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Use of Gene Cloning to Determine Polarity of an Operon: Genes *carAB* of *Escherichia coli*

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A gene-cloning approach was used to determine the transcription polarity of the carbamoylphosphate operon (*carAB*) of *Escherichia coli*. In agreement with the accompanying paper (J. Bacteriol. **143**:914-920, 1980), our results lead to the conclusion that *carA* is the proximal gene of the *carAB* operon.

The control region of the *carAB* operon, which encodes carbamoylphosphate synthase (EC 2.7.2.9), is of peculiar interest because it interacts with at least two regulatory macromolecules to produce the pattern of gene expression known as cumulative repression (6, 10, 11, 13). The accompanying paper (5) discloses the polarity of transcription of the *carAB* cluster but at the same time shows how difficult the interpretation of polar effects may be. In the present paper, the conclusions of Gigot et al. (5) are supported by the results of a totally different approach; plasmids carrying only the *carA* gene are shown to carry the control element of the cluster since the extrachromosomal *carA* copies are still repressible by arginine and uracil. A preliminary account of this work has appeared previously (4).

MATERIALS AND METHODS

Bacterial and phage strains. The bacterial and bacteriophage strains used in this work are listed in Table 1. Genetic symbols are from Bachmann et al. (1).

Phage assays and phage propagation. The procedures used for the propagation and assay of phages have been described previously (6, 8).

Heteroduplex formation and electron microscopy. The preparation of phages and the heteroduplex procedure have been described in detail (6, 8). Length measurements are expressed in kilobases (kb).

DNA restriction, cloning, and plasmid construction. All of the technical details and references concerning restriction endonuclease digestion, DNA fragment isolation, cloning, transformation, and construction and isolation of plasmids have been described by Crabeel et al. (3).

Growth of cells. The cells used for the enzyme assays were grown as described in the accompanying paper (5).

Enzyme determinations. Carbamoylphosphate synthase (EC 2.7.2.9), aspartate carbamoyltransferase (EC 2.1.3.2.), and ornithine carbamoyltransferase (EC 2.1.3.3) were assayed as described previously (6, 9, 12). The *carA* gene product (i.e., the small subunit of carbamoylphosphate synthase) was assayed by the in vitro complementation procedure described in the accompanying paper (5).

Chemicals. Restriction endonucleases were purchased from Miles Laboratories, Kankakee, Ill. All of the reagents for the enzyme assays were from Sigma Chemical Co., St Louis, Mo.

RESULTS AND DISCUSSION

Phage vectors and heteroduplex mapping of the *car* locus. The previously described λ d*car*37-9 transducing phage (6) (Fig. 1) carries a chromosomal segment of 5.1 kb, which is hardly larger than the 4.75 kb necessary to encode the two subunits of carbamoylphosphate synthase (17).

Phage λ d*car*53 was also isolated from λ 199 by the method of Schrenk and Weisberg (15). Heteroduplex molecules between λ d*car*37-9 and λ d*car*53 DNAs (Fig. 1) revealed that the two phages underwent the same type of substitution; the *car* region is carried in the same orientation on both phages, but some extra material is present on λ d*car*53 between *car* and the *att* site. λ d*car*37-9 and λ d*car*53 thus originated from prophages inserted at different sites but underwent seemingly identical excision events.

The *carB8* deletion encompasses all of the 56 known *carB* markers but does not alter the expression of *carA* (9). It could be localized on heteroduplex molecules between λ d*car*37-9 and a λ d*car*53B8 derivative obtained by preparing a *carB8*-λ *carB8* homogenate from strain Jef8 lysogenized with phage λ d*car*53. The deletion is 2.6 kb long and represents approximately 75% of the *carB* gene. The data are summarized in Fig. 2.

The almost symmetrical location of *carB8* within the chromosomal segment carried by λ

TABLE 1. *List of strains*

Organism	Genotype/phenotype	Source or reference
Bacteria		
P4XB2	Hfr P4X *metB argR* $(\lambda)^+$	This laboratory
Jef8 λ^+	Hfr *metB thr carB8* $(\lambda)^+$	9
Jef8 λ^-	Hfr *metB thr carB8*, cured of λ	9
Mi178	Hfr *metB thr carA178* $(\lambda)^+$	9
C600-4	F^- *hsdR hsdM*$^+$ *thi pro carA178 recA*	This laboratory
Phages		
λ 199	*cI857 susS7 sus xis6 b515 b519*	R. Weisberg
λ nin5	*N7 N53 cI857 nin5*	2
λ d*car*37-9	λ d*carAB*	8
λ d*car*53	λ d*carAB*	This work
λ d*car*53B8	λ d*car*53 carrying the *carB8* deletion	This laboratory
Plasmid pMB9	ColE1 Tc	P. Wensink

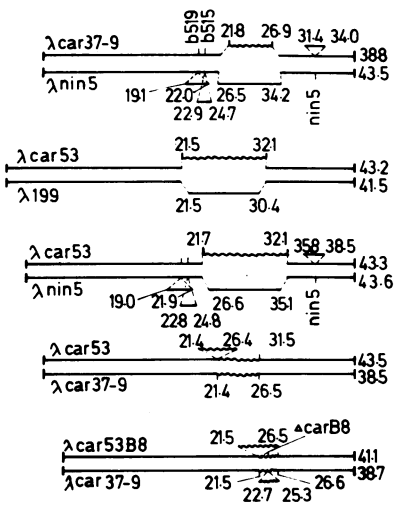

FIG. 1. *Schematic diagrams of the heteroduplexes studied. Coordinates are in kilobases. Bacterial sequences are shown as wavy lines. Dashed lines join points which are physically connected in the heteroduplexes.*

d*car*37-9 does not allow us to conclude, on this basis alone, on which side of the deletion the *carA* gene is situated. The orientation of *carAB* on the phage can in fact be deduced from a purely topographical analysis accompanying the construction of a *carA* plasmid vector (see below).

Construction of plasmid vectors. By using the appropriate restriction enzyme cuts, it was possible to isolate *carA* and determine whether the cloned gene retained the normal regulation pattern of the whole operon.

A comparison of *Eco*RI digests of λ d*car*37-9 and λ 199 revealed that segments D and C (15) were replaced by two segments (x and y) with lengths of 5.8 and 3.8 kb, respectively. Digestions of λ d*car*53 still gave a 5.8-kb fragment, but a new one 8.8 kb long replaced the shorter piece. Consequently, (i) the extra chromosomal DNA present on λ d*car*53 does not bear an *Eco*RI site and (ii) the order of the fragments on λ d*car*37-9 DNA is A'-y-x-E-F (Fig. 3). Besides, the cut separating y from x is in the space covered by the *carB8* deletion. This is demonstrated by the *Eco*RI restriction pattern of λ d*car*53B8; the 8.8- and 3.8-kb fragments are replaced by a unique piece 13 kb long (i.e., 8.8 + 5.8 − 2.6, the length of the *carB8* deletion). Thus, either x or y should carry the entire *carA* gene.

The colicinogenic factor pMB9 carries a gene conferring tetracycline resistance to its host cell. Besides, it bears only one *Eco*RI-specific site and is thus particularly suitable for cloning experiments. pMB9 and λ d*car*37-9 DNAs were digested with *Eco*RI, mixed, and ligated (see above). This DNA was used to transform Mi178, a *carA* mutant synthesizing an active *carB* subunit at a normal rate (5). Tetracycline-resistant colonies were selected on rich medium; about

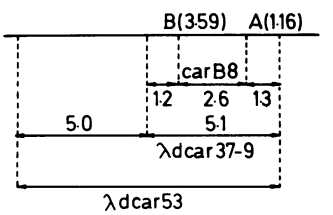

FIG. 2. *Physical map of the car region. The upper line indicates the orientation of car genes. The numbers are the lengths of the genes, as estimated from the molecular weights of the carA (42,000) and carB (130,000) proteins (17). The other lines show, respectively, the positions of deletion carB8 and of the chromosomal fragments carried by λ dcarAB 37-9 and λ dcarAB53. All distances are in kilobases.*

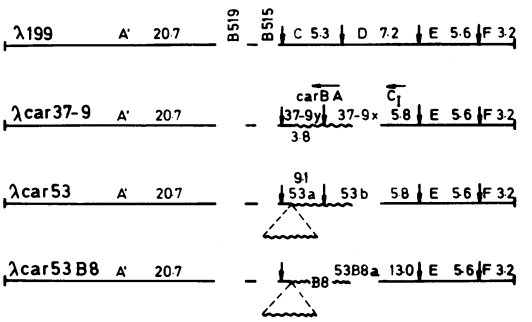

FIG. 3. *Physical map of the λ dcar phages used in this study. EcoRI cuts are indicated by arrows. Lengths are in kilobases.*

2% of the resistant clones did not require arginine or uracil for growth and could thus carry a pMB9-*carA* composite plasmid. Two of them (PGW1 and PGW2) were analyzed further.

Structure of plasmid vectors. To identify which of the x and y *car* fragments is joined to pMB9 DNA in the two plasmids, their DNAs were digested with *Eco*RI, and the fragments were examined on agarose slab gels.

Two bands were obtained from the PGW1 digest. One migrated to the same position as pure *Eco*RI-digested pMB9 DNA (5.6 kb long). The other was indistinguishable from the band given by fragment x. That it indeed contained fragment x was confirmed by the fact that PGW1 made its host cell immune to phage λ; Fig. 3 shows that fragment x was expected to carry the repressor gene. The analysis of PGW1 thus established that on λ d*car*37-9 the genes are arranged in the order *A-J-att-BAcar-C-R*.

The structure of PGW1 was examined further by electron microscopy. The length of the plasmid was 11.2 ± 0.2 kb, thus agreeing with the sum of the two fragments observed on gels; thus, only one copy of each fragment is included in the plasmid. Heteroduplex molecules between PGW1 and λ d*car*37-9 (data not shown) also provided evidence that part of the *car* region is present on PGW1.

A similar analysis of the structure of PGW2 was conducted. It demonstrated that this plasmid is composed of one pMB9 equivalent, two *car* x fragments, and a 3.2-kb contaminant of chromosomal origin (Fig. 4). However, the orientation of the *carA* gene with respect to the pMB9 sequence (and thus to the plasmid promotors) is different in the two vectors. Therefore, host cells carrying either of the two plasmids were used to study *carA* expression.

The orientation of the λ c gene and of *carA* in PGW1 was determined by restriction mapping, using *Hin*dIII, which is known to cut pMB9 DNA at a well-defined site, close to or in the promotor of the *tet* gene (14) (Fig. 4).

Expression of the *carA* gene on PGW1 and PGW2. *recA* carriers of PGW1 and PGW2 were investigated for *carA* expression in unsupplemented minimal medium and in the presence of both arginine and uracil (Table 2). The specific activity of the *carA* gene product was assayed by complementation with an extract of strain Mi178 (*carA178*). The specific activity of native carbamoylphosphate synthase was estimated as well; this value is of course limited by

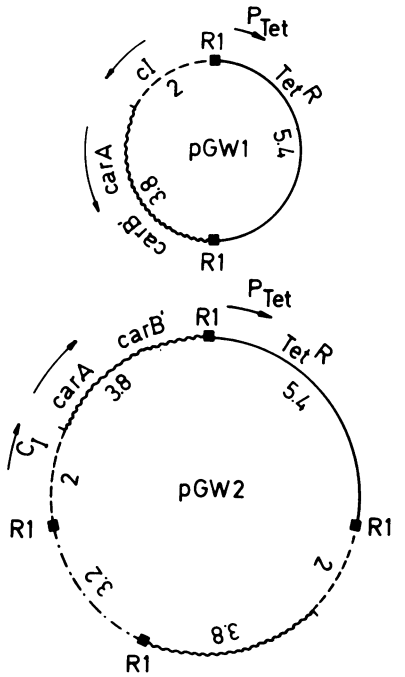

FIG. 4. *Structures of plasmids PGW1 and PGW2.* ——, *Plasmid DNA;* -----, *lambda DNA;* ∿∿, *carAB DNA;* -●-●-●-, *undetermined chromosomal fragment (in PGW2).*

TABLE 2. *Expression of the carA gene in PGW1 and PGW2 plasmid carriers*

Strain	Additions to minimal medium[a]	Enzyme sp act[b]			
		Carbamoylphosphate synthase (complete)[c]	*carA* gene product[d]	Ornithine carbamoyltransferase	Aspartate carbamoyltransferase
C600-4 (PGW1)	None	1.10	17.95	52.0	5.4
	Arginine + uracil	0.41	7.44	5.6	1.9
C600-4 (PGW2)	None	0.69	5.75	21.7	4.2
	Arginine + uracil	0.06	0.73	3.3	0.7

[a] All media contained 25 μg of tetracycline per ml.
[b] Enzyme specific activities are expressed as units (micromoles of product formed per hour) per milligram of protein.
[c] Glutamine-dependent activity of carbamoylphosphate synthase.
[d] The small subunit was assayed by in vitro complementation with cell extracts of mutant *carA178*.

the expression of the unique resident chromosomal *carB* gene. In unsupplemented minimal medium, carriers of PGW1 and PGW2 plasmids synthesized about 16- and 8-fold excesses of *carA* product, respectively. The consistently higher *carA* specific activity in the PGW1-harboring strain was paralleled by a higher level of complete carbamoylphosphate synthase (two to three times as much as in PGW2 carriers). This suggests that in the cells harboring PGW1 the number of *car* genes is already high enough to limit the efficiency of repression of all *car* genes present, including the one on the chromosome. This interpretation is supported by the fact that arginine and uracil, at the concentrations used, fully repressed the synthesis of both *carA* and complete carbamoylphosphate synthase in PGW2 carriers, whereas in the PGW1 host the repression was only partial. The higher levels of ornithine and aspartate carbamoyltransferases in PGW1, whether arginine and uracil are added or not, are consistent with this interpretation.

The average number of plasmids present in the cells has been determined in minimal medium and in condition of repression by the method of Womble et al. (18). The estimates obtained (PGW1, about 25; PGW2, about 7) were not influenced by arginine and uracil, indicating that the observed variations of *carA* specific activity reflect a true repression phenomenon.

It is worth mentioning, in support of the present demonstration, that in vitro transcription of gene *carA* on both PGW1 and PGW2 molecules is repressible to the same extent by partially purified arginine repressor in the presence of arginine (7).

The reason why PGW2 carriers produce lower levels of *carA* product is not clear. PGW2 is about twice as large as PGW1, and the number of plasmids per cell is, not unexpectedly, lower in PGW2 than in PGW1 carriers. In addition, the unknown 3.2-kb-long chromosome fragment of PGW2 might interfere with the expression of one of the two exemplars of the *car* region carried by this plasmid.

The present data show that *carA* is the proximal gene of the *carAB* operon and therefore confirm the conclusions of the accompanying paper by Gigot et al. (5). It is clear that this cloning approach to the determination of transcription polarity could be applied to other gene clusters as well.

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