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Molecular Cloning of Four Tricarboxylic Acid Cycle Genes of *Escherichia coli*

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A fragment of DNA (3.1 kilobases [kb]) from a ColE1 *Escherichia coli* DNA hybrid plasmid containing the bacterial citrate synthase gene (*gltA*) was subcloned in both orientations into phage λ vectors by in vitro recombination. The resulting phages were able to transduce *gltA* and, as prophages, complemented the lesion of a *gltA* mutant, showing that a functional *gltA* gene is contained in the 3.1-kb fragment. The segment of *E. coli* DNA cloned in these λ *gltA* phages was extended in vivo by prophage integration and aberrant excision in the *gltA* region. Plaque-forming derivatives, carrying up to three additional tricarboxylic acid cycle genes, succinate dehydrogenase (*sdh*), 2-oxoglutarate dehydrogenase (*sucA*), and dihydrolipoamide succinyltransferase (*sucB*), were isolated and characterized by their transducing and complementing activities with corresponding mutants, and the order of the genes was confirmed as *gltA-sdh-sucA-sucB*. Physical maps of a variety of the transducing phages showed that the four tricarboxylic acid cycle genes are contained in a 12.8-kb segment of bacterial DNA. The four gene products, plus a possible succinate dehydrogenase small subunit, were identified in postinfection labeling studies, and the polarities of gene expression were defined as counterclockwise for *gltA* and clockwise for *sdh*, *sucA*, and *sucB*, relative to the *E. coli* linkage map.

In *Escherichia coli* four of the genes encoding tricarboxylic acid (TCA) cycle enzymes are clustered between 16.0 and 16.5 min in the linkage map as follows: *phr-dctB*gltA-cbt-sdh-supG-lysT-sucAB-tolAB-nadA*. . . (2). The *gltA* and *sdh* genes encode citrate synthase and succinate dehydrogenase, respectively, and the *sucA* and *sucB* genes (the *suc* operon) encode the E1 and E2 components of the 2-oxoglutarate dehydrogenase complex. This clustering may be functionally significant although three other TCA cycle genes, *mdh*, specifying malate dehydrogenase; *icd*, specifying isocitrate dehydrogenase; and *lpd*, specifying the common E3 component of the pyruvate and 2-oxoglutarate dehydrogenase complexes, are situated elsewhere (2), and the genes for three other TCA cycle enzymes, aconitase, fumarase, and succinyl coenzyme A synthetase, have yet to be located.

Citrate synthase catalyzes the condensation of acetyl coenzyme A with oxaloacetate to form citrate, the first step in the TCA cycle. Mutants are characterized by their inability to grow on glucose minimal medium unless it is supplemented with L-glutamate or L-proline (1). The enzyme has a subunit molecular weight of 46,000 (23, 25). Succinate dehydrogenase catalyzes the interconversion of succinate and fumarate, and mutants are able to grow on fumarate but not succinate minimal medium. The *E. coli* enzyme has proved difficult to purify, but it is known to have a major subunit with a molecular weight of about 70,000 and one or more minor subunits with molecular weights of 26,000 to 31,000, and possibly a smaller subunit (7, 24). The 2-oxoglutarate dehydrogenase complex catalyzes the conversion of 2-oxoglutarate to succinyl coenzyme A. It comprises three types of subunit, 2-oxoglutarate dehydrogenase (E1), dihydrolipoamide succinyl-transferase (E2), and lipoamide dehydrogenase (E3), with a stoichiometry of 12 (E1):24 (E2):12 (E3) (22). The molecular weights of the subunits have been reported as: E1, 94,000 to 97,000; E2, 42,000 to 54,000; and E3, 56,500 (22, 23, 26). This complex is analogous to the pyruvate dehydrogenase complex, which has specific E1 and E2 components encoded by the two *ace* genes (*aceE*, *aceF*) of the *ace* operon at 2.6 min in the *E. coli* linkage map (2). The E3 subunit, which catalyzes the reoxidation of the reduced lipoamide coenzyme in both complexes is encoded by a single gene, *lpd*, linked to the *ace* operon but possessing its own promoter (14, 18).

Transduction studies with phage P1 have giv-

en cotransduction frequencies of 97% for *sdh* and *gltA* and 90% for *sdh* and *sucA* (8, 15), corresponding to maximum intergenic distances of 1.0 kilobase (kb) and 3.5 kb, respectively (29). From the sizes of the gene products, the lengths of the corresponding genes can be estimated as: *gltA*, 1.3 kb; *sdh*, 2.0 kb; *sucA*, 2.7 kb; and *sucB*, 1.4 kb, a total of approximately 7.4 kb. The four genes should thus be contained in an 11.9-kb fragment of bacterial DNA, which in turn should be clonable in lambda vectors. The aim of this work was to clone the *sdh* and *sucA,B* genes by allowing λ to integrate in their vicinity and generate λ *sdh* and λ *sucA,B* by aberrant excision. For this purpose, a fragment from a ColEI-*gltA*$^+$ hybrid plasmid (11) was subcloned into λ, and the corresponding prophage was established in the *gltA* region of the *E. coli* chromosome. As well as enabling further studies on the genes, their products, and their expression, the work was aimed at comparing the nucleotide sequences of the *sdh* gene with that reported for fumarate reductase (*frd*) (S. T. Cole, Eur. J. Biochem., in press), an enzyme which also catalyzes the interconversion of fumarate and succinate, and the *sucA* and *sucB* genes with those encoding the analogous *aceE* and *aceF* genes, which have been cloned (14) and are currently being sequenced in this laboratory.

MATERIALS AND METHODS

Bacteriophages. The phages used were: λNM540 (Δ*sr*Iλ1-2 *shn*λ3$^+$ *att*$^+$ *imm*^{21} *nin5* *shn*λ6°) and λNM761 (Δ*sr*Iλ1-2 *shn*λ3$^+$ Δ*att-red* *imm*^{21} *nin5* *shn*λ6°) as *Hin*dIII insertion and replacement vectors, and λNM781 (Δ*sr*Iλ1-3 *c*I857 *nin5*) and NM816 (28) as *Eco*RI replacement vectors, and their derivatives. Pools of recombinant phages, obtained by insertion of *Eco*RI and *Hin*dIII fragments of *E. coli* CR63 DNA in the corresponding vectors, were constructed (λNM761, λNM816) or were kindly provided by N. E. Murray (λNM540, λNM781). The *lpd* transducing phages used in subcloning were: λG83, a derivative of λNM781, described by Guest and Stephens (14), and λG105, a derivative of λNM540, constructed by P. E. Stephens. Phage λG105 has a 5.4-kb *Hin*dIII-*Eco*RI fragment, containing the *E. coli lpd* gene, inserted between *sr*Iλ1/2 and *shn*λ3 in λNM540 (14). Other phages, λ *b2c imm*$^{\lambda}$, λ *b2c imm*^{21}, λ *imm*^{434}, λ *c*I$^-$ *imm*^{434}, λ *h80 del9c imm*$^{\lambda}$ and λ *vir*, were used in lysogen selection and for routine testing of immunity and sensitivity.

Bacterial strains. The bacterial strains are listed in Table 1. Strain C600 was used for routine phage propagation and assays, and C600(λ *imm*^{21}), C600 (P2), W1485E(λ), and Ymel/λ were used for testing phage phenotypes. A copy of the Clarke and Carbon (5) colony bank containing synthetic ColE1-*E. coli* hybrid plasmids in strain JA200 was kindly provided by R. A. Cooper.

Media and general methods. The rich medium used for routine subculture and growth was L broth, with glucose (1 g/liter) included only for strains carrying TCA cycle mutations (14). A peptone medium containing tryptone-peptone (10 g/liter; BBL Microbiology Systems), NaCl (5 g/liter), and agar (Difco Laboratories) was used for phage assays and selection. The minimal medium E of Vogel and Bonner (27) was used with glucose (11 mM), potassium succinate (40 mM), potassium fumarate (40 mM), or potassium acetate (40 mM) as the carbon source and supplemented as required with glutamate (2 mM), succinate (2 mM), thiamine (5 mg/liter), uracil (35 mg/liter), L-tryptophan (30 mg/liter), biotin (0.1 mg/liter), and nicotinamide (10 mg/liter) for nutritional selection and testing of phenotypes. Media were solidified, where required, with agar, 15 g/liter or 10 g/liter for plates and 6.5 g/liter for top layers.

The general methods of Murray et al. (21) and Borck et al. (4) were used for the preparation of plating cells,

TABLE 1. Strains of *E. coli* K-12

Strain	Genotype[a]	Source/reference
C600	*thr-1 leuB6 thi-1 supE44 tonA21 lacY1*	N. E. Murray
C600(λ *imm*^{21})		λ *imm*^{21} lysogen of C600
C600(P2)		P2 lysogen of C600
W1485E	F$^+$ *supE42 iclR*	C. Yanofsky
W1485E(λ)		λ lysogen of W1485E
Ymel/λ	F$^+$? *mel-1 supF58*	
KS302	Hfr *sup* Δ(*gal-bio*)	K. Shimada
JRG599	*metB1 met-105 relA1 thyA56 azi tsx-87 ton? pps-1* Δ(*aroP-lpd*)*74*	Formerly KΔ18 (17)
JRG1003	*sdh0 frdA11 trpA9671 iclR trpR rpsL195*	Cole and Guest (6)
JRG72	*sucA1 supE42 iclR*	Creaghan and Guest (9)
JRG153	*sucB17 iclR trpR*	Formerly Wsuc17 (15)
W620	*thi-1 pyrD36 gltA6 galK30 rpsL129 supE44*	E. L. Wollman
H80	Hfr *thi-1 ton* Δ(*nadA-*λ*gal*)*506*	W. Epstein
KB5	Hfr *thi-1* Δ(*tolA-chlA*)	B. Rolfe
CR63	F$^+$ *supD60* λ^r	N. E. Murray
JA200	F$^+$ *thr-1 leuB6 thi-1 ara-14 lacY1 galK2 galT22 trpE63 recA56 xyl-5 mtl-1 supE44*	R. A. Cooper (5)

[a] All strains are F$^-$ and λ$^-$ unless stated otherwise.

phage stocks, phage assays, and other routine procedures.

Phage lysates and DNA preparation. High-titer phages for DNA preparation were made by infecting 200- or 400-ml cultures of exponentially growing C600 with phage at a multiplicity of infection of 1, as described previously (6). After purification on a CsCl gradient, samples intended for DNA preparation were digested with pronase as described by Loenen and Brammar (19). The DNA was extracted with phenol to remove proteins (6) and dialyzed against TE buffer.

Restriction endonuclease digestion of DNA and agarose gel electrophoresis. The methods used have been described previously (6). All gels were horizontal 0.8% agarose slab gels, and electrophoresis was carried out for approximately 18 h at a constant voltage of 20 mV. The sizes of the standard fragments were based on the restriction map of Davids et al. (10). The restriction enzymes were kindly provided by K. and N. E. Murray (*Bgl*II, *Xho*I) or purchased from Boehringer Co. Ltd. (*Eco*RI, *Sma*I), Uniscience Ltd. (Bethesda Research Laboratories, Inc; *Sal*I, *Sst*I), and C. P. Laboratories Ltd. (New England Biolabs, Inc; *Bam*HI; *Hin*dIII).

DNA ligation. DNA restriction fragments with cohesive ends were ligated with T4 DNA ligase. To approximately 2 μg of DNA in 90 μl of restriction buffer (Tris-hydrochloride, 10 mM [pH 7.5]; $MgCl_2$, 10 mM; 2-mercaptoethanol, 10 mM; NaCl, 50 or 100 mM) was added 10 μl of 10× T4 ligase buffer (Tris-hydrochloride, 0.66 M [pH 7.2]; EDTA, 0.01 M; $MgCl_2$, 0.1 M; dithiothreitol, 0.1 M; ATP, 1 mM) and T4 ligase (kindly provided by K. Murray). The mixture was incubated at 10°C for 5 to 6 h and then at 4°C for 16 to 18 h before being used in transfection.

Transfection. Lambda genomes were packaged in vivo by the transfection of *E. coli* C600, using a slight modification of the method of Wilson and Murray (28).

Genetic methods. Transduction was according to Cole and Guest (6), and the Clarke-Carbon colony bank was screened for F^+-mediated conjugation and complementation of *sdh* and *suc* mutants as described previously (11). Transductants and conjugants were selected on appropriate media: $GltA^+$, glucose minimal medium; Nad^+, glucose medium supplemented with acid-hydrolyzed casein (0.2% [wt/vol]); Sdh^+, succinate- acetate- peptone medum; $SucA^+$ and $SucB^+$, glucose- acetate- peptone medium; Tol^+, L agar plus sodium deoxycholate (0.375% [wt/vol]), after a 3-h period for expression (3).

Analysis of polypeptides formed after UV irradiation and phage infection. Polypeptides expressed from λ transducing phages were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography after UV-irradiated *E. coli* S159 and its λ imm^{λ} and λ imm^{21} lysogens were infected as described previously (13). Bacteria were grown in M9 minimal medium (20) containing maltose (0.2%) for the λ-sensitive strain or potassium DL-lactate (50 mM) for the lysogens. Phages were added at a multiplicity of infection of 10, and L-[^{35}S]methionine (Radiochemical Centre, Amersham, England) was added to a final concentration of 40 to 140 μCi/ml. The acrylamide concentration in the gels was 10% (wt/vol), and the unlabeled proteins used as standards for M_r determination were: β-galactosidase (116,000); phosphorylase *b* (94,000); conalbumin (type I, 78,000); bovine serum albumin (68,000); ovalbumin (43,000); lactate dehydrogenase (35,000); carbonic anhydrase (29,000); trypsin inhibitor protein (21,500); and cytochrome *c* (12,500).

RESULTS

Attempts to clone the *sdh* and *sucA,B* genes. Two procedures for direct cloning of the *sdh* and *sucA,B* genes were tested. First, pools of artificially constructed recombinant λ phages containing *Hin*dIII and *Eco*RI fragments of *E. coli* DNA in the appropriate vectors, λNM540 and λNM761 (*Hin*dIII) and λNM781 and λNM816 (*Eco*RI), were screened for their ability to transduce *sdh*, *sucA*, and *sucB* mutants. Nutritional selections, using minimal media containing succinate and acetate as carbon and energy sources for *sdh* transduction and glucose and acetate for *sucA* and *sucB*, and a weakly selective peptone medium were applied. No transducing activity was observed with *sucA* or *sucB* recipients or with the *sdh* recipients except on peptone medium where λ *frdA* transducing phages partially complement the *sdh* lesion (12). Second, the colony bank of hybrid ColE1-*E. coli* DNA plasmids, constructed by Clarke and Carbon (5), was screened by conjugation for complementation of *sdh* and *sucA* mutants. No $SucA^+$ exconjugants were detected, and the only clone (pLC16-43, pGS1) giving Sdh^+ exconjugants proved to contain the fumarate reductase gene (*frd*), which partially replaces *sdh* when present in higher copy number (12).

An alternative and indirect strategy for cloning the genes was attempted. This involved cloning in λ vectors a gene such as *gltA*, which is close to the *sdh* and *suc* genes, and using this to generate phages carrying the desired genes by prophage integration and aberrant excision in the *gltA* region. Accordingly, the four recombinant phage pools were screened for the ability to transduce the *gltA* mutant W620, but no λ *gltA* transducing phage were detected. However, three ColE1-$gltA^+$ hybrid plasmids, pLC26-17 (pGS7), pLC27-18 (pGS8), and pLC31-28 (pGS9), were found to complement the *gltA* lesion (11). These plasmids, representing a total of 19.4 kb of *E. coli* chromosome, shared a 9.5-kb region, which must contain the *gltA* gene (11), and part of it was subcloned into a λ vector to direct prophage integration to the *gltA* region of the chromosome.

Construction of λ *gltA* transducing phages. The ColE1-$gltA^+$ plasmids possessed single *Eco*RI and *Hin*dIII restriction targets, spanning a 3.1-kb fragment of their common region. This was subcloned, in two orientations, in lambda by using appropriate digests of λNM540 and two λ *lpd* transducing phages (λG83 and λG105) to provide the vector arms. The cloning strategy is based on screening recombinant phages for the

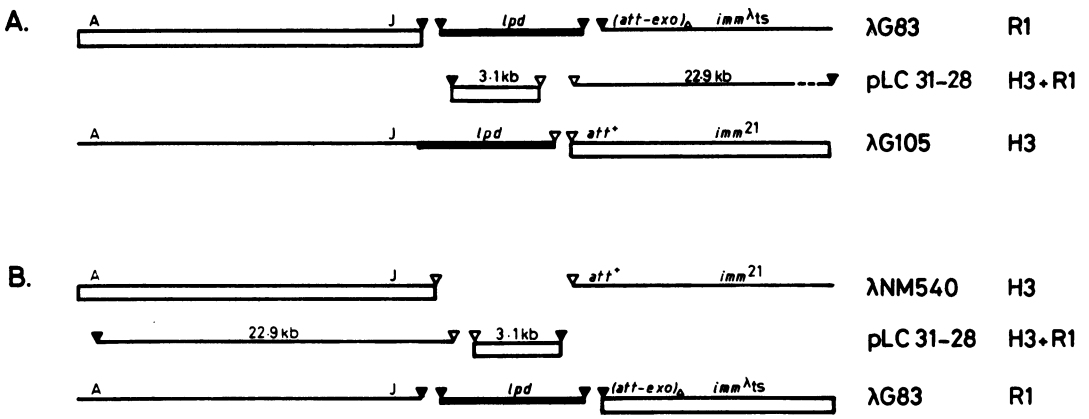

FIG. 1. Diagram illustrating the strategy for subcloning the 3.1-kb *Eco*RI plus *Hin*dIII fragment of pLC31-28, into phage λ in opposing orientations, A and B. Restriction sites are indicated by symbols: ▽, *Hin*dIII; ▼, *Eco*RI. Double lines indicate fragments which must join to achieve subcloning. Thickened lines indicate bacterial fragments already present in the phages.

desired immunity and replacement of the *lpd* fragment (Fig. 1). For one orientation (A), a threefold molar excess of doubly digested pLC31-28 was mixed and ligated with *Eco*RI-digested λG83 and *Hin*dIII-digested λG105. After transfection, phages forming turbid plaques at 37°C (imm^{21}) that were unable to transduce an *lpd* mutant (JRG599) to Lpd$^+$ were isolated. Similarly, for the other orientation (B), the digested plasmid was mixed and ligated with *Hin*dIII-digested λNM540 and *Eco*RI-digested λG83, and phages with temperature-sensitive immunity (imm^{λ}_{ts}) and unable to transduce *lpd* were isolated. The residual plasmid fragment (22.9 kb) was judged too large to be packaged into active phages. The presence and orientations of the 3.1-kb fragment in phages of the selected phenotype were confirmed by restriction analysis with *Eco*RI and *Hin*dIII, singly and in combination. One phage of each type was retained for further work: λG112 (att^+ imm^{21}) and λG113 ($att\text{-}exo_{\Delta}$ imm^{λ}_{ts}). The genomes of these phages were estimated as 40.1 kb (λG112) and 40.6 kb (λG113). These are at the lower end of the viable range (37 to 51.5 kb); there is therefore ample room for the incorporation of additional *E. coli* DNA without loss of λ functions.

In transduction tests with strain W620 (*gltA*) as recipient, lysates of λG112 and λG113 gave GltA$^+$ transductants on glucose minimal medium at a very high frequency (1 per 3.5 PFU, λG112) or lower frequencies (1 per 10^4 PFU, λG113, and 1 per 10^2 PFU, λG113 with a homoimmune helper). This indicates that all or most of the *gltA* gene is contained in the subcloned 3.1-kb fragment. The lower transduction frequency with λG113 probably stemmed from its integration deficiency, which limited transduction to *rec*-mediated integration or replacement events in the 3.1-kb region of homology. To establish whether the phage-cloned *gltA*$^+$ gene is expressed from its own promoter, lysogenic and dilysogenic derivatives of W620 were prepared nonselectively on L agar and then tested for complementation of the *gltA* lesion by being streaked on glucose minimal medium in the absence of glutamate. All the monolysogens had a GltA$^+$ phenotype and, for the integration-proficient λG112, this indicates that the *gltA*$^+$ gene in the 3.1-kb fragment is expressed from its own promoter rather than from flanking phage promoters (which are silent in the prophage). In the case of the integration-deficient λG113, the *gltA* gene could be linked to a bacterial promoter during prophage formation. However, dilysogenic derivatives, constructed with λ imm^{434} to mediate λG113 insertion at the bacterial *att*λ site, were also GltA$^+$ and were restored to GltA$^-$ by curing. This confirmed that the *gltA* gene was cloned with its own promoter in the 3.1-kb *Hin*dIII-*Eco*RI fragment of λG112 and λG113, now designated λ *gltA* phages.

Extension of λ *gltA* transducing range to include the *sdh*, *sucA* and *sucB* genes. Plaque-forming phages carrying the *sdh*, *sucA*, and *sucB* genes were isolated by in vivo extension of the *gltA* region cloned in λG112 and λG113 (Fig. 2). The λ *gltA* phages were first established as prophages by recombination in the homologous *gltA* region of strain KS302 (an *att*λ deletion mutant used to prevent λ *int*-mediated integration of λG112 at *att*λ). Lysates induced by UV irradiation or thermal induction of several lysogens were then screened for their ability to transduce JRG1003 (*sdh*, *frd*) to Sdh$^+$. The presence of λ *sdh* transducing phages was detected by the formation of "galaxies" or trans-

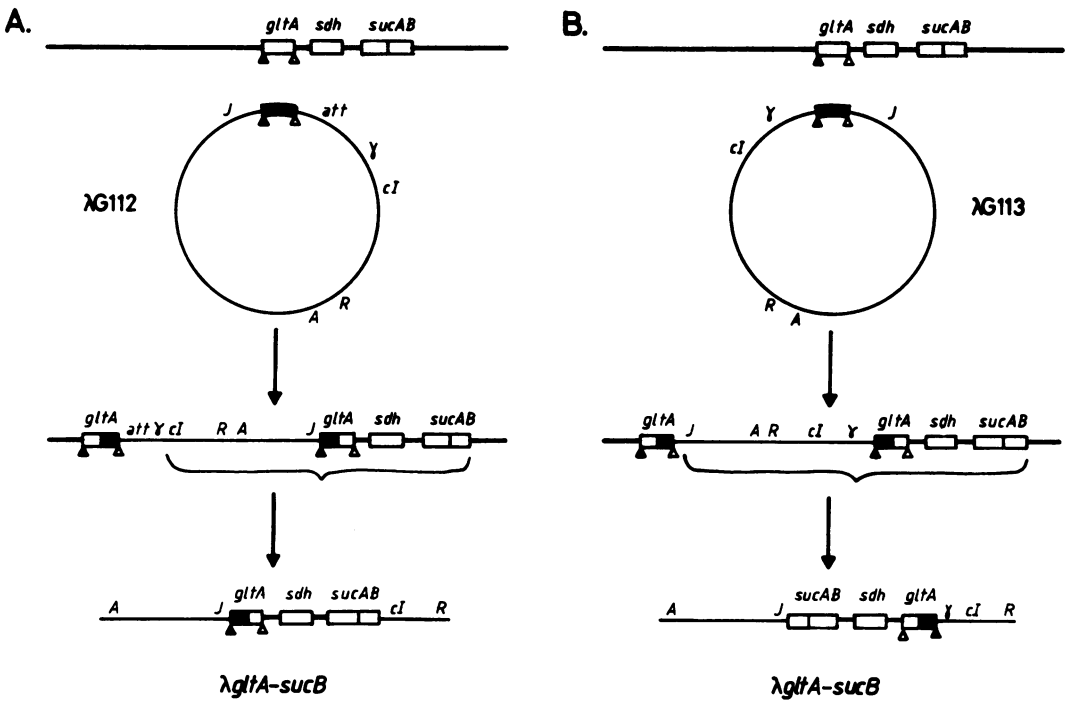

FIG. 2. Diagrammatic representation of the extension of the transducing ranges of λ *gltA* by integration in the region of DNA homology, followed by aberrant excision. The two orientations of the cloned *gltA* gene (filled box) are represented in (A) and (B): ▲, *Eco*RI; △, *Hin*dIII. The phages corresponding to the two orientations and the orientation of the *gltA* fragment relative to the bacterial chromosome (thick line) are defined by: (i) Spi⁻ enrichment of *sdh* transducing phages in orientation A (λG112) and (ii) restriction analysis which shows that all extensions occur in the direction of the *Hin*dIII site.

duction plaques with densely turbid centers, using peptone as the selective medium (12). Several of the induced lysates were active, and independent λ *sdh* transducing phages were readily isolated and purified through further rounds of transduction.

Lysates derived from λG112 were enriched for Spi⁻ phages by propagation on C600(P2), and this led to a simultaneous enrichment of λ *sdh* phages. In one case the titers of λ *sdh* transducing phages and Spi⁻ phages became equal after a 25-fold enrichment, indicating that all the Spi⁻ phages are transducing phages and vice versa. The Spi⁻ phenotype arises from the loss of the *exo*, *bet*, and *gam* functions, and their replacement by extension from *gltA* to *sdh* indicates that the rightward extension in λG112 corresponds to the clockwise direction on the *E. coli* linkage map, as shown in Fig. 2. This in turn identifies the orientation of the 3.1-kb cloned fragment relative to the chromosome (Fig. 2) and confirms the earlier correlation of physical and linkage maps based on indirect genetic evidence (11). It follows that the formation of λ *sdh* transducing phages from λG113 involves leftward extension of the phage-cloned *gltA* region (Fig. 2).

Transducing phages (λ *gltA-sdh*) derived initially from both parental λ *gltA* phages were tested for their ability to transduce the *sucA* and *sucB* genes, using appropriate recipients and the peptone selective medium (Table 2). By far the most common class consisted of λ *gltA sdh sucA sucB* phages, although some phages containing only two or three of the four genes were obtained. All four of the Spi⁻ derivatives of λG112 that were tested belonged to the predominant class (λ *gltA-sucB*). A few phages transduced the *suc* mutants at relatively low frequencies, suggesting that they contain only part of the corresponding *suc* gene (Table 2). The progression of transducing activities is clearly consistent with the gene order, *gltA-sdh-sucA-sucB*, established previously (9).

Serious problems of instability were experienced during nonselective and bulk propagation of many of the phages. The λG112 derivatives with short extensions were particularly prone to revert to the parental type, and the longer extensions proved impossible to maintain in the

TABLE 2. Classes of phages with extended transducing ranges

Phage type	Transduction				No. from each parent phage	
	gltA	*sdh*	*sucA*	*sucB*	λG112	λG113
λ *gltA sdh*	+	+	−	−	4	1
λ *gltA sdh* (*sucA*)	+	+	±[a]		2	0
λ *gltA sdh sucA*	+	+	+	−	1	1
λ *gltA sdh sucA* (*sucB*)	+	+	+	±[a]	1	0
λ *gltA sdh sucA sucB*	+	+	+	+	18	4

[a] ±, Low frequency of transduction.

λG113 derivatives. In consequence, a rather limited selection of phages was retained for further analysis. These are listed in Table 3, together with the genes cloned, the phage genes deleted, and the approximate sizes of the bacterial insert and the total phage genome. Included is a phage (λG136) which originally contained all four TCA cycle genes but lost its *gltA* and *sdh* transducing activity during nonselective propagation.

The phage-cloned *sdh*, *sucA*, and *sucB* genes were shown to be accompanied by their promoters by demonstrating that the nutritional defects of corresponding mutants were complemented in lysogens and dilysogens constructed under nonselective conditions. Since all the phages are integration deficient, the use of λ*imm*^{434} to provide an integration site where phage-cloned genes are expressed only when accompanied by their own promoters was particularly important. The Sdh$^+$ phenotype was recognized by growth on minimal medium with succinate or acetate as the sole carbon and energy source, and the SucA$^+$ and SucB$^+$ phenotypes were recognized by growth on glucose minimal medium without a succinate supplement and the ability to use acetate as substrate. The mutant phenotypes were also shown to be restored after the transducing prophages were cured with a heteroimmune phage.

The λ *gltA-sucB* transducing phages were tested for transduction of the *tolA*, *tolB*, and *nadA* genes, which are located clockwise to *sucB*. No *tolAB* or *nadA* transduction was detected with strains KB5 and H80 as recipients. Thus, it would appear that the extensions have not reached as far as the *tol* and *nadA* genes. The transducing activities of the phages with respect to *cbt*, *supG*, and *lysT* were not investigated.

Restriction analysis of transducing phage DNA. The DNAs from seven phages with extended transducing ranges were digested with a total of eight restriction endonucleases, both singly and in combination, and analyzed by quantitative agarose gel electrophoresis. A physical map of the *gltA-sucB* region of the *E. coli* chromosome was then deduced from the restriction maps of the transducing phage genomes (Fig. 3). The cloned fragments overlap and extend beyond the segment cloned in the original ColE1-*gltA*$^+$ plasmids. The sizes of the inserts were calculated from the cumulative sizes of the small restriction fragments although the exact point of fusion between bacterial and phage DNA could not usually be determined (Fig. 3, Table 3). The total sizes of the transducing phage genomes were determined for the λG112 derivatives (42.0 to 48.2 kb), but this was not possible for the λG113 derivatives due to the paucity of useful restriction targets in the left arm of the vector. The coordinates of the restriction targets in the longest cloned fragment were: Sa_1, 0.4; Bg_1, 0.55; St_1, 2.4; Ba_1, 2.6; $H3_1$, 3.2; Xh_1, 5.7; St_2, 6.65; Ba_2, 7.1; Bg_2, 9.5; Bg_3, 10.5; Sm_1, 12.8; Sa_2, 12.9; and Bg_4, 15.1 (in kb, relative to RI_1; Fig. 3). The bacterial inserts of phages which trans-

TABLE 3. Characteristics of λ *gltA* transducing phages and derivatives transducing *sdh*, *sucA*, and *sucB*

Strain	Parent	Genes cloned	Phage genotype[a]	Bacterial insert (kb)	Total phage size (kb)
λG112	—	*gltA*	*att*$^+$ *int*$^+$ *xis*$^+$	3.1	40.1
λG120	λG112	*gltA sdh*	Δ(*att-xis* or *exo*)	7.1–8.7	42.0
λG119	λG112	*gltA sdh*	Δ(*att-int* or *xis*)	7.1–9.5	43.5
λG117	λG112	*gltA sdh sucA sucB*	Δ(*att-int* or *xis*)	10.7–12.8	47.7
λG116	λG112	*gltA sdh sucA sucB*	Δ(*att-int* or *xis*)	12.9–15.1	49.8
λG118	λG112	*gltA sdh sucA sucB*	Δ(*att-cIII*)	16.7	48.2
λG113	—	*gltA*	Δ(*att-exo*)	3.1	40.6
λG121	λG113	*gltA sdh*	Δ(*att-exo*)	7.1–9.5	ND[b]
λG136	λG113	*sucA sucB*	Δ(*att-exo*)	7.0–9.3	ND

[a] Phage genotypes are based on tests for *int-xis*, *exo*, *gam*, and restriction analysis of the point of fusion between the bacterial insert and phage DNA.

[b] Not determined because the lack of restriction sites in the λ left arm precludes accurate sizing of the λG113 derivatives.

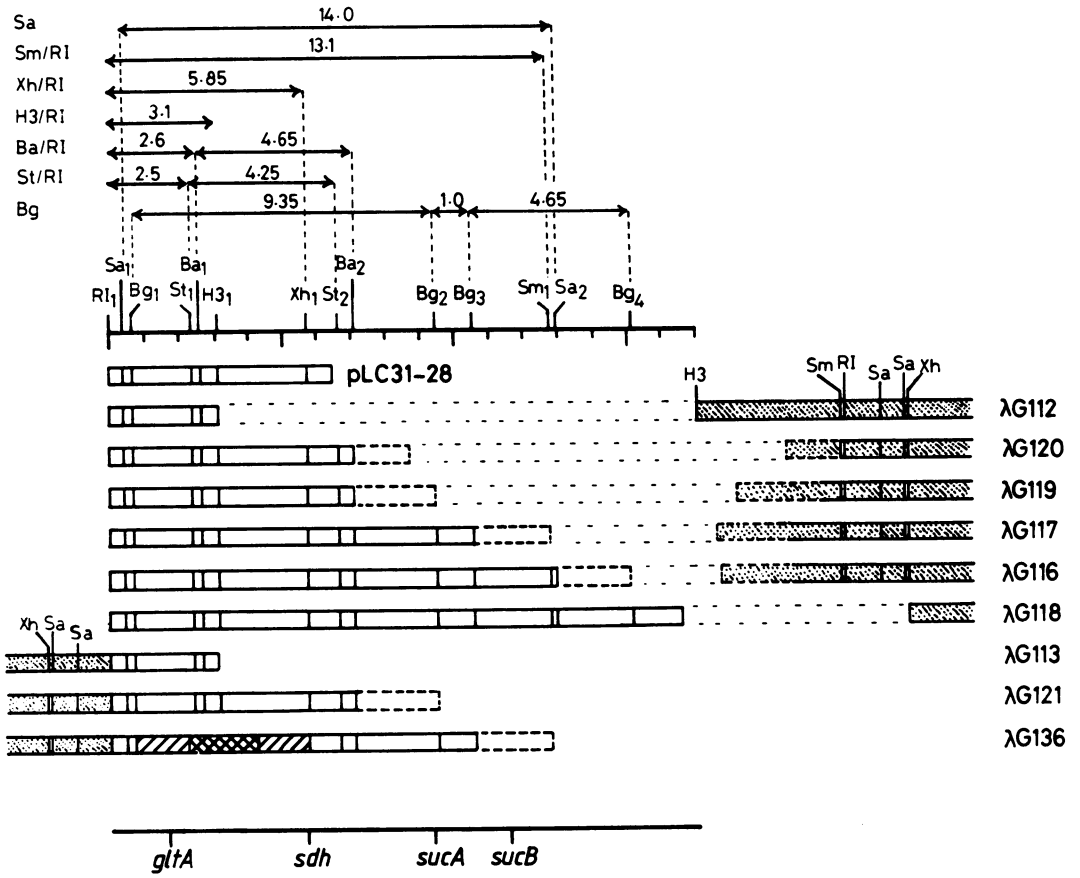

FIG. 3. Restriction maps of the λ *gltA* phages, λG112 and λG113, and the transducing phages produced by their in vivo extension. The cloned bacterial fragments, ▭, and segment of the λ right arms, ▭, are shown, but the λ left arms are not shown because lack of restriction sites precludes any conclusions as to their extent in the λG113 series. Dashed boxes, ▭, indicate uncertain limits of bacterial or phage DNA. The limits of the 3.5-kb deletion in λG136 are indicated by ▭, and ▭ represents the segment that must be deleted. The orientation of the bacterial fragments is drawn such that left to right corresponds to clockwise on the *E. coli* chromosome, and in the λG113 series, this results in the reversal of the orientation of the vector arms. The overlapping end of the ColE1-*gltA*$^+$ plasmid, pLC31-28, from which the 3.1-kb fragment was subcloned, is included for comparison. A summary of the restriction sites is shown, together with a scale (1 division = 1 kb) from which their coordinates may be read. The abbreviations used for the restriction sites are: Ba, *Bam*HI; Bg, *Bgl*II; H3, *Hin*dIII; RI, *Eco*RI; Sa, *Sal*I; Sm, *Sma*I; St, *Sst*I; and Xh, *Xho*I. A map indicating the approximate positions of the genes *gltA*, *sdh*, *sucA*, and *sucB* is included.

duced *sdh* but not *sucA* extended to between the Ba_2 and Bg_2 sites, and phages transducing *sucA* and *sucB* had extensions beyond Bg_3. The restriction analysis of λG136 (λ *sucA sucB*) confirmed the conclusion from transduction experiments that the *gltA* and *sdh* region had been deleted, because a segment containing the St_1, Ba_1, and $H3_1$ sites was missing.

Postinfection labeling of phage-directed polypeptides. Phages, representative of different transduction classes and containing bacterial genes inserted with opposite polarities, were used in postinfection labeling to identify the products of genes in the *gltA* to *sucB* region and to deduce their transcription polarities. The phages were: λG112 (λ *gltA*) and derivatives λG112 (λ *gltA sdh*) and λG116 (λ *gltA sdh sucA sucB*); and λG113 (λ *gltA*) and derivatives λG121 (λ *gltA sdh*) and λG136 (λ *sucA sucB*).

(i) Polypeptides synthesized in λ-immune hosts. The expression of lambda-cloned bacterial genes was investigated by labeling polypeptides after infection of homoimmune lysogens of *E. coli* S159. Host cultures were irradiated to reduce bacterial gene expression, and lysogens were used to limit protein synthesis to bacterial genes cloned with their own promoters. The lysogens were S159 (λ imm^{λ} ind^{-}) for λG113 and its

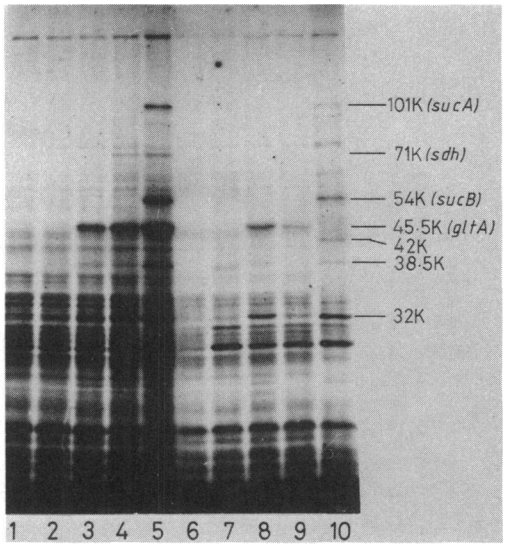

FIG. 4. Autoradiogram of [^{35}S]methionine-labeled polypeptides produced after infection of homoimmune hosts, S159 (λ *imm*$^{\lambda}$ *ind*$^{-}$) and S159 (λ *imm*^{21}), with transducing phages of the λG112 and λG113 series and fractionation in sodium dodecyl sulfate-polyacrylamide gel. Labeling was for 30 min. The λ *nadC* phages, λG75N and λG78N, were included as controls. The phages, corresponding to individual tracks, are: 1 and 6, uninfected bacteria; 2, λG75N (λ *nadC*); 3, λG112 (λ *gltA*); 4, λG120 (λ *gltA sdh*); 5, λG116 (λ *gltA sucB*); 7, λG78N (λ *nadC*); 8, λG113 (λ *gltA*); 9 λG121 (λ *gltA sdh*); 10, λG136 (λ *sucA sucB*).

derivatives and S159 (λ *imm*^{21}) for the λG112 series; two λ *nadC* transducing phages, λG78N and λG75N (15), were used as controls. When the host cells were grown in maltose or glucose medium, expression of the cloned genes was low, and no *sdh* product could be detected, probably due to catabolite repression of TCA cycle enzymes. Furthermore, postinfection expression was almost completely abolished when complex medium, such as L broth or peptone broth, was used, but an M9 salts medium with lactate allowed much better expression of the cloned genes, and this was used in subsequent experiments with lysogenic hosts.

All the λ *gltA* transducing phages (λG112, λG120, λG116, λG113, and λG121) directed the synthesis of one specific polypeptide, readily identified by its size (M_r 45,500) as citrate synthase, the *gltA* product (Fig. 4). Likewise, the *sdh* gene product was identifiable as a weakly labeled polypeptide (M_r 71,000) with λG120 and λG116, but not with λG121, where incorporation was too weak for detection (Fig. 4). This corresponds to the large flavoprotein subunit, but the small subunit (if present) could not be detected. The two *sucA,B* transducing phages (λG116 and λG136) gave labeled polypeptides corresponding to the E1 component (M_r 101,000) and E2 component (M_r 54,000) of the 2-oxoglutarate dehydrogenase complex (Fig. 4). The results also confirmed that the *gltA* and *sdh* genes are deleted in λG136. Additional polypeptides, M_r 42,000, 38,500, 32,000, and possibly one or more of lower M_r, were also detected with some phages compared with λ *nadC*, but their origin could not be deduced.

(ii) Polypeptides synthesized in a λ-sensitive host. Expression of the cloned genes was also investigated by infecting the λ-sensitive host, S159, after growth in maltose minimal medium and UV irradiation. Under these conditions phage promoters are active and, depending on orientation, the expression of cloned genes will be enhanced or retarded by the powerful leftward promoter, p_L (in the early phase of infection), or influenced by rightward transcription from p'_R (during the late phase of infection). Polypeptide synthesis was analyzed during early (3 to 13 min) and late (23 to 33 min) phases (Fig. 5).

Early-phase incorporation into citrate synthase (M_r 45,500) was only found with λG112 and its derivatives (λG120 and λG116), although late-phase incorporation was observed with all of the λ *gltA* phages, including λG113 and λG121, in which the *gltA* gene is inserted with opposite orientation (Fig. 5). This indicates that the *gltA* gene is cloned with a leftward orientation in λG112 and its derivatives (λ *gltA*^{l}). By contrast, the *gltA* gene in the λG113 series must have the rightward orientation (λ *gltA*^{r}) because expression appears to be inhibited until transcription from p_L is switched off.

The *sdh* gene product (M_r 71,000) was labeled with λG121 in the early and late periods but only in the late period with λG120 and λG116 (Fig. 5). Thus, the *sdh* gene has a leftward orientation in the λG113 derivative λG121 and a rightward orientation in λG120 and λG116. This means that *sdh* is expressed with the opposite polarity to *gltA*. A polypeptide (M_r 28,000) exhibiting a labeling pattern similar to that of the 71,000 *sdh* gene product could be the small subunit of succinate dehydrogenase (Fig. 5). This interpretation is complicated by the presence of an early-labeled polypeptide of similar size expressed by all members of the λG112 series and λG75N. The interfering polypeptide is probably the product of the *exo* gene, which is deleted in the λG113 series and λG78N. Further support for this interpretation comes from λG118, an *exo*-deleted phage, which produces the 28,000 polypeptide only in the late phase (data not shown).

Incorporation into the *sucA* and *sucB* gene products (M_r 101,000 and 54,000) was observed only in the early period with the λG113 deriva-

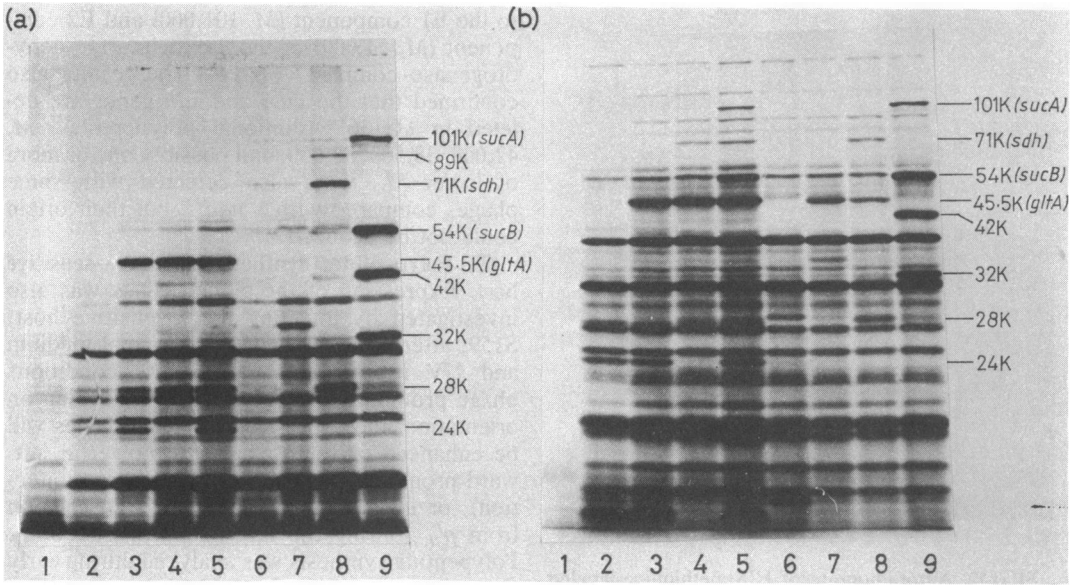

FIG. 5. Autoradiograms of the labeled polypeptides produced after infection of a λ-sensitive host by transducing phages in the λG112 and λG113 series, and fractionation in sodium dodecyl sulfate-polyacrylamide gel. Labeling was for 10 min either (a) early, 3 to 13 min, or (b) late, 23 to 33 min. The phages, corresponding to individual tracks, are: 1, uninfected bacteria; 2, λG75N (λ *nadC*); 3, λG112 (λ *gltA*^{l}); 4, λG120 (λ *gltA*^{l} *sdh*^{r}); 5, λG116 (λ *gltA*^{l} *sdh*^{r} *sucA*^{r} *sucB*^{r}); 6, λ78N (λ *nadC*); 7, λG113 (λ *gltA*^{r}); 8, λG121 (λ *gltA*^{r} *sdh*^{l}); 9, λG136 (λ *sucA*^{l} *sucB*^{l}).

tive, λG136, but in the late period with both λG116 and λG136 (Fig.5). This indicates that the *sucA* and *sucB* genes are expressed with the same polarity as the *sdh* gene, and it also confirms earlier findings that the *suc* operon has a clockwise polarity relative to the *E. coli* linkage maps (8). The conclusions are summarized in Fig. 6, which shows the relative polarities of the *gltA*, *sdh*, and *suc* genes in the two series of transducing phages. The phages can be designated as follows: λG112, λ *gltA*^{l}; λG120, λ *gltA*^{l} *sdh*^{r}; λG116, λ *gltA*^{l} *sdh*^{r} *sucAB*^{r}; λG113, λ *gltA*^{r}; λG121, λ *gltA*^{r} *sdh*^{l}; λG136, λ *sucAB*^{l}. The sizes of the four TCA cycle gene products derived from seven different gels were: *gltA*, 45,500 ± 1,200; *sdh*, 71,000 ± 1,500; *sucA*, 10,100 ± 2,500; and *sucB*, 54,000 ± 1,300.

The experiments also showed several other

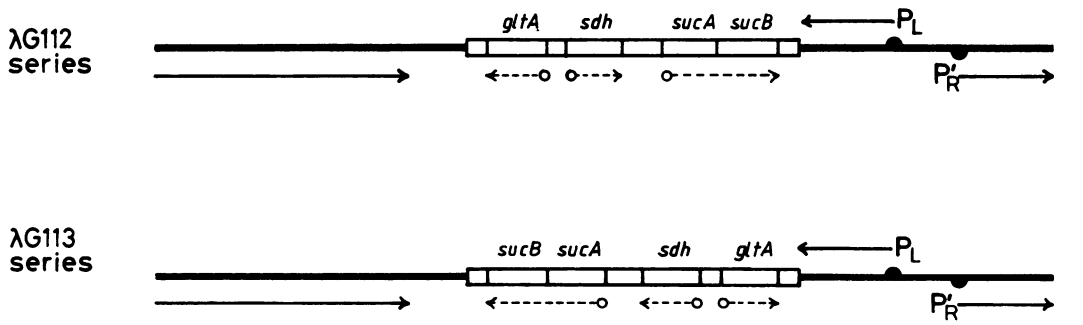

FIG. 6. Diagram illustrating the orientation and polarity of expression of the cloned genes *gltA*, *sdh*, *sucA*, and *sucB* in the λG112 and λG113 series. The orientation of the cloned segments with respect to the *E. coli* chromosome is such that left to right in the λG112 series corresponds to clockwise in *E. coli*, and vice versa in the λG113 series. Vector DNA is indicated by thick single lines, and bacterial DNA is indicated by double lines. The phage promoters, p_L (early) and p'_R (late), which may influence the expression of the cloned bacterial genes in λ-sensitive hosts, are indicated by filled semicircles. Solid arrows indicate the direction of phage transcription, and dashed arrows indicate the polarity of transcription of the bacterial genes.

specifically labeled polypeptides not found in the controls, notably products of M_r 35,000 with λG113, M_r 89,000 with λG121, and M_r 42,000 and 32,000 with λG136. All were found in both early and late periods, indicating the same transcriptional polarity as the *sdh* and *suc* genes. They are probably the products of gene fusions, arising from prophage excision or deletion, rather than intact bacterial genes, because they appear to be unique to the specified phages and no corresponding products were observed with λG118, which contains the longest bacterial insert. Other polypeptides (e.g., M_r 24,000) probably represent phage gene products that are encoded by some, but not all, of the phages.

DISCUSSION

A 3.1-kb *Hin*dIII-*Eco*RI fragment derived from a ColE1-*gltA*$^+$ plasmid was subcloned into λ vectors in both orientations and shown by transduction, complementation, and postinfection labeling to contain a functional *gltA* gene. The cloned fragment was extended in vivo by prophage integration into the *gltA* region of the *E. coli* chromosome, followed by aberrant excision to generate phages capable of transducing the *sdh*, *sucA*, and *sucB* genes. In most cases the ability to transduce particular genes was paralleled by the ability to synthesize the gene products (in immune hosts) and to complement the corresponding mutations, indicating that the genes had been cloned together with their own promoters. The products of all four genes were readily identifiable by size, and these were in good agreement with previously reported values.

The phages with extended bacterial inserts fell into three main categories, λ *gltA sdh*, λ *gltA sdh sucA*, and λ *gltA sdh sucA sucB*, and thus confirmed the relative order of the four genes: *gltA-sdh-sucA-sucB* (8). Further investigation of a selection of these phages also established the orientation of the 3.1-kb fragment with respect to the *E. coli* chromosome, such that the *Eco*RI-*Hin*dIII direction (rightwards in λG112) corresponded to a clockwise orientation in the *E. coli* linkage map. This was suggested by the properties of the Spi$^-$ derivatives of λG112 (Fig. 2) and confirmed by restriction analysis, which showed that in transducing phages derived from λG112 the 3.1-kb fragment had extended rightwards, whereas in those derived from λG113 the extension was to the left (Fig. 3). Of the three categories of phages, the majority were λ *gltA sdh sucA sucB*, and these contained at least 10.7 kb of bacterial DNA, resulting in genome lengths approximating those of the wild type. The predominance of these phages probably stems from the small size of the parental phages, λG112 and λG113, and suggests that phages having the wild-type genome size are preferentially selected.

The sizes of the cloned bacterial fragments ranged from 7.1 to 16.7 kb. The 3.1-kb fragment containing *gltA* needed extending to 7.1 to 8.7 kb, to between Ba_2 and Bg_2, to bring in *sdh*, and to 10.7 to 12.8 kb, to between Bg_3 and Sm_1, to bring in *sucA* and *sucB* (Fig. 3). The estimated sizes of the four genes (and a gene for the small subunit of succinate dehydrogenase, if present) could easily be accommodated by these lengths of DNA. None of the phages appeared to contain *nadA* or *tolA,B*, which are immediately clockwise to *sucB* in the *E. coli* linkage map even though the bacterial insert of λG118 extends at least 3.9 kb beyond the region encoding the *suc* genes. The restriction map derived from the transducing phages overlaps that of the ColE1-*gltA*$^+$ plasmid, pLC31-28 (11), by approximately 6.5 kb, thus extending the *E. coli* restriction map by about 10 kb, to give a total length of approximately 30 kb in the *gltA* region. The finding that this segment contains only single *Eco*RI and *Hin*dIII restriction targets, situated at least 13 to 14 kb from the ends, could explain why phages containing the *gltA*, *sdh*, or *sucA,B* genes were not found in the recombinant phage pools constructed with vectors of limited capacity.

From the way in which the expression of the cloned genes in λ-sensitive hosts was influenced by early and late phage promoters, the polarities of the four genes were deduced (Fig. 6). The *gltA* gene is transcribed in a counterclockwise direction, whereas *sdh*, *sucA*, and *sucB* are transcribed in a clockwise direction relative to the *E. coli* linkage map. Tentative evidence for the presence of a small subunit (28,000) of succinate dehydrogenase, expressed with the same polarity as the large subunit (71,000) was also obtained.

Work now in progress is aimed at further localizing the *sdh* gene(s) and the *suc* genes, for nucleotide sequence analysis and further studies on their expression.

ACKNOWLEDGMENT

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