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# Direction of Transcription of the Regulatory Gene *araC* in *Escherichia coli* B/r

**(protein synthesis *in vitro*/clockwise transcription/L-arabinose/positive control)**

GARY WILCOX, JAMES BOULTER, AND NANCY LEE

Section of Molecular Biology and Biochemistry, Department of Biological Sciences, University of California, Santa Barbara, Calif. 93106

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**ABSTRACT** **The protein product of the regulatory gene *araC* can be synthesized in a cell-free, protein-synthesizing system programmed with a λ*paraC*$^+$*B* DNA template. Hybrid, renatured phage DNA molecules prepared with DNA from phages λ*paraC*$^+$*B* and λ*paraC3B* (*araC3* is a nonsense mutation) were used to program the cell-free synthesis of the *araC* protein. The findings observed lead to the conclusion that the codogenic strand of the *araC* gene is on the light strand of the phage DNA. The *araB* gene is on the heavy strand, as determined by DNA·RNA hybridization. Thus, with regard to the standard *E. coli* map, *araC* is transcribed in a clockwise direction, whereas transcription of the *araBAD* operon has a counterclockwise orientation. The technique described should allow one to determine the direction of transcription of any gene that can be incorporated into the genome of a specialized transducing phage.**

The three structural genes of the L-arabinose *BAD* operon of *Escherichia coli* B/r, *araB*, *araA*, and *araD*, code for the structure of three proteins, L-ribulokinase, L-arabinose isomerase, and L-ribulose 5-phosphate-4-epimerase, respectively. Polarity of the operon has the orientation $\xrightarrow{araB\ araA\ araD}$, with the controlling elements at the end of *araB* farthest from *araA*. The expression of the *araBAD* operon is controlled by *araC*, which is closely linked to the structural genes of the *araBAD* operon but is not in this operon. The region between *araB* and *araC* contains the controlling sites for the *araBAD* operon: the initiator (*araI*), the promoter ($araP_{BAD}$), and the operator (*araO*). *araO* is located between the *araI*–$araP_{BAD}$ sites and *araC*. The order of *araI* and $araP_{BAD}$ is not known. The product of the regulatory gene *araC* is a protein that interacts with the *araO* site on the DNA in the absence of L-arabinose. In the presence of L-arabinose, *araC* protein is removed from *araO* and converted into an activator that interacts at the *araI* site to allow initiation of transcription of the *araBAD* operon (Fig. 1; for review see refs. 1 and 2).

There is no existing evidence from which one can deduce the direction of transcription of the *araC* gene. Although many operons in *E. coli* are known to be transcribed in a counterclockwise direction, at least two operons have divergent transcription—the *argECBH* cluster (3) and the biotin locus (4). The only regulatory gene for which the direction of transcription is known in *E. coli* is the *lac i* gene. Both genetic (5) and biochemical (6) evidence demonstrate that the *lac i* gene is transcribed in a counterclockwise direction, the same direction as the *lac* operon.

In this paper we describe a technique that allows us to demonstrate that the *araC* gene is transcribed in a clockwise direction. This is opposite to the direction of transcription of the *araBAD* operon. The technique used could, in principle, be applied in the determination of the orientation of transcription of any gene.

Abbreviations: H-strand and L-strand, heavy and light strand, respectively, of λ DNA.

## MATERIALS AND METHODS

*Bacteria and Bacteriophage.* Unless otherwise noted, all bacterial strains used in this study are derivatives of *E. coli* K-12 that carry the *ara–leu* region of *E. coli* B/r strain UP 1000. Details of the construction of the heat-inducible, lysis-defective, plaque-forming, *ara*-transducing phage λ*paraC*$^{ts}$*67B* (NL 20-806) and its derivatives, λ*para* C$^+$B (NL 20-820) and λ*para* C3B (NL 20-822), will be described elsewhere (J. Boulter and N. Lee, manuscript in preparation). Fig. 1 shows

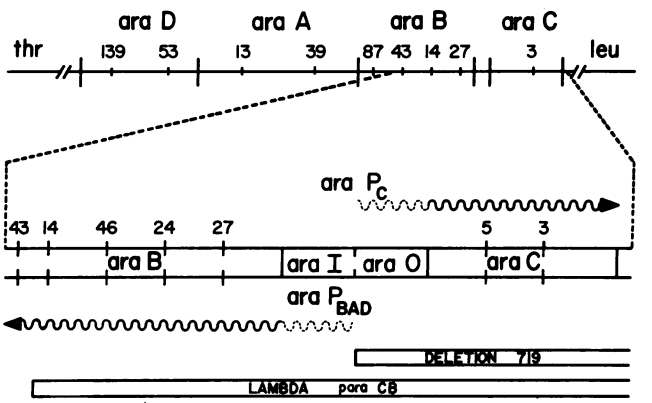


FIG. 1. The L-arabinose operon (*araBADIOC*) and neighboring regions of *Escherichia coli* B/r. Genes *araD*, *araA*, and *araB* code for the L-ribulose 5-phosphate 4-epimerase, L-arabinose isomerase, and L-ribulokinase, respectively. Gene *araC* is the positive control element required for the expression of the L-arabinose operon. The *numbers* represent point mutants (1). The transcription products and the direction of synthesis of the promoter-proximal portion of the *araB* gene and the *araC* gene are represented by wavy lines (⇝); the *dashed portion* at the 5′ terminus of each transcription product is included as there is no evidence, as yet, that any portion of the *ara* controlling sites is transcribed. The plaque-forming *ara*-transducing phage, λ*paraCB* (NL 20–806), used in the hybridization studies harbors about 50% of the *araB* gene (from *araB14* to the right), the *ara* controlling sites, and all of *araC*. In order to distinguish among the various promoters in the L-arabinose system, we use subscripts containing the letters of the genes transcribed from that promoter. For example, the promoters for the *araC* gene and the *araBAD* operon will be designated $araP_C$ and $araP_{BAD}$, respectively. Abbreviations: *thr*, threonine; *leu*, leucine; *araO*, the operator site; *araI*, the initiator site.

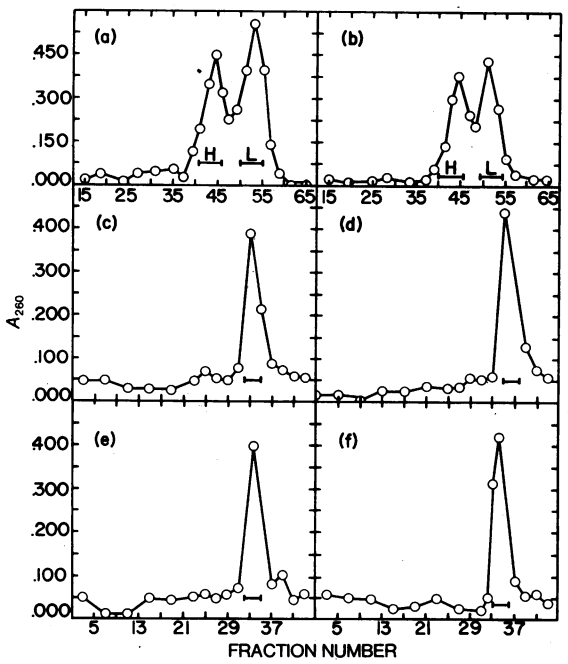


FIG. 2. (*a*) Separation of the heavy (*H*)- and light (*L*)-strands of the plaque-forming *ara*-transducing phage λ*paraC*$^+$*B* by centrifugation to equilibrium in CsCl gradients. The *bars* (⊢) indicate the fractions pooled. (*b*) Separation of the H- and L-strands of the plaque-forming *ara*-transducing phage λ*para C*$^3$*B* by centrifugation to equilibrium. CsCl sedimentation equilibrium gradient profile of: (*c*) $C_H{}^+C_L{}^+$ renatured native duplex DNA, (*d*) $C_H{}^3C_L{}^3$ renatured native duplex DNA, (*e*) $C_H{}^+C_L{}^3$ renatured hybrid duplex DNA, and (*f*) $C_H{}^3C_L{}^+$ renatured hybrid duplex DNA. Preparation of the samples is described in *Materials and Methods*. Fractions were collected dropwise from a hole punched in the bottom of the tube.

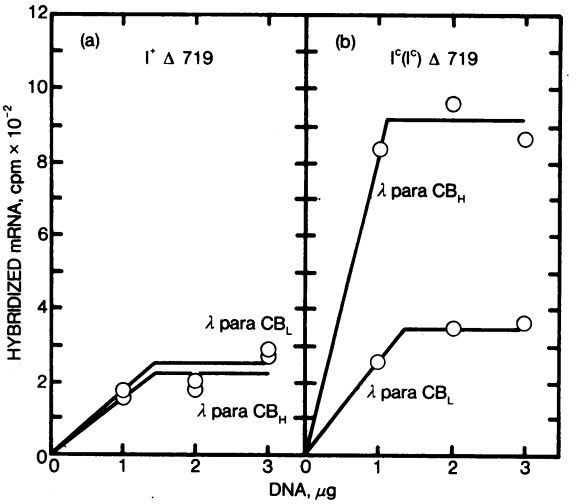


FIG. 3. Strand specificity of [$^3$H]RNA synthesized *in vivo* as measured by hybridization to increasing amounts of DNA. Pulse-labeled [$^3$H]RNA was isolated from (*a*) *araI*$^+$Δ*719* and (*b*) *araI*$^{c110}$*I*$^{c44}$Δ*719* as described in *Materials and Methods* and used in liquid hybridization assays with the indicated amounts of purified λ*paraC*$^+$*B* H-strand and L-strand DNA. Aliquots of the [$^3$H]RNA solutions were mixed with 1 μg, 2 μg, and 3 μg of the appropriate DNA in a total volume of 0.45 ml of 10 mM Tris·HCl, 0.5 M KCl, pH 7.3. The samples were then incubated for 4.5 hr at 67°. At the end of this time, the samples were quickly chilled in an ice-bath. The samples were diluted with 10 ml of 0.3 M sodium chloride, 0.03 M sodium citrate, pH 7.0, and filtered slowly through nitrocellulose filters. The filters were treated with RNase for 1 hr at room temperature, washed several times with 0.3 M sodium chloride, 0.03 M sodium citrate, dried, and counted. The number of cpm hybridized to λ*paraC*$^+$*B* H- or L-strand in excess of λ helper H- or L-strand is considered to be *ara* mRNA.

that the λ*para C*$^+$*B* phage carries the promoter-proximal half of the *araB* gene, *ara* controlling sites, and all of *araC*. *araI*$^+$-Δ*719* (SB 1095), *araI*$^{c110}$*I*$^{c44}$Δ*719* (SB2336), and the strains used for the preparation of components for the cell-free protein-synthesizing system have been described (7–9).

*Growth of Phage Lysates and Purification of Phage.* A 500-ml culture of *E. coli* K-12 *ara*Δ*766* (NL 20-028) was grown overnight in a 1-liter Kluyver flask at 37°. The cells were harvested by centrifugation at 5,860 × *g* for 20 min and resuspended in an equal volume of buffer containing (w/v): 1% $K_2HPO_4$–$KH_2PO_4$ (pH 7.0), 0.02% $MgSO_4 \cdot 7H_2O$, and 0.1% $(NH_4)_2SO_4$. The cells were then infected with the desired phage at a multiplicity of 0.1 plaque-forming phage per cell and incubated with slow shaking for 15 min at 37°. Aliquots of 100–200 ml of the infected cells were diluted into fresh, warmed tryptone–yeast extract medium (1–2 liters) and grown in Kluyver flasks with aeration for 2.5–3 hr. At the end of this time the culture was treated with chloroform and centrifuged at 7400 × *g* for 30 min to remove bacterial debris. The supernatant was adjusted to contain 0.5 M NaCl, and the phage were removed by addition of 10% (w/v) polyethylene glycol. The concentrated phage pellet was resuspended in 40–80 ml of 10 mM Tris·HCl, 10 mM $MgSO_4$, pH 7.5 by slow stirring overnight at 4°. The phage were purified by centrifugation in CsCl block gradients followed by centrifugation in CsCl to equilibrium. All *ara* transducing phage were plaque-forming and appeared as a single band in CsCl equilibrium gradients, with a density greater than that for the parental λcI857S7 phage.

*Separation and Renaturation of* λ*para DNA Strands.* The separation of heavy (H)- and light (L)-strands of λ*paraC*$^+$*B* and λ*paraC3B* by centrifugation to equilibrium in CsCl (10, 11) in a Spinco 25.1 rotor is illustrated in Fig. 2*a* and *b*. Each of the purified, separated strands (referred to in the text as $C_H{}^+$, $C_L{}^+$, $C_H{}^3$, and $C_L{}^3$) was treated in the following way. About 100 μg of $C_H{}^+$ DNA was incubated for 2.5 hr at 67° with equal amounts of $C_L{}^+$ or $C_L{}^3$ DNA. The same annealing schedule was used for $C_H{}^3$ DNA. At the end of the incubation period the samples were removed and immediately chilled in an ice bath. Each of the four samples was then rebanded in CsCl equilibrium gradients in a Spinco SW65 rotor to remove any single-stranded material remaining after renaturation. $A_{260}$ profiles for the renatured native duplexes, $C_H{}^+C_L{}^+$ and $C_H{}^3C_L{}^3$, and the renatured hybrid duplexes, $C_H{}^+C_L{}^3$ and $C_H{}^3C_L{}^+$, are presented in Fig. 2*c–f*, respectively. The pooled, purified duplexes were dialyzed extensively against 10 mM Tris-acetate buffer, pH 8.2, before use. Each of the separated strands was also subjected to renaturation, as described above, and used as control templates in the cell-free protein synthesizing system.

*Messenger RNA·DNA Hybridization Assay.* Fifty-milliliter cultures of *araI*$^+$Δ*719* and *araI*$^{c110}$*I*$^{c44}$Δ*719* were grown overnight in 1% casein hydrolysate medium containing 50 μg/ml

TABLE 1. *Template complementation studies*

| Complementing DNA | L-Arabinose isomerase (units/ml) | β-galactosidase ($A_{420}$) |
|---|---|---|
| λ*paraC*$^+$, native | 0.133 | 0.783 |
| λ*paraC*$_H{}^+C_L{}^+$, renatured | 0.113 | 0.753 |
| λ*paraC*$^3$, native | 0.002 | 0.790 |
| λ*paraC*$_H{}^3C_L{}^3$, renatured | 0.003 | 0.770 |
| λ*paraC*$_H{}^+C_L{}^3$, renatured | 0.001 | 0.720 |
| λ*paraC*$_H{}^3C_L{}^+$, renatured | 0.085 | 0.718 |
| λ*paraC*$_H{}^3$ | 0.004 | 0.434 |
| λ*paraC*$_L{}^3$ | 0.002 | 0.571 |
| λ*paraC*$_H{}^+$ | 0.004 | 0.478 |
| λ*paraC*$_L{}^+$ | 0.003 | 0.605 |
| None | 0.002 | 0.798 |
| λ*h80daraC*$^+A^-$, native | 1.02 | 0.740 |

The conditions for protein synthesis have been described (9). Each reaction mixture contained 19 μg/ml of λ*h80daraC*$^-A^+$ (Δ*766*) DNA, 6.5 μg/ml of λ*h80dlac* DNA, and 14 μg/ml of the specified complementing DNA. Protein synthesis occurs at 37° for 80 min and is initiated by the addition of fraction S30. At the end of the synthesis period, the synthesis mixture is vigorously mixed in a Vortex mixer and divided as follows: 25 μl is added to 1 ml of β-galactosidase assay mixture and incubated at 20° for 10 hr; 125 μl of L-arabinose isomerase assay mixture is added to the remaining 125 μl of protein synthesis mixture and incubated for 10 hr at 30°. The last entry in the table, λ*h80daraC*$^+A^-$ DNA, is included for comparison with our previous work (9). The 10-fold increase in L-arabinose isomerase synthesis when the λ*h80daraC*$^+A^-$ template is compared with the λ*paraC*$_H{}^+C_L{}^+$, native template is probably due to the fact that the *araC*$^+$ allele of the λ*paraC*$^+B$ phage has been selected by reversion to *araC*$^+$ from *araC*$^c$ and may not be a true revertant (J. Boulter and N. Lee, manuscript in preparation). Wild-type *araC* protein is more stable in the cell-free system than any alleles that have been tested (e.g., *araC*$^c$).

of L-tryptophan. In the morning the cells were diluted to a culture density of $1.65 \times 10^8$ cells per ml in 100 ml of the same medium. When the cultures had reached a density of $8.25 \times 10^8$ cells per ml, 10-ml subcultures were taken and labeled for 1 min in 125-ml flasks containing 200 μCi of [$^3$H]uridine (28 Ci/mmole). At the end of the labeling period, the cells were killed and the [$^3$H]RNA was extracted by the method of Rose, Mosteller, and Yanofsky (12). The [$^3$H]RNA was quickly frozen in a dry ice–acetone bath and stored at −86° until use. The hybridization of constant amounts of [$^3$H]RNA to various amounts of $C_H{}^+$, $C_L{}^+$, $C_H{}^3$, and $C_L{}^3$ DNA was performed as described by Lee *et al.* (11), and the results are presented in Fig. 3.

## RESULTS AND DISCUSSION

It is often the case that a regulatory gene is not part of the operon it controls [the *hut* system in *Salmonella typhimurium* is an exception (13)]. Insofar as monocistronic regulatory genes seem to be the rule rather than the exception, one frequently cannot determine the direction of transcription on the basis of polarity studies with nonsense and deletion mutations. Perhaps the most useful genetic method for determining the direction of transcription of a regulatory gene is the isolation and subsequent mapping of mutations that produce more of the desired regulatory gene product, as has been done for the *lac i* gene (14, 5). Alternatively, if the appropriate transducing phages are available, one can, by biochemical methods that have been described (6), hybridize labeled RNA to the separated strands of the transducing phage DNA and thereby deduce the direction of transcription. However, the extremely low constitutive levels of regulatory proteins that are present for some (15–17), and perhaps many, metabolic pathways make it difficult to distinguish the desired RNA from the background nonspecific RNAs. In light of the above, we have sought an alternate methodology for determining the direction of transcription of the regulatory gene, *araC*, in *E. coli* B/r.

Many techniques are available for the isolation of specific transducing phages in *E. coli* (18). The isolation of a φ*80dara* transducing phage (19) and subsequent recombination with a lambda phage containing a temperature-sensitive allele of the prophage repressor gene (20) made possible the development of cell-free protein-synthesizing systems to study regulation of the L-arabinose *BAD* operon (21, 22, 9). It has been shown that the *araC* protein can be synthesized in the cell-free system programmed with a λ*h80daraC*$^+A^-$ DNA template and that it will stimulate the synthesis of L-arabinose isomerase from a λ*h80daraC*$^-A^+$ DNA template present in the same reaction mixture, i.e., a *trans* acting positive regulatory protein is synthesized *in vitro* (9). A λ*paraC*$^+B$ transducing phage has been isolated that carries about 50% of the *araB* and the entire *araC* gene (see Fig. 1).

The experimental procedure used in the present study consisted of separating the DNA strands from λ*paraC*$^+B$ and λ*paraC3B* (*araC3* is a nonsense mutation) phages and then renaturing the single-stranded DNAs in all possible combinations. We then ask which of the renatured DNAs can direct *araC* protein synthesis in the cell-free system, as measured by the synthesis of L-arabinose isomerase from a λh*80daraC*$^-A^+$ DNA template also present in the synthesis mixture. As an internal control to rule out the possibility that some of the renatured DNAs severely inhibit the cell-free system, we have measured the synthesis of β-galactosidase from a λ*h80dlac* DNA template, which was also present in the synthesis mixture.

*The Sense Strand of the araC Gene Is on the Light Strand of the Phage.* It can be seen in Table 1 that the native or re, natured $C_H{}^+C_L{}^+$ DNA produces a product, the *araC* protein which can stimulate the synthesis of L-arabinose isomerase from the λ*h80daraC*$^-A^+$ DNA template. When the cell-free system contains a native or renatured *C3* DNA template, no L-arabinose isomerase is produced. The same result is obtained when the hybrid $C_H{}^+C_L{}^3$ DNA is used. However, when the hybrid DNA contains the light strand of the λ*paraC*$^+B$, phage, $C_H{}^3C_L{}^+$ DNA, *araC* protein is synthesized in the cell-free system, as indicated by an amount of L-arabinose isomerase synthesis, which is about 80% that obtained with the renatured $C_H{}^+C_L{}^+$ DNA. To insure that the separated strands, which had been subjected to renaturing conditions, do not contain DNA that carries the sense strand of the *araC* gene, the separated strands were used to program the cell-free system. These strands reduced the synthesis of β-galactosidase at most 2-fold, but there is no significant stimulation of L-arabinose isomerase synthesis by any one of them. Thus, the pooled strands do not contain for example, any native *araC*$^+$ DNA. It is interesting to note that the $C_L{}^+$ DNA, although it contains the *araC* sense strand, is not active in the cell-free system. It seems likely that the denatured DNA is either not transcribed or destroyed by nucleases.

TABLE 2. *Comparison of the native and renatured DNA templates*

| Complementing DNA | Incubation system | L-Arabinose isomerase (units/ml) |
|---|---|---|
| $\lambda paraC_H{}^+C_L{}^+$, native | Complete | 0.135 |
| $\lambda paraC_H{}^+C_L{}^+$, native | Complete, minus L-arabinose | 0.010 |
| $\lambda paraC_H{}^+C_L{}^+$, native | Complete, minus cyclic AMP | 0.012 |
| $\lambda paraC_H{}^+C_L{}^+$, native | Complete, plus 0.27 M D-fucose | 0.012 |
| $\lambda paraC_H{}^3C_L{}^+$, renatured | Complete | 0.085 |
| $\lambda paraC_H{}^3C_L{}^+$, renatured | Complete, minus L-arabinose | 0.007 |
| $\lambda paraC_H{}^3C_L{}^+$, renatured | Complete, minus cyclic AMP | 0.011 |
| $\lambda paraC_H{}^3C_L{}^+$, renatured | Complete, plus 0.27 M D-fucose | 0.010 |

Protein synthesis was as described in the *legend* to Fig. 1, except that the $\lambda h80dlac$ DNA template was omitted from the synthesis mixture. At the end of the synthesis period, 150 μl of L-arabinose isomerase assay mixture is added to the synthesis mixture and incubated for 10 hr at 30°.

Further support for the conclusion that the light strand is the codogenic strand of the *araC* gene on the $\lambda paraC^+B$ phage DNA is provided by comparing the native $C_H{}^+C_L{}^+$ DNA and the hybrid renatured $C_H{}^3C_L{}^+$ DNA in the cell free system. It can be seen in Table 2 that the product produced by these two DNAs requires L-arabinose and cyclic AMP to stimulate the synthesis of L-arabinose isomerase. D-Fucose, an anti-inducer of the L-arabinose *BAD* operon both *in vivo* (23) and *in vitro* (9, 22), inhibits the synthesis of L-arabinose isomerase when either DNA template is present. Thus, the product produced by either the native or renatured hybrid DNA has both L-arabinose and cyclic AMP dependence and D-fucose inhibition, characteristic properties of *araC* protein, providing additional evidence that the light strand of the phage DNA contains the sense strand of the *araC* gene.

*The Sense Strand of the araB Gene Is on the Heavy Strand of the Phage.* The fragment of the *araB* gene carried by the transducing phage allows us to determine which strand carries the codogenic strand of the promoter-proximal portion of the *araBAD* operon. $^3$H-Labeled RNA was extracted from strains $araI^{c110}I^{c44}\Delta 719$ and $araI^+\Delta 719$. The $araI^cI^c$ mutation results in constitutive expression of the *araBAD* operon in $araC^-$ strains, which corresponds to about 20% of the fully induced wild-type level (8). The mRNA extracted from the $araI^{c110}I^{c44}\Delta 719$ strain should contain measurable amounts of *araB* message and little, if any, *araC* message, since $ara\Delta 719$ removes all known point mutations in the *araC* gene (see Fig. 1). On the other hand, the $araI^+\Delta 719$ strain produces very little *araB* and little, if any, *araC* message. In Fig. 3 we see the results of annealing $^3$H-labeled RNA extracted from $araI^{c110}I^{c44}\Delta 719$ and $araI^+\Delta 719$ strains to the separated strands of $\lambda paraC^+B$ phage DNA. The *araB* mRNA hybridizes to the heavy strand of the transducing phage DNA, indicating that the sense strand of the *araB* gene is on the heavy strand of the phage DNA. The level of *ara* mRNA observed is what one would expect from the amount of constitutive expression in the $araI^cI^c\Delta 719$ strain and the length of the *araB* fragment on the $\lambda paraC^+B$ phage. Thus, the direction of transcription of the *araBAD* operon must be opposite to the direction of transcription of the *araC* gene. The *araBAD* operon is transcribed in a counterclockwise direction on the standard *E. coli* map, as deduced from genetic experiments (1, 2). Therefore, the *araC* gene must be transcribed in a clockwise direction. This places the promoter, $araP_C$, and, if there are any, the controlling elements of the *araC* gene, in the region between *araB* and *araC*. The results of complicated experiments *in vivo* involving a heteroimmune superinfecting phage have been interpreted as evidence that *araC* has the same orientation as *araBAD* (24). However, we feel that this interpretation is not correct and that our experiments provide a direct demonstration that *araC* and *araBAD* have opposite orientations.

Although *araB* and *araC* show very close genetic linkage (1), the region between them is very complex. At least three macromolecules, RNA polymerase, CGA, protein, and *araC* protein, are required for initiation of transcription of the *araBAD* operon. In addition, in the absence of L-arabinose, *araC* protein is a repressor which interacts with the *ara* operator to prevent expression of the *araBAD* operon. The picture becomes more complicated now that *araC* is known to be transcribed in a clockwise direction because the promoter for *araC* must also be in this region. Perhaps the repressor form of *araC* protein, by interacting with the *ara* operator, controls transcription of the *araC* gene as well as the *araBAD* operon. Is there divergent transcription from independent promoters controlled by a single operator? Our present efforts are directed towards determining controls associated with the synthesis of *araC* protein and an ordering of the controlling sites already known to exist between *araB* and *araC*.

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