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Divergent Orientation of Transcription from the Arginine Gene *ECBH* Cluster of *Escherichia coli*

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Ribonucleic acid (RNA) isolated from *Escherichia coli* W3350 (F^-, $argE^+C^+B^+H^+$), in the absence of L-arginine, hybridizes with the separated leftward (*l*) and rightward (*r*) transcribing strands of the arginine transducing phage hϕ80*darg*$E^+C^+B^+H^+$ $ppc^+imm^{\lambda cI857}$ deoxyribonucleic acid (DNA) with a ratio of 30:70, respectively. In the presence of L-arginine and its intermediates, L-ornithine and L-citrulline, RNA transcriptions from both the strands of the *argECBH* cluster were repressed. The derepressed RNA, when hybridized with the separated strands of hϕ80*dargEC*-I imm^{λ} phage DNA (the arginine genes are inversely inserted in this phage), which has a deletion in gene *E* and extends to gene *C* of the *argECBH* cluster, showed no leftward transcription, whereas the rightward transcription was reduced to about 40% of that when the DNA carrying the entire *ECBH* cluster was used for hybridization. The hybridization results thus demonstrate that (i) the regulation of the *argECBH* gene cluster in *E. coli* is under transcriptional control, (ii) the orientation of transcription is divergent, (iii) *E* gene transcribes anticlockwise, whereas the rest of the genes, *C*, *B*, and *H*, transcribe clockwise, and (iv) the position of the promoter(s) and operator(s) is located between the *E* and *C* genes of the *argECBH* cluster.

Arginine biosynthesis in *Escherichia coli* K-12 is mediated by nine structural genes, four of which are linked to form the *argECBH* cluster situated at 77 min of the *E. coli* chromosome (15). These genes are under the repressive control of *argR* gene product (3). The *argR* gene is located at a separate site on the chromosome (7, 12, 14). The evidence that the expressions of *argCBH* genes are controlled coordinately with each other, but noncoordinately with that of *argE*, has been reported previously (5). Jacoby (11), Elseviers et al. (4), and Bollon and Vogel (2) have presented genetic evidence that, like the biotin genes of *E. coli* (8), the *argECBH* cluster is transcribed divergently. They have suggested further, from the analysis of *cis*-dominant regulatory mutations in the cluster, that the transcription proceeds from an internal promoter-operator complex situated between *argE* and *argC* genes. These findings led us to investigate the transcription from the *argECBH* cluster and characterize the messenger ribonucleic acid with respect to its orientation by hybridizing with the separated strands of arginine transducing phage deoxyribonucleic acid (DNA). Our results show that the expression at the genes of the *argECBH* cluster in *E. coli* is under transcriptional control and the orientation of transcription is divergent—the gene *E* transcribes leftward (anticlockwise), whereas the *CBH* genes transcribe rightward (clockwise, Fig. 2).

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used were: *E. coli* W3350 (F^-, arg^+, gal^-, bio^+) obtained from the laboratory; the double lysogens M5 (hϕ80 $imm^{\lambda cI857}$, hϕ80 *darg*$E^+C^+B^+H^+ppc^+$ $imm^{\lambda cI857}$), which carries a defective arginine transducing phage, and MN42 λ^- (hϕ80 $imm^{\lambda cI857}$, hϕ80 d*argEC*-I ppc^+ imm^{λ}), which carries a defective arginine transducing phage with a deletion in *argE* and extending into *argC* genes. The orientation of the *argCBH* cluster in the transducing phage is the reverse of the *arg* cluster in *E. coli* chromosome, that is, the cistrons are oriented as *argHBC* in the transducing phage (our observation and W. Maas' personal communication). These two lysogens were kindly supplied by N. Glansdorff.

Chemicals and reagents. All chemicals used, including L-arginine monohydrochloride (BDH Bio-Chemical), L-(1)-ornithine monohydrochloride (Baker), and L-citrulline (Sigma Chemical Co.), are commercially available.

Preparation of the helper hϕ80 $imm^{\lambda cI857}$ and the defective arg phage hϕ80 darg $imm^{\lambda cI857}$. The lysogens were grown to an optical density (OD) of 0.12 (2.4

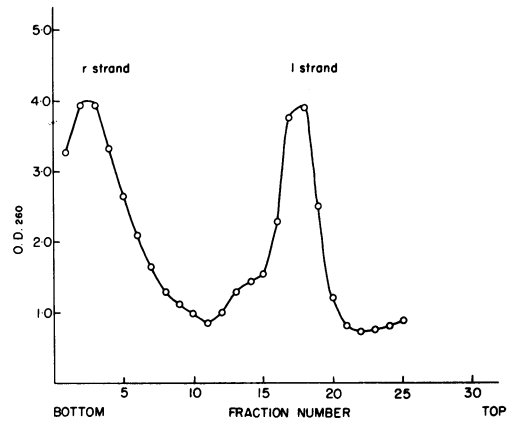

FIG. 1. *Fractionation of the l and r strands of hφ80 dargECBH imm$^{\lambda cI857}$ DNA by binding with the ribopolymer poly U in the ratio of three parts poly U to 1 part phage DNA (wt/wt). Fractions (90 μliters) were collected after centrifugation of the denatured poly U-bound DNA at 30,000 rpm for 48 h in a Spinco SW50.1 rotor at 4 C.*

× 10^8 cells/ml) at 30 C in nutrient broth in the presence of 0.05 M $MgSO_4$. They were then induced at 43 C for 15 min and subsequently grown for 2 to 4 h at 37 C with vigorous shaking till lysis was complete. The bacteriophages were purified by CsCl gradient centrifugation as described by Guha et al. (8). The band of the transducing phage was 7 mg/cm^3 heavier than that of the helper phage. The two bands were separated by puncturing the bottom of the tube and collecting 90-μliter fractions. The OD at 260 nm (OD_{260}) was taken for each of the fractions. The top helper phage fraction (hφ80 *imm*$^{\lambda cI857}$) and the bottom transducing phage fractions (hφ80 *darg imm*$^{\lambda cI857}$) were pooled in such a way that there was very little cross-contamination. The two fractions were purified further by a second cycle of purification through CsCl gradient centrifugation.

Separation of the complementary strands of phage DNA. The method for separating phage DNA complementary strands used was that of Hradecna and Szybalski (10). The phage in CsCl was dialyzed against 10^{-3}M ethylenediaminetetraacetic acid (EDTA), pH 8.5. The complementary *l* and *r* strands from hφ80 *imm*$^{\lambda cI857}$ phage DNA were separated by binding with the ribopolymer poly U, G (Miles Laboratory, Elkhart). DNA strands of hφ80 *dargE*$^+$*C*$^+$*B*$^+$*H*$^+$ *imm*$^{\lambda cI857}$ and hφ80 *dargEC*-1 *imm*$^{\lambda}$ phages were separated by binding with the ribopolymer poly U (Miles Laboratory, Elkhart) in the ratio of 1 part of DNA to 3 parts of poly U (wt/wt). To an approximate volume of 0.4 ml of hφ80 *darg imm*$^{\lambda cI857}$ phage suspension in 10^{-3}M EDTA containing 100 μg of DNA equivalent (1 OD_{260nm} = 50 μg of phage DNA equivalent), 0.3 ml of poly U from a stock solution (1 mg/ml in distilled water) was added. The total volume was made to 1 ml, and 5 μliters of 10% Sarkosyl (Geigy) and 5 μliters of 1 M NaOH were added. The mixture was kept in a boiling-water bath for 5 to 7 min and then chilled immediately. Approximately 4 ml of a saturated solution of CsCl was added, and the refractive index of the mixture was adjusted to 1.402. The CsCl solution containing the poly U-bound denatured DNA was now centrifuged at 30,000 rpm for 48 h in a Spinco SW50.1 rotor. Fractions of 90 μliters were collected by puncturing the bottom of the tube and OD_{260} of each fraction was taken. The two fractions containing *l* and *r* strands were separated well enough for the preparative work (as shown in Fig. 1). The strands were also separated by using the ribopolymer poly U,G instead of poly U. In this case, the density difference between the heavy and the light (*r* strand and *l* strand, respectively) fractions was only 5 to 7 mg/cm^3, as determined by the Spinco model E analytical centrifuge. Since this density difference was found to be very small, hφ80 *darg* DNA strands used in all the hybridization experiments were separated by binding with poly U.

The *l* and *r* fractions of the hφ80 *imm*$^{\lambda cI857}$ and hφ80 *darg imm*$^{\lambda cI857}$ phage DNA were pooled and self-annealed to avoid any cross-contamination and to dissociate the ribopolymers from the strands. The strands were checked by hybridizing with [^{3}H]ribonucleic acid (RNA) isolated from the lysogen CSH 43(hφ80 *imm*$^{\lambda cI857}$), obtained from the Cold Spring Harbor Laboratory, uninduced and late (28 to 30 min) after induction. Regardless of the type of ribopolymers used for the separation of the strands, the uninduced RNA was hybridized only with the *l* strand of both the helper and hφ80 *darg* DNAs, whereas 80% of the late (28 to 30 min) RNA after induction hybridized with the *r* strand of the phage DNA. The complementarity of the *l* and *r* fractions were verified further by the capability of annealing together when the *l* and *r* fractions were mixed and kept at 65 C for 2 h.

Isolation of RNA. RNA was isolated from the bacterial strain W3350. About 40-ml cultures were grown at 37 C in minimal medium containing M9 buffer (1) supplemented with vitamin B_1 (2 μg/ml), glucose (2%), and all the essential L-amino acids except L-arginine, L-glutamic acid, L-glutamine, L-ornithine, and L-citrulline. (The medium will henceforth be referred to as "arginine-less medium.") For the isolation of [^{32}P]RNA, M9 buffer was replaced by 0.15 M NaCl in 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 7.2. At a cell density around 2 × 10^8 cells per ml, the cells were centrifuged and suspended in either 0.15 M NaCl in 0.01 M Tris-hydrochloride, pH 7.2, for [^{32}P]RNA or in 4 ml of M9 buffer for [^{3}H]RNA. After starvation for about 10 min in either of the buffers, the cells were again centrifuged in 4 ml of arginine-less medium. A 1-ml amount each of these resuspended cells was distributed to each of 9 ml of fresh minimal medium containing either (i) L-arginine (100 μg/ml), (ii) L-ornithine (100 μg/ml), (iii) L-citrulline (100 μg/ml), or (iv) unsupplemented minimal medium. After about 10 min of incubation, either ^{32}P (5 μCi/ml as $NaH_2{}^{32}PO_4$ in water (the specific activity of ^{32}P was 500 mCi/mmol; New England Nuclear Corp.) or [^{3}H]uridine (25 μCi/ml, New England Nuclear Corp.) was added for a period of 2 min, the pulse was quenched by pouring in frozen Tris buffer or M9

medium, and RNA was subsequently isolated by the technique of Godson (6).

Thawed cells were centrifuged at 8,000 rpm for 8 min at 4 C and suspended in 0.4 ml 25% sucrose (in 0.01 M Tris-hydrochloride, pH 6.8), followed by the addition of 0.1 ml lysozyme-ethylenediaminetetraacetic acid(EDTA) solution (5% lysozyme in 0.1 M EDTA).

A lysing mixture containing 0.1 ml of 5% Brij 58 in 0.1 M Tris-hydrochloride, pH 7.2, per ml, 0.1 ml of 0.1 M $MgSO_4$ per ml, 0.05 ml of 1 mg of deoxyribonuclease (Worthington Chemicals, ribonuclease [RNase] free) per ml, and 0.4 ml of 1% sodium deoxycholate in 0.1 M Tris-hydrochloride, pH 8.6, was added to the cells, and the suspension was agitated to get complete lysis. RNA was extracted by addition of 0.1 ml of 2% Macaloid and 1.5 ml of water-saturated phenol, followed by centrifugation at 10,000 rpm for 10 min. Phenol extraction was performed three times. The radioactivity in trichloroacetic acid-precipitable fractions from the aqueous layer after final phenol extraction was determined.

Hybridization procedure. The liquid hybridization technique of Nygaard and Hall (14) and modified by Guha et al. (9) was employed. Excess *l*- or *r*-strand DNA (keeping DNA-RNA as 3:1 in all experiments) was mixed with RNA isolated under various growth conditions. The mixture, together with 0.3 ml of phenol-saturated 2 × SSC (0.3 M NaCl-0.03 M sodium citrate, pH 7.2) was incubated at 60 C for 4 h. The mixture was then passed through 2 × SSC-equilibrated nitrocellulose filters (type B6 course, Schleicher & Schuell) treated with RNase (20 μg of pancreatic RNase per ml and 10 U of T_1 RNase per ml) for 30 min at 37 C. The filters were then rewashed with 2 × SSC and dried. Radioactivity trapped in the filters was determined by Packard Scintillation Counter (model 3375) with toluene-2,5-diphenyloxazole-1,4-bis-(5-phenyloxazolyl)-benzene as the scintillation fluid.

RESULTS AND DISCUSSION

Table 1 shows that about 2.5% of the [^{32}P]RNA isolated from *E. coli* W3350 in arginine-less medium was hybridizable with the *l* strand of φ80 d*argE*$^+$*C*$^+$*B*$^+$*H*$^+$ *ppc*$^+$ *imm*$^\lambda$ phage DNA, whereas 5% of the radioactivity was annealed with the *r* strand, giving the ratio of *l*:*r* as 33:67. In the presence of L-arginine (100 μg/ml) both *l* and *r* specific RNA were completely repressed. When derepressed RNA labeled with [^{3}H]uridine, hybridized with the separated strands of hφ80 d*argE*$^+$*C*$^+$*B*$^+$*H*$^+$ phage DNA, the ratio of hybridization to the *l* and *r* strands was 26:74, respectively (Table 2). Although the strand ratio was found to be more or less the same as that obtained with [^{32}P]RNA, the efficiency of hybridization was considerably reduced. The results in Tables 1 and 2 represent the observations of several experiments. To rule out the possibility that the high efficiency of hybridization was not due to nonspecific, radioactive cellular materials other than DNA-RNA hybrids which were retained in the filter, the RNA after isolation was characterized with respect to DNA and phospholipid contaminants. No such contaminant was observed in any of our preparations. We do not have any satisfactory explanation for such a high efficiency of hybridization with [^{32}P]RNA.

In the presence of L-ornithine or L-citrulline (Tables 1 and 2, lines 3 and 4), the *l* RNA transcription was repressed completely, whereas *r* RNA transcription was repressed 80 to 95% in presence of L-ornithine and more than 98% in presence of L-citrulline. This has also been observed from our hybridization result (Table 2, figures in parenthesis) with [^{3}H]RNA isolated from strain MA 123, which is an arginine auxotroph (*argG*$^-$, obtained from Dr. Maas).

When [^{3}H]RNA isolated from the strain W3350 in absence of L-arginine was hybridized with the separated *l* and *r* strands of hφ80 d*argEC*-1 *imm*$^\lambda$ phage DNA in which the arginine gene cluster is inversely oriented and where

TABLE 1. *Hybridization of [^{32}P]RNA isolated from depressed and repressed argECBH cluster of E. coli with isolated DNA strands*[a]

Co-repressor in the medium (a)	Amt hybridized with DNA (%)				Corrected amt hybridized with *argECBH* DNA (%)		*l* + *r* (%)	*l*:*r* (%)
	l strand		*r* strand		*l* strand	*r* strand		
	φ80d*arg* (b)	φ80 (c)	φ80d*arg* (d)	φ80 (e)	(b-c)	(d-e)		
No co-repressor (arginine-less)	4.12	1.60	5.80	0.60	2.52	5.20	7.72	33:67
L-Arginine (100 μg/ml)	0.41	0.40	0.41	0.41	0	0	0	
L-Ornithine (100 μg/ml)	0.68	0.69	1.68	0.64	0	1.04	1.04	0:100
L-Citrulline (100 μg/ml)	0.04	0.04	0.26	0.08	0	0.18	0.18	0:100

[a] Input radioactivity: 12,000 to 15,000 counts/min. All hybridizations were done in DNA excess (DNA-RNA = 3:1).

TABLE 2. *Hybridization of [³H]RNA isolated from derepressed and repressed argECBH cluster of E. coli with isolated DNA strands of the arginine transducing phage*[a]

Co-repressor in the medium (a)	Amt hybridized with DNA (%)				Corrected amt hybridized with *argECBH* DNA (%)		*l* + *r* (%)	*l*:*r* (%)
	l strand		*r* strand		*l* strand	*r* strand		
	φ80d*arg* (b)	φ80 (c)	φ80d*arg* (d)	φ80 (e)	(b–c)	(d–e)		
No co-repressor (arginine-less)	0.300 (0.360)	0.230 (0.315)	0.427 (0.481)	0.230 (0.316)	0.070 (0.045)	0.197 (0.165)	0.267 (0.210)	26:74 (22:78)
L-Arginine (100 μg/ml)	0.234 (0.309)	0.235 (0.314)	0.222 (0.310)	0.231 (0.311)	0 (0)	0 (0)	0 (0)	— (—)
L-Ornithine (100 μg/ml)	0.229 (0.312)	0.231 (0.316)	0.240 (0.316)	0.230 (0.318)	0 (0)	0.010 (0)	0.010 (0)	0:100 (0)
L-Citrulline (100 μg/ml)	0.232 (0.315)	0.234 (0.315)	0.240 (0.330)	0.235 (0.313)	0 (0)	0.005 (0.027)	0.005 (0.027)	0:100 (0:100)

[a] Input radioactivity: 450,000 to 500,000 counts/min. All hybridizations were done in DNA excess (DNA-RNA = 3:1). The figures in the parenthesis are the percentage of [³H]RNA isolated from *E. coli* MA 123 (*argG*⁻) hybridized with the phage DNA. For isolation of RNA from MA 123, the cells were grown in presence of 50 μg of L-arginine per ml. When the OD_{550} reached 0.12, the cells were harvested and washed to get rid of excess arginine. The co-repressors were added to the washed cells which were suspended in arginine-less medium.

the *argE* gene is completely deleted, no hybridizable *r* RNA was observed (Table 3, line 1). The radioactivity annealed with the *l* strand of the same phage (*CBH* genes) was found to be reduced considerably due to the fact that the deletion of the *E* gene extends into the *C* gene and, perhaps, extends further, as indicated by our transcription results.

The above results show that the divergence within the *argECBH* cluster begins somewhere between genes *E* and *C* and that the gene *E* is transcribed anticlockwise, or leftwards, and genes *CBH* are transcribed clockwise or rightward (Fig. 2). This divergent orientation of transcription from the *argECBH* cluster in *E. coli* is in agreement with the genetical evidences (4, 11). Similar results have also been obtained

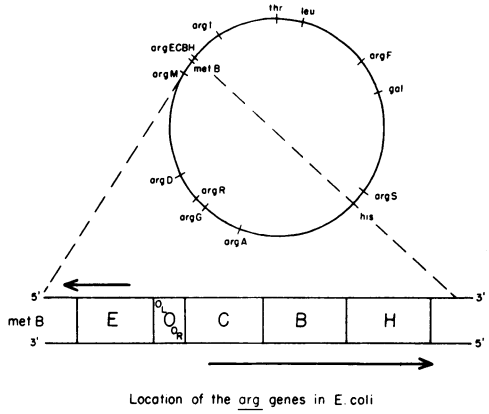

FIG. 2. *As indicated in the figure.*

TABLE 3. *Hybridization of [³H]RNA isolated from derepressed and repressed argECBH cluster of E. coli with the separated strands of φ80 dargEC-1 phage DNA*[a]

Co-repressor in the medium (a)	Amt hybridized with DNA (%)				Corrected amt hybridized with argEC-1 DNA (%)		*l* + *r* (%)	*l*:*r* (%)
	l strand		*r* strand		*l* strand (CBH genes)	*r* strand (E gene)		
	φ80d*arg* (b)	φ80 (c)	φ80d*arg* (d)	φ80 (e)	(b–c)	(d–e)		
No co-repressor (arginine less)	0.325	0.270	0.270	0.271	0.055	0	0.055	100:0
L-Arginine (100 μg/ml)	0.271	0.272	0.270	0.270	0	0	0	

[a] Input radioactivity: 450,000 to 500,000 counts/min. All hybridizations were done in DNA excess (DNA-RNA = 3:1). The page was obtained and purified after heat inducing MN42 λ-1 (hφ80 d*argEC*-1 ppc⁺*imm*$^{\lambda}$, hφ80 *imm*$^{\lambda c/857}$), (see Materials and Methods). The orientation of the arginine genes in this phage DNA is inverse.

TABLE 4. *Hybridization of [³H]RNA isolated from derepressed and repressed argECBH cluster of E. coli with isolated DNA strands of an arginine transducing phage*[a]

Co-repressor in the medium (a)	Amt hybridized with DNA (%)				Corrected amt hybridized with *argECBH* DNA (%)		*l* + *r* (%)	*l*:*r* (%)
	l strand		*r* strand		*l* strand	*r* strand		
	φ80d*arg* (b)	φ80 (c)	φ80d*arg* (d)	φ80 (e)	(b-c)	(d-e)		
No co-repressor (arginine less)	0.384	0.231	0.290	0.230	0.153	0.060	0.213	70:30
L-Arginine (100 μg/ml)	0.230	0.230	0.230	0.232	0	0	0	0

[a] Input radioactivity: 450,000 to 500,000 c.p.m. All hybridizations were done in DNA excess (DNA-RNA = 3:1). The phage was obtained after heat induction of MN42 λ⁻(hφ80 d*argE*⁺*C*⁺*B*⁺*H*⁺ *ppc*⁺ *imm*^λ, hφ80 *imm*^λ*cI857*) double lysogen. The density difference between the helper and the defective phage was 5 mg/cm³. The *l* and *r* strands of this defective arginine transducing phage were separated by binding with the ribopolymer poly U and characterized by hybridizing with the noninduced and late (28 to 30 min) [³H]RNA isolated from CSH 43 (hφ80 *imm*^λ*cI857*) lysogen. RNA from the noninduced lysogen hybridized only with the *l* strand, whereas the late RNA hybridized with the *l* and *r* strands in the ratio of 25:75.

by Pouwels et al. (personal communication) from their transcription studies using arginine transducing phage DNA in which the orientation of the *ECBH* cluster is inverted. We have also hybridized [³H]RNA isolated from *E. coli* in absence of arginine with this inversely oriented hφ80 d*argE*⁺*C*⁺*B*⁺*H*⁺ phage DNA and obtained similar results (Table 4).

Our transcription results support the enzyme activities of the repressed and derepressed *argECBH* cluster under various conditions as reported by Bollon and Vogel (2). The complete repression of *l* RNA transcription in presence of L-citrulline corroborates with their findings when they have found repression of *argE* gene activity by L-citrulline in an arginine auxotroph, with limited supply of arginine in the medium, as in our case (we had an arginine-less medium). They have observed further that the repression by citrulline is dependent on the condition of arginine restriction in the medium. This is perhaps true for other intermediates like L-ornithine. The *argE* gene product, acetyl ornithinase, coverts *N*-acetyl ornithine to ornithine, and citrulline is produced from ornithine by ornithine transcarbamylase, the *argF* gene product. If the nature of the repression of the *argCBH* genes in the presence of intermediates is carefully analyzed from our transcription data, we observe that the *CBH* genes are repressed in presence of ornithine and citrulline in a variable amount although there is a complete repression of the *E* gene transcription. It appears that, unlike other operons, *argECBH* cluster can be repressed by its intermediates and there is a gradient of repression by the intermediates, which perhaps is related to the sequence in the biosynthetic pathway.

The above results show that the *arg* mRNA transcription from the *arg ECBH* cluster is divergently oriented as in the biotin locus of *E. coli* (8) and the expression of the genes is under transcriptional control. The position of the operator in the *argECBH* cluster is proposed to be between genes *E* and *C* of the cluster.

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