyzed the structure of its control region. The data establish that *carAB* is preceded by two promoters in tandem, respectively controlled by arginine and the pyrimidines.

The complete sequence of the *carA* gene is presented; the structure of *carB* is discussed elsewhere (15). The translational start of *carA* was found to be atypical, consisting of one of two adjacent codons, UUG or AUU.

MATERIALS AND METHODS

***Escherichia coli* Strains.** The strains P4X (Hfr *metB*), P4XB2 (Hfr *metB argR*) and JEF8 (Hfr *metB thr carB8*) have been described (2, 6).

Growth Conditions. Cultures were grown in minimal medium 132 or in enriched medium 853 as described in ref. 6.

DNA Preparation. Phage and plasmid DNA preparations, and the isolation and separation of restriction fragments were as described by Piette *et al.* (10).

Restriction, Ligation, and Transformation. These were done according to ref. 11.

DNA Sequence Analysis. An 856-base-pair (bp) *Hpa* II/*Hin*dIII fragment from pMC50, or a *Sau*3A digest thereof, was cloned in M13 mp8 or M13 mp9 (16) and the sequences of the cloned fragments were determined by the dideoxy method (17). Fragments analyzed are shown in Fig. 1. The sequence of the *Hpa* II/*Hin*dIII fragment was also determined by the method of Maxam and Gilbert (18), using the strategy indicated in the same figure. The results obtained were in complete accord. The sequence of the *carA* structural gene was determined by the method of Maxam and Gilbert (18).

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Abbreviations: kbp, kilobase pair(s); bp, base pair(s).

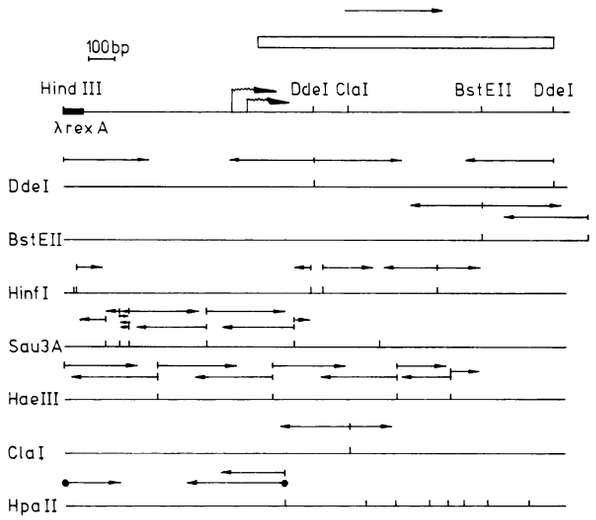

FIG. 1. Strategy for the determination of the nucleotide sequence of the control region of the *carAB* operon and the *carA* structural gene. Fragments analyzed with the dideoxy method are represented by ●→; those determined using the Maxam and Gilbert method are represented by |→. The arrow points in the direction of the sequence analysis. The heavy line at the extreme left represents λ DNA (97 nucleotides). Transcription starts are indicated by wavy arrows. The position of the structural gene is indicated by an open box on top of the figure.

pMC40 was digested with *Hin*dIII and *Eco*RI to yield three fragments of 4.3 (vector), 3.6, and 2.7 kilobase pairs (kbp). The 3.6-kbp fragment was digested with *Dde* I, which gave four fragments of 1.1, 0.95, 0.9, and 0.6 kbp. The 0.95 and 0.91-kbp *Dde* I fragments were used for secondary cleavage and 5'-end-labeling. The *Eco*RI/*Hin*dIII 3.6-kbp fragment was also digested with *Bst*EII and *Acc* I; three fragments of 1.6, 1.5, and 0.4 kbp were obtained. The 1.6-kbp DNA fragment was used for secondary digestion and 5'-end-labeling as indicated in Fig. 1.

Nuclease S1 Mapping of the *car* Transcripts. *In vivo* produced mRNA was mapped using the S1 endonuclease mapping method (19). The probe was a 5'-end-labeled single-stranded 805-bp *Hpa* II/*Hin*fI fragment extending from nucleotides +112 to −693 (cf. Figs. 1 and 2). Preparation of RNA, hybridization conditions and S1 digestion were as described by Piette *et al.* (12). The probe was hybridized with 100 μg of total RNA extracted from the following strains: JEF8 strain containing the pMC50 plasmid grown in minimal medium 132; P4X (wild type) grown in medium 853; P4XB2 (*argR*) grown in medium 853; P4X grown in minimal medium and P4X grown in minimal medium plus uracil (50 μg/ml). After S1 digestion of the DNA·RNA hybrids, the products were separated on an 8% polyacrylamide sequencing gel. The Maxam and Gilbert A+G and T+C reaction products were loaded in adjacent lanes to locate the 5' ends of the transcripts on the sequence ladder.

RESULTS

Cloning and Nucleotide Sequence of *carA*. The transducing phage λ37-9 carries a 5.1-kbp-long chromosomal segment, 4.7 kbp of which are required to accommodate the coding region of the *carAB* operon (20). Plasmids pMC40 and pMC50 were constructed by cloning into pBR322 a 5.6-kbp fragment delineated by endonuclease *Hin*dIII-specific sites located on λ DNA on both sides of the 5.1-kbp chromosomal fragment. The two plasmids carry this fragment in opposite orientations. These plasmids were used to determine the sequence of the *carA* gene according to the strategy depicted in Fig. 1. Part of this sequence has been independently confirmed by Bouvier *et al.* (21).

Sequence of the Control Region and Localization of Transcription Start Sites. The longest open-reading frame that might correspond to the *carA* gene starts at a GTG codon located at the 5' distal end of an imperfect palindrome (Fig. 2, right box). The two adjacent palindromes shown in Fig. 2 have sequences similar to arginine operators (11, 12, 22, 23). The reading frame initiated at the GTG codon terminates at a TAA codon 1195 nucleotides downstream. The correctness of this frame assignment has been confirmed by a gene fusion experiment (21) joining *lacZ* to *carA* at the *Hae* III site present around nucleotide +55 in Fig. 2. On the basis of current consensus sequences (24, 25), several putative promoters could be detected upstream from the putative arginine operators; two of these, indicated in Fig. 2 as P1 and P2, were found to be properly located with respect to the 5' termini of the transcripts determined by nuclease S1 mapping. The probe used in the S1 experiments was a DNA fragment extending from the first *Hpa* II site found in the open-reading frame (nucleotide +112 in Fig. 2) to a *Hin*fI site 805 nucleotides upstream in λ DNA (Fig. 1). The location of the 5' termini (see below) was in accord with the conclusions reached by Bouvier *et al.* (21) on the basis of primer extension experiments.

Since the *carAB* operon is cumulatively repressed, each of the two promoters might act as a separate regulatory site. We carried out S1 analyses on RNA preparations obtained from cells grown under conditions known to affect expression of the operon: (*i*) the JEF8 *carB8* mutant carrying plasmid pMC50 and grown in minimal medium; (*ii*) the wild-type strain P4X grown in minimal medium; (*iii*) P4X grown in minimal medium supplemented with excess uracil (50 μg/ml); (*iv*) P4X grown in enriched medium 853, which contains excess arginine (100 μg/ml) and growth-supporting (but limiting) amounts of pyrimidines (11 μg/ml); and (*v*) the *argR* mutant P4XB2 grown in medium 853.

The results (see Fig. 3) of S1 analyses permit the following conclusions: (*i*) Two promoters, both active in minimal medium, appear to be used in transcription of *carAB*. In the upstream promoter P1, the sequence T-T-G-A-C-T exhibits a good fit to the −35 consensus sequence of prokaryotic promoters (T-T-G-A-C-A), and the sequence C-A-G-A-A-T matches four bases out of six in the −10 consensus sequence (T-A-T-A-A-T) (24, 25). The downstream promoter P2 displays the sequences T-T-G-A-T-T (−35 region) and T-A-A-T-A-T (−10 region), both of which match their consensus sequences quite well.

(*ii*) P1 is inactive in excess uracil (compare lanes 4 and 5 in Fig. 3) and slightly active in medium 853 (compare lanes 3 and 4) where the concentration of pyrimidines (11 μg/ml) is known to be insufficient to promote complete repression (unpublished observations).

(*iii*) P2 is strongly active in the *argR* mutant as compared to the wild type (compare lanes 2 and 3); it is weak in medium 853 (compare lanes 3 and 4) where arginine is in excess (100 μg/ml).

(*iv*) The −10 region of P1 is followed by a short G+C-rich sequence, as in promoters that respond negatively to guanosine tetraphosphate (ppGpp) (26). Following this sequence one finds an octamer (T-T-T-G-C-C-A-G) that resembles the right-hand half (T-T-T-G-C-C-G-G) of an imperfect palindrome located beyond the −10 region of one of the putative promoters (P2) of the *pyrBI* operon (27, 28). Another sequence (C-A-T-A-T-C-T) found in P1 of *carAB* is also present in the control region of *PyrBI* near putative promoter P1.

(*v*) The −10 region of promoter P2 of *carAB* is included within a canonical arginine operator (11, 12, 22, 23). Three nucleotides downstream lies a second, more degenerated version of such a sequence.

The Sequence of *carA* and the Putative Translational Start. The most striking feature of the *carA* message is the lack of a

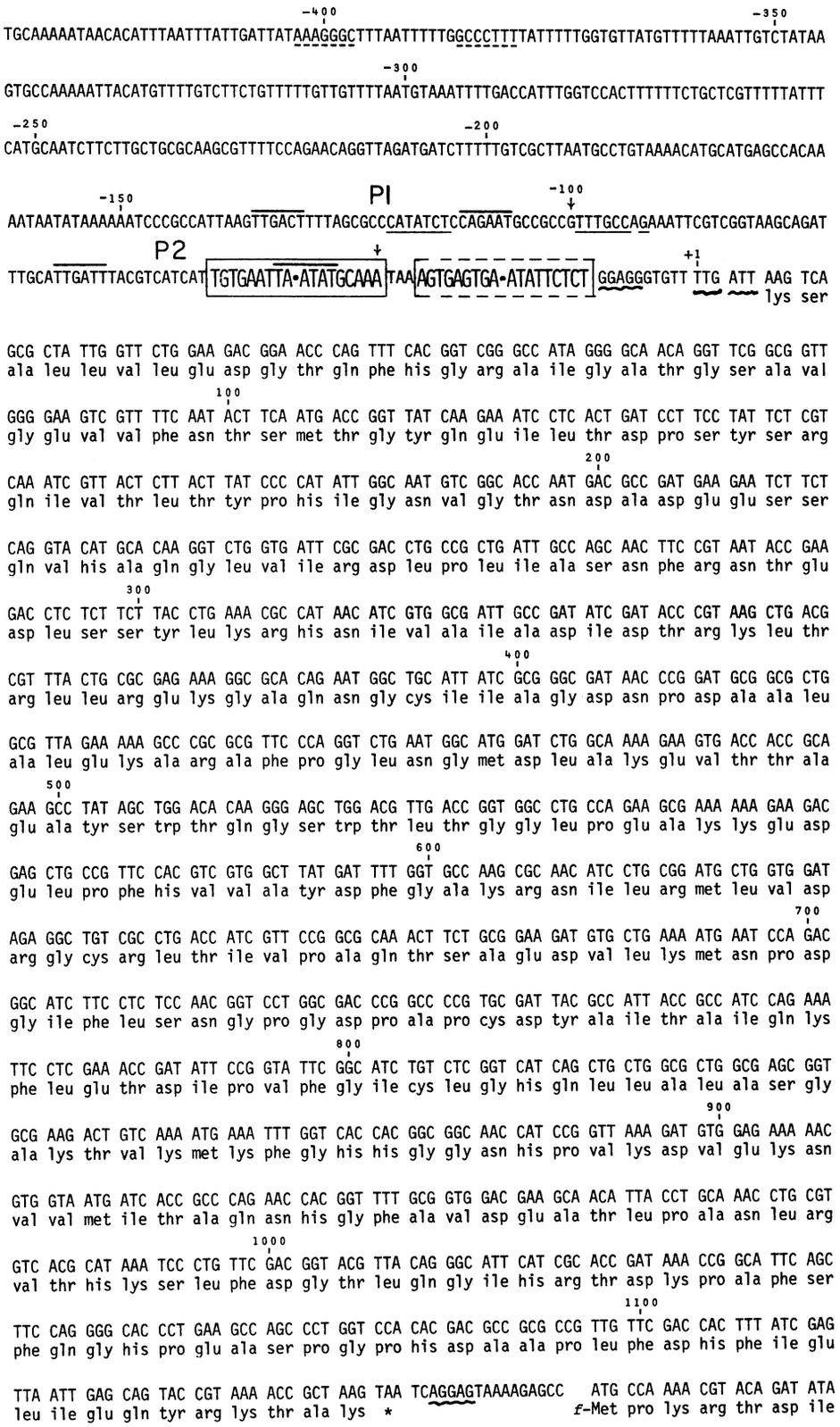

FIG. 2. The *carA* gene. The symmetric elements of a possible transcriptional terminator shown by Bouvier *et al.* (21) to mark the end of the *dapB* gene are indicated by broken underlining. The −35 and −10 regions of the two *car* promoters (P1 and P2) are indicated by a solid overline, and transcriptional starts (see Fig. 3) are shown by the arrows. Operators (in large type) are boxed, the more conserved operator with a solid line. Homologies within and beyond P1 with the control region of *pyrBI* are underlined with a solid line. Wavy lines indicate Shine–Dalgarno sequences and possible translational starts for *carA*. The translational stop codon is indicated by an asterisk. The last part of the sequence is the beginning of the *carB* gene.

typical translational start. The GTG codon mentioned in a previous section is located only two to four nucleotides from the 5′ end of the messenger initiated at P2 and is thus not preceded by a Shine–Dalgarno sequence (29). Nor is there a Shine–Dalgarno sequence in the 5′ leader of the transcript initiated at P1. The next in-phase candidates are the adjacent

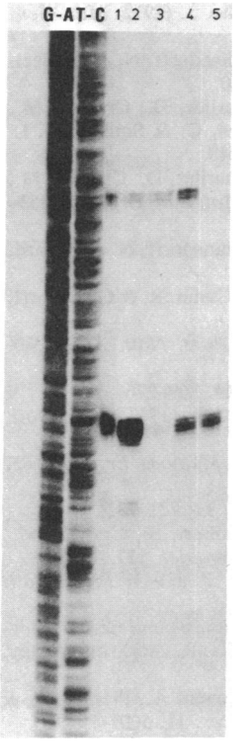

FIG. 3. S1 endonuclease mapping of the *carAB* transcripts. The probe was the 805-bp *Hpa* II/*Hin*fI fragment extending from nucleotides +112 to −693. The two left lanes correspond, respectively, to the G+A and T+C reactions. Lanes: 1, fragment protected by hybridization with RNA of JEF8 (*carB8*) strain containing pMC50; 2, fragment protected with RNA of p4XB2 (*argR*$^-$) grown on enriched medium 853; 3, fragment protected with RNA of P4X (*argR*$^+$) grown on enriched medium 853; 4, fragment protected with RNA of P4X grown on minimal medium; 5, fragment protected with RNA of P4X grown on minimal medium with uracil. No bands were observed when the probe was digested with nuclease S1 in the absence of RNA.

codons UUG and AUU, which are located 5 and 8 nucleotides, respectively, from a typical Shine–Dalgarno sequence (G-G-A-G-G).

The carA protein has been purified to homogeneity but the NH_2-terminal amino group of this protein was found to be inacessible to Edman degradation. This terminal blockage is under current investigation.

DISCUSSION

The present data reveal the molecular basis of the phenomenon previously defined as cumulative repression of carbamoyl-phosphate synthetase synthesis. Transcription of *carAB* is initiated at two tandem promoters that differ in their regulatory properties.

The upstream promoter (P1) is specifically regulated by pyrimidines. However, its sequence offers no evident clues to the mechanism of this regulation. Features typical of attenuation control (leader peptide, ρ-independent terminator) are not found in the P1 region. This picture contrasts with the organization of the control region of the *pyrBI* operon (27, 28) which is also repressed by pyrimidines but appears to be controlled by attenuation (27, 28). The control regions of *carAB* and *pyrBI* are not without similarity, however. Roof *et al.* (27) have pointed out that the most probable *pyrBI* promoter (P2 in ref. 28) immediately precedes an imperfect palindrome of composition C-C-G-G-A-C-A-A-T-T-T-G-C-C-G-G; seven nucleotides beyond the −10 region of promoter P1 of *carAB* we find the octamer T-T-T-G-C-C-A-G. Thus, the relative positions of the octamer and of the −10 region are almost identical in both promoters. *carA* P1 also contains the sequence C-A-T-A-T-C-T, which is found in front of the −10 region of *pyrBI* promoter P1 (28). These sequence similarities suggest that *pyrBI* and *carAB* may respond to a common regulatory factor [a regulatory protein related to the pyrimidine pathway or RNA polymerase itself, as suggested by Jensen *et al.* (30) in the case of *pyrBI*]. Previous data from our laboratory (20) have suggested the existence of a regulatory factor common to *pyrBI* and *carAB*: the presence of a large number of copies of *carAB* in *E. coli* cells results in partial derepression of *pyrBI*, as though this common factor was titrated out by *carAB* DNA. A number of regulatory mutants affecting the expression of *carAB* have been obtained from strains harboring *carA–lacZ* gene fusions (unpublished results). Their study may lead to identification of the sequences involved in the pyrimidine-specific control.

Between the −10 region of *carAB* P1 and the octamer T-T-T-G-C-C-A-G, there is a G+C-rich region, which suggests that P1 may be negatively controlled by ppGpp (26). A similar G+C-rich domain precedes the *pyrBI* P2 promoter, which is negatively affected by ppGpp (28, 31). Partial inhibition of *carAB* by ppGpp could be visualized as part of the stringent response; starvation of an amino acid would not only turn off the synthesis of RNA but would also affect the synthesis of RNA precursors.

The downstream promoter (P2) is under arginine control, as indicated by its high activity in an *argR* mutant and by its weak activity in the wild-type strain grown in excess arginine. P2 overlaps in 18-nucleotide-long imperfect palindrome that matches the consensus sequence

$$\begin{matrix}A\\T\end{matrix}\text{-A-T-G-A-A-T-A-}\begin{matrix}A\\T\end{matrix}\text{-T-N-A-T-N-C-A-N-T}$$

of the operator site ("arginine boxes") of several other genes of the arginine regulon (11, 12, 22, 23). *argE*, *argCBH*, *argF*, and *argI* are controlled by repressor interactions at pairs of *arg* boxes separated by three nucleotides (22, 23); it is probably significant, therefore, that the *arg* box overlapping P2 is followed by a second, more degenerate one. As discussed elsewhere (23), the efficiency of the control exerted by arginine via the *argR* product appears to be influenced by the composition and the number of *arg* boxes in the control region. Transcription of *carAB* at promoter P2 is regulated by arginine over a 30-fold range (see Table 1; compare specific activities of the *argR*$^-$ strain grown in the presence of uracil and of *argR*$^+$ cells provided with both arginine and uracil). The 30-fold repression of *carAB* is comparable to that of *argE* and *argCBH*, which are 40- and 60-fold repressible, respectively (23). Studies of mutants with altered regulation of *carAB* will indicate which sequences are important in arginine-mediated control at P2.

How is the cumulative pattern of *carAB* repression actually brought about? *carAB* is controlled mainly at the level of transcription (13, 14). The P1–P2 DNA segment could be long enough to accommodate two adjacent molecules of RNA polymerase. Cleavage inhibition experiments (32) indeed show that RNA polymerase covers a segment of DNA 44–50 nucleotides upstream to about 20 nucleotides downstream of the transcription start point. Simultaneous binding of adjacent polymerase molecules to the *carAB* control region may therefore be possible, because 20 base pairs downstream of the P1 start brings us to a point 48 base pairs upstream of the P2 start. Irrespective of whether polymerase binds independently or alternately at promoters P1 and P2, the following hypothesis appears plausible to explain cumulative repression of *carAB* expression.

Under conditions of simultaneous derepression of the arginine and pyrimidine pathways, both promoters would be active. In excess pyrimidines, P1 would become inactivated by a mechanism that remains unclear. In excess arginine, P2 would become repressed; P1 would be accessible but to express *carAB*, the P1-bound polymerase would have to displace operator-bound arginine repressor. Indeed, several lines of evidence suggest that transcription initiated upstream of a bound repressor molecule is much less sensitive to repression than when polymerase and repressor compete for overlapping sites. The earliest observation in this respect is that of Reznikoff *et al.* (33) on *trp–lac* fusions. Recently, we have shown that transcription initiated at a secondary promoter located outside the control region of the *argECBH*

divergent operon is only weakly repressible (12, 23). Besides, it is possible that, in the absence of pyrimidines, P1-bound polymerase destabilizes P2-bound repressor. The model of cumulative repression that we propose predicts that mutants negatively affected at P1 should be sensitive to arginine, while mutants impaired at P2 should be uracil sensitive.

Other genes or operons display alternative transcription start points. The *gal* operon contains two overlapping promoters, both of them controlled by the same repressor (34, 35). The adenylate cyclase, *deo*, and rRNA operons exhibit tandem, well spaced-out promoters (36, 37). In principle, such genetic arrangements are well suited for fine tuning of gene activity by independent regulatory signals. In the case of the *carAB* operon, the physiological significance of a dual-promoter structure is particularly clear-cut. From the mechanistic point of view, it will be interesting to compare the different systems when more becomes known about the DNA–protein and protein–protein interactions involved in their functioning.

The exact translational start point of the *carAB* messenger RNA remains conjectural because of the unamenability of the *carA* gene product to Edman degradation. The only possible initiation codons to be preceded at a canonical distance by a Shine–Dalgarno sequence are the adjacent codons UUG and AUU, both of which have been recognized to function *in vivo* as initiating codons in exceptional cases (38–41). Either codon is followed by an adenine, which might facilitate interaction with formylmethionyl-tRNA, either by an interaction with the invariant uridine present on the 5′ side of the anticodon (42) or by contributing to the stacking energy of the codon–anticodon interaction (43, 44). In the case of UUG, an interaction with one or two of the adenine nucleotides present on the 3′ side of the anticodon loop might be envisaged: UUG is preceded by two uridine residues on *carAB* messenger RNA. The amino acid composition of a protein starting at UUG or AUU would be in good agreement with the composition determined by Trotta *et al.* (45), assuming a M_r of 42,000.

We acknowledge the help of Myriam Hoeykens in determining the sequence of the *carA* control region. We are grateful to Henri Grosjean, Patrick Stragier, Jean Bouvier, and Jean-Claude Patte for fruitful discussion and for disclosing unpublished results. This work was supported by grants from the Fonds de la Recherche Fondamentale Collective-Fonds voor Kollectief Fundamenteel Onderzoek and from a Concerted Research Action between the Free Universities of Brussels and the Belgian State, and by Grant GM 25846 (to C.J.L.) from the National Institutes of Health. J.P. is a fellow of the Belgian Instituut voor Wetenschappelyk Onderzoek in Nyverheid en Landbouw.

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