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Activation of a Cryptic Pathway for Threonine Metabolism via Specific IS*3*-Mediated Alteration of Promoter Structure in *Escherichia coli*†

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The *tdh* operon of *Escherichia coli* consists of two genes whose products catalyze sequential steps in the formation of glycine and acetyl coenzyme A from threonine. The operation of the *tdh* pathway can potentially confer at least two capabilities on the cell: the first is to provide a biosynthetic source of glycine, serine, or both that is an alternative to the conventional (triose phosphate) pathway; the second is to enable cells to utilize threonine as the sole carbon source. The latter capability is referred to as the Tuc$^+$ phenotype. In wild-type *E. coli*, the *tdh* operon is expressed at levels that are too low to bestow the Tuc$^+$ phenotype, even in leucine-supplemented media, where the operon is induced eightfold. In eight Tuc$^+$ mutants, the expression of the *tdh* operon was elevated 100-fold relative to the uninduced wild-type operon. The physical state of the DNA at the *tdh* locus in these Tuc$^+$ strains was analyzed by Southern blotting and by DNA sequencing. In eight independent isolates the mobile genetic element IS*3* was found to lie within the *tdh* promoter region in identical orientations. In six cases that were examined by DNA sequencing, IS*3* occupied identical sites between the −10 and −35 elements of the *tdh* promoter. The transcription start points for the wild-type *tdh* promoter and one IS*3*-activated *tdh* promoter were identical. In effect, the repeatedly observed transposition event juxtaposed an IS*3*-borne −35 region and the *tdh*-specific −10 region, generating a hybrid promoter whose utilization led to elevated, constitutive expression of the *tdh* operon. This is the first case of promoter activation by IS*3* where the site of transcription initiation is unaltered.

In *Escherichia coli*, the conversion of threonine to other metabolites can be initiated by at least three enzymes. Biosynthetic threonine dehydratase (EC 4.2.1.16) converts threonine to 2-ketobutyrate and NH_4^+ (51). The carbon skeleton of 2-ketobutyrate is used for the production of isoleucine. Biodegradative threonine dehydratase catalyzes the same reaction but plays no role in the synthesis of the branched-chain amino acids (17). Finally, threonine dehydrogenase (EC 1.1.1.103) catalyzes the NAD^+-dependent oxidation of threonine to 2-amino-3-ketobutyrate (AKB) (8).

AKB coenzyme A (CoA) ligase, the second enzyme in the pathway initiated by threonine dehydrogenase, converts AKB to glycine and acetyl CoA (Fig. 1). Threonine dehydrogenase and AKB CoA ligase have been purified to homogeneity from *E. coli* and extensively characterized (8, 14, 34). The genes for these two enzymes make up the *tdh* operon (Fig. 2). The complete primary structures of the two enzymes are known (3, 4). Threonine dehydrogenase activity has been detected under all growth conditions so far examined (9, 36, 37). Leucine has been shown to induce threonine dehydrogenase activity sevenfold (36, 37). This pathway has been shown to be operational in reversing the phenotypes of glycine or serine auxotrophs under some growth conditions (19, 36, 40).

Although the *tdh* pathway can potentially allow cells to utilize threonine as a carbon source, a wild-type *E. coli* K-12 is phenotypically Tuc^0: it cannot utilize threonine as a carbon source. Apparently, in normal cells the activities of the *tdh* operon enzymes are too low to allow for such growth. Mutants of *E. coli* K-12 able to utilize threonine as the sole carbon source (the Tuc$^+$ phenotype) display elevated levels of threonine dehydrogenase and AKB CoA ligase (8, 10). Presumably, the acetyl CoA generated via the *tdh* pathway furnishes both carbon and energy.

We have characterized a mutational event that accounts for the hyperexpression of the *tdh* operon in eight independent Tuc$^+$ strains. In each case, the increased expression of the *tdh* operon could be attributed to the presence of an IS*3* element in the promoter region of the operon. The insertion event created a hybrid promoter that is not only more efficient than the wild-type *tdh* promoter, but is also unregulated.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains, bacteriophage strains, and plasmids used are listed in Table 1. The transduction protocol used to construct strain SP971 was similar to that described by Miller (32).

Media. Basal medium was salts mix E of Vogel and Bonner (52). Solid media contained 1.5% Bacto Agar (Difco Laboratories). The following compounds were included when appropriate: L-threonine as a carbon source (0.2%), glucose (0.4%), amino acids (40 mg/liter unless otherwise specified), and ampicillin (25 mg/liter). All minimal media contained vitamin B_1 (1 mg/liter) and biotin (0.1 mg/liter). Either L broth (30) or nutrient agar (31 g/liter; Difco) was used as complete medium.

DNA preparations. Plasmid DNA and M13 replicative form DNA were isolated by the alkaline lysis procedure of Ish-Horowicz and Burke (28). Small preparations of plasmid and replicative form DNA were made by a scaled-down version of the alkaline lysis procedure. Cells were transformed by the method of Chung and Miller (12) or Cohen et

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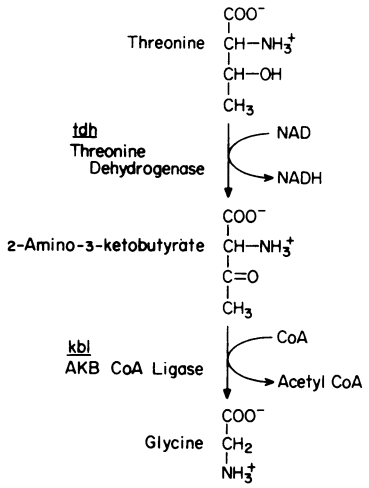

FIG. 1. The *tdh* pathway. L-Threonine is converted to glycine and acetyl CoA in two sequential steps. Threonine dehydrogenase catalyzes the oxidation of threonine to AKB. AKB CoA ligase converts AKB to glycine and acetyl CoA. This pathway has been shown to be operational in the reversal of the phenotype of some glycine (19, 36) and serine (40, 41) auxotrophs.

al. (13). Single-stranded M13 templates were prepared by the procedure of Sanger et al. (45).

Chemicals and reagents. Restriction endonucleases, T4 DNA ligase, DNA polymerase I large fragment (Klenow), and −20 and −40 oligodeoxynucleotide sequencing primers were purchased from New England Biolabs. [α-^{35}S]dATP (1,200 Ci/mmol) and [α-^{32}P]dATP (800 Ci/mmol) was purchased from Amersham. Special-purpose oligodeoxynucleotides used for sequencing or for the construction of deletion mutations were synthesized on an Applied Biosystems machine. N^4-Hydroxy-dCTP was a gift from Hans Weber.

Isolation of Tuc$^+$ mutations. Single-colony isolates of *E. coli* PS1236 were inoculated into screw-cap tubes containing 5 ml of minimal medium with 2% L-threonine as a carbon source. The tubes were incubated with shaking at 37°C until turbidity appeared (usually 10 to 14 days). The Tuc$^+$ mutants were purified by single-colony isolation on minimal agar plates with 2% threonine as the carbon source. To insure independent isolation of mutations, only one isolate per tube was kept. The optimum growth conditions for the Tuc$^+$ mutants are with 0.5% L-threonine and 0.005% Casamino Acids. Threonine will also serve as a nitrogen source for Tuc$^+$ strains.

Construction of M13mp18/p. A promoterless derivative of M13mp18 was made by oligodeoxynucleotide-directed deletion. Ten nanograms of a 30-mer (ATGGTCATAGCTGT TATTGCGTTGCGCTCA) was annealed to 0.1 μg of M13mp18 single-stranded DNA template at 90°C in 1.5× Klenow buffer (10 mM Tris, 5 mM Mg^{2+} [pH 8.0]) and then slowly cooled to room temperature. The oligodeoxynucleotide was extended in the presence of 0.5 mM deoxynucleotide triphosphates and 5 U of DNA polymerase (Klenow) for 15 min at room temperature. The reaction mixture was directly transformed into strain JM101. Phage from colorless plaques were isolated and their DNA was sequenced to verify that the correct deletion (from coordinates 1177 to 1279 of the *lac* operon, using the *Hin*cII site of *lacI* as coordinate 1) had taken place.

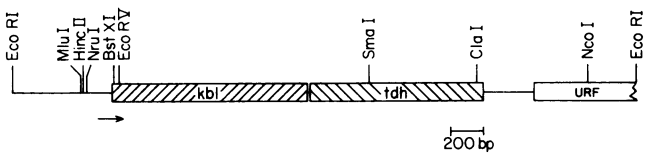

FIG. 2. Physical map of the *tdh* operon. The *tdh* operon encodes the genes for threonine dehydrogenase (*tdh*) and AKB CoA ligase (*kbl*). It is not known if the unidentified open reading frame is part of the operon. The operon was originally cloned as a 3.6-kbp *Eco*RI fragment. The *tdh* operon lies at the 81-min region of the *E. coli* chromosome (39). Relevant restriction sites are shown.

DNA sequence analysis. DNA sequences were determined by the method of Sanger et al., modified for the use of [α-^{35}S]dATP as the labeling nucleotide (6). Recombinant M13 phage were used as template. The −20 and −40 17-nucleotide M13 primers (from New England Biolabs) were used. In some cases, custom-made oligodeoxynucleotides were used as primers. Both Klenow DNA polymerase (New England Biolabs) and modified T7 DNA polymerase (Sequenase, from U.S. Biochemicals) were used in the sequencing reactions.

Southern blotting analysis. Southern analysis was performed by the method of Southern (49) except that the electrophoretically separated DNA fragments were transferred to nylon membranes (Sartorius Nylon 66) using 0.4 N NaOH. ^{32}P-labeled probes were made by primer extension using single-stranded DNA from an M13 clone containing the *Eco*RI-*Hin*cII (435-base-pair [bp]) fragment (Fig. 2) (27). Hybridizations were carried out in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 65°C. The final high-stringency wash was done at 65°C with 0.2× SSC for 15 min.

RNA isolation. RNA was prepared from 250 ml of cells grown on casein hydrolysate medium to approximately 1/3 log phase, using the procedure of Salser et al. (44). The RNA, in the form of an ethanol slurry, was stored at −20°C, and samples were withdrawn, centrifuged on a Microfuge, and suspended for primer extension.

Primer extension. A 60-ng sample of a 17-residue oligodeoxynucleotide (designated AKB 1), TGATAAAATTCTC CACG, complementary to *tdh* mRNA, was coprecipitated with either 20 or 40 μg of RNA. The pellet was suspended in 10 μl of double-distilled H_2O, heated to 100°C for 2 min, and then quickly cooled in ice-water. The extension reaction was done as reported by Curtis (15) except that the reaction volume was increased to 20 μl, the incubation was done at 42°C, and only dATP (0.5 mM final concentration) was added in the chase solution. The reaction was stopped by the addition of 4 μl of sequencing stop solution, heated to 100°C, and quickly cooled on ice. A 3.5-μl sample of the reaction was loaded on a 6.0% acrylamide–6 M urea sequencing gel. The extension products were run next to a sequence ladder generated from the same oligodeoxynucleotide primer, using as template the clone M13mp18/p p-121 (see Results).

Construction of λ *tdh-lacZ* lysogens. *tdh* promoter fragments were subcloned from the M13mp18/p constructs into pMLB1034 by using restriction endonucleases *Eco*RI and *Bam*HI (see Fig. 4 and 7). The wild-type *tdh* promoter clone, in M13mp18/p, is designated M13mp18/p p-121 (see Results). The pMLB1034 plasmid derivatives were transformed into strain CSH26 (λRZ11). Lac$^+$ λ recombinants were isolated as described by Yu and Reznikoff (56). The plating indicator for Lac$^+$ phage was strain NK5031. High-titer lysates of Lac$^+$ λ recombinants were used to lysogenize strain BW3912. Two independently isolated lysogens of each promoter construction were used for β-galactosidase assays.

TABLE 1. Bacterial strains, phage strains, and plasmids

Designation	Relevant genotype or description	Reference or source
Strain		
PS1236	Δ(*lacZ*)*169 araD139*, *thi-1*, *rpsL*, *relA*; also designated MC4100	J. Beckwith
PS2016	As PS1236 but Tuc$^+$	This work
PS2040	As PS1236 but Tuc$^+$	This work
PS2041	As PS1236 but Tuc$^+$	This work
PS2042	As PS1236 but Tuc$^+$	This work
PS2043	As PS1236 but Tuc$^+$	This work
PS2045	As PS1236 but Tuc$^+$	This work
PS2071	As PS1236 but Tuc$^+$	This work
NK5031	Δ(*lacZ*)MM5265 *gyrA supF*	(31)
BW3912	Δ(*lacZ*)*169 pho-510 thi*	B. Wanner
W1485	Wild type	
SBD76	As W1485 but Tuc$^+$	E. Dekker
SH205	HfrC *phoA8 glpD3 glpR2 relA1 ton22 zah-735*::Tn*10* Δ(*argF-lac*)*U169*	(47)
SP971	As SBD76 but Δ*lac zah-735*::Tn*10*	This work
JM101	Δ(*lac-pro*) *thi rpsL hsdR4 endA sbcB supE44* F′ *traD36 proA*$^+$*B*$^+$ *lacI*^{q} Δ[(*lacZ*)M15]	
CSH26	*ara* Δ(*lac pro*) *thi*	(32)
Lambda phages		
λRZ11	As λplac5 (*c*I857 *S*am7) but promoterless *lacZ*	(56)
λRZ11 p-121	As λRZ11 but *tdh-lacZ*	This work; see Fig. 7
λRZ11 2042	As λRZ11 but (*tdh*::IS*3*)-*lacZ*; *tdh*::IS*3* from PS2042	This work; see Fig. 7
λRZ11 971	As λRZ11 but (*tdh*::IS*3*)-*lacZ*; *tdh*::IS*3* from SP971	This work; see Fig. 7
λRZ11 N4-2	As λRZ11 p-121 but C→T change at −34 from *tdh* transcriptional start point	This work; see Fig. 7
λRZ11 IS3(−35)	As λRZ11 p-121 but −35 hexamer changed from TCGTCG to TTGCTG	This work; see Fig. 7
M13 phages		
mp18/p	mp18 but promoterless *lacZ*α *tdh-lacZ*α fusion	This work; Materials and Methods
mp18/p p-121		
mp18/p 2042	(*tdh*::IS*3*)*lacZ*α fusion; *tdh*::IS*3* from PS2042	This work; see Fig. 4
mp18/p 971	(*tdh*::IS*3*)*lacZ*α fusion; *tdh*::IS*3* from SP971	This work; see Fig. 4
mp18/p N4-2	As mp18/p p-121 but C→T change at −34 of *tdh* promoter	This work; see Materials and Methods and Fig. 4
mp18/p IS3(−35)	As mp18/p p-121 but −35 hexamer changed from TCGTCG to TTGCTG	This work; see Materials and Methods and Fig. 4
Plasmids		
pDR121	*kbl*$^+$ *tdh*$^+$ Amp^r	(41)
pMLB1034	*lacZ* Amp^r	(5)
pMLB p-121	*lacZ*$^+$ Amp^r	This work; see Materials and Methods and Fig. 7
pMLB2042	*lacZ*$^+$ Amp^r	This work, see Materials and Methods and Fig. 7
pMLB971	*lacZ*$^+$ Amp^r	This work, see Materials and Methods and Fig. 7

The pMLB1034 derivatives carrying the IS*3*(−35) and N4-2 versions of the *tdh* promoter were not genetically stable, despite the fact that these promoters proved to be similar in strength to the IS*3*-activated promoters. The cause of the instability is unknown. To circumvent this instability, certain intermediate constructions were made that generated plasmids able to undergo homologous recombination with λRZ11. A 427-bp fragment of DNA was deleted from mp18/p p-121 by digestion with endonucleases *Eco*RI and *Mlu*I. The ends of the fragments were made flush with Klenow enzyme and deoxynucleotide triphosphates. After ligation and transformation of the mixture, a clone with the 427-bp deletion was obtained. This clone was designated p-121 ∇Mlu. The *Eco*RI site was regenerated during this manipulation. The *tdh* promoter fragment of p-121∇ Mlu was subcloned into pMLB1034 by using the *Eco*RI and *Bam*HI restriction sites to make pMLB∇Mlu. A fragment from pMLB∇Mlu carrying the *tdh* promoter and *lacZ*α was subcloned into pBR322 by using the *Eco*RI and *Cla*I restriction sites, generating a derivative named pBR∇Mlu. Finally, *Eco*RI-*Bst*XI promoter-bearing fragments of M13mp18/p N4-2 and mp18/p IS*3*(−35) were cloned into the *Eco*RI-*Bst*XI sites of pBR∇Mlu. These derivatives, expressing *lacZ*α instead of the *lacZ* gene, were genetically stable and able to undergo recombination with λRZ11 as described above.

Construction of IS*3*(−35) and N4-2 *tdh* promoter derivatives. An M13mp18/p (promoterless) derivative of IS*3*(−35) was constructed in two steps by oligodeoxynucleotide-directed mutagenesis. A 30-residue oligodeoxynucleotide (CAAACTTATAGGTCGGATAACGCGTTAACA) was annealed to M13mp18/p p-121 and extended as described above. This oligonucleotide was designed so as to mediate the deletion of six nucleotides corresponding to the −35 hexamer. The resulting deletion construct was predicted to have poor promoter activity. The DNA molecules in the extension reaction were transformed into strain JM101. Single-stranded DNA from several clear plaques was sequenced to verify structure. The same procedure was ap-

plied to this intermediate construct to reinsert six nucleotides corresponding to the −35 hexamer of the *tdh*::IS*3* promoter, using an oligodeoxynucleotide having the sequence ACTTATAGGTCGAGCAAGATAACGCGTTA.

The N4-2 *tdh* promoter mutant was isolated using the dCTP analog N^4-hydroxy-dCTP (54). Twelve nanograms of a 17-residue oligodeoxynucleotide primer (AKB 1) was annealed to 1.0 μg of single-stranded template corresponding to the construct M13mp18/p p-121. The template-primer combination was extended in the presence of 1× Klenow buffer, 0.5 mM dithiothreitol, 250 μM each dATP and dGTP, 375 μM N^4-hydroxy-dCTP, and 5 U of DNA polymerase (Klenow) for 15 min at 37°C. The reaction was continued for 30 min following the addition of all four deoxynucleotide triphosphates to final concentrations of 0.75 mM, and the mixture was then transformed into *E. coli* JM101. Upon plating, 7 plaques out of 40,000 showed a more distinctive Lac$^+$ phenotype. The single-stranded DNA from these isolates was purified and subjected to DNA sequencing. Four of the seven had the same C→T change at position −34 (see Fig. 5). These isolates were subsequently subcloned into the vector M13mp18/p to eliminate any possible carryover of mutations from the original vector. One of the N4-2 isolates was cloned into pMLB1034 and recombined with λRZ11 as described above.

Enzyme assays. Threonine dehydrogenase was assayed by measuring NAD$^+$ reduction by coupling to a dye mixture. Cells were separated from their growth medium by centrifugation and suspended in 0.033 M CAPS buffer (pH 10.5) containing 0.003% CETAB (hexadecyltrimethylammonium bromide). The reaction mixture contained 0.5 ml of 0.33 M CAPS (pH 10.5), 0.2 ml of dye-NAD$^+$ mixture, and 0.1 ml of 0.2 M L-threonine in 0.2 M CAPS buffer (pH 10.5) and lysed cells. The dye mixture contained 5 parts of 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyl tetrazolium chloride (3.2 mg/ml), 1 part of phenazine methosulfate (0.4 mg/ml), 1 part of 0.2% gelatin, and 1 part of NAD$^+$ (20 mg/ml). Assays were done in duplicate with a blank tube lacking threonine. All the components except threonine were added to the tubes, which were preincubated at 37°C for 5 min. The reaction was initiated by adding threonine. After color had developed, the reaction was stopped by adding 0.2 ml of 0.67 N HCl. The amount of formazan produced was estimated spectrophotometrically at 520 nm, using a molar extinction coefficient of 11,800. The protein content of the lysed cell extract was estimated by reading the A_{650} of the culture before harvesting. The assay was linear over a wide range of cell densities (0.05 to 1.2) and times of incubation (10 min to 4 h). β-Galactosidase was determined as described (32). Cells were grown on 0.2% glycerol–1× salts in either the presence or absence of 30 μM leucine. Assays were done in quadruplicate and have standard errors of ≤10%.

RESULTS

Isolation of Tuc$^+$ strains. Wild-type Tuc^0 *E. coli* K-12 strains cannot grow on L-threonine as the sole carbon source. Mutants can be selected, however, which have acquired the ability to utilize L-threonine as a carbon source (Tuc$^+$ phenotype). Eight Tuc$^+$ strains, obtained from E. Dekker or isolated as part of this study, were all shown to have elevated levels of threonine dehydrogenase (Table 2; 9). The parental Tuc^0 strain, PS1236, expresses the *tdh* gene at a low level. There is a ninefold increase in threonine dehydrogenase upon leucine supplementation (Table 2). All the Tuc$^+$ mutants have a 100-fold elevated level of threonine dehydrogenase which is not further increased by growth in the presence of leucine. The three typical Tuc$^+$ strains (Table 2) have about the same threonine dehydrogenase specific activity, which does not vary in growing cells and is independent of the carbon source (K. Igo, unpublished data).

TABLE 2. Threonine dehydrogenase levels in typical Tuc$^+$ mutants

Strain	Sp act[a] after addition to minimal medium:	
	None	Leucine (5 μg/ml)
PS1236 (wild type; Tuc^0)	0.36	3.4
PS2016	30.5	30.3
PS2040	34.2	34.5
PS2041	28.3	26.9

[a] The specific activity is expressed as nanomoles of reduced formazan produced per minute per A_{650} unit of bacterial culture (see Materials and Methods). Each assay was done in duplicate. The specific activity values are the average of two independent trials.

Demonstration that IS*3* is present in all Tuc$^+$ *tdh* promoters. Genomic Southern blotting analysis was performed to gain information about the structures of the promoter regions of the *tdh* operons in the Tuc$^+$ strains. Appropriately digested chromosomal DNA from Tuc$^+$ strains was probed with labeled DNA specific for the *tdh* promoter. Each Tuc$^+$ strain gave rise to a *tdh* promoter fragment 1.2 kbp larger than that of the Tuc^0 control strain (Fig. 3). The promoter-bearing DNA fragment of the Tuc^0 control had the size (600 bp) predicted from the known sequence of the wild-type *tdh* operon promoter region (3). The parental Tuc^0 strain, PS1236, also gave rise to a 600-bp promoter-bearing fragment (data not shown).

DNA fragments carrying the *tdh* promoter regions of six of the eight Tuc$^+$ strains were cloned and analyzed structurally. The cloning scheme utilized a promoterless version of M13mp18 (see Materials and Methods, Fig. 4, and reference 53). Positive clones for each of the six Tuc$^+$ strains were obtained and directly sequenced. Each of the six clones shared the following features: (i) the presence of an IS*3* element between positions −19 and −20 from the *tdh*

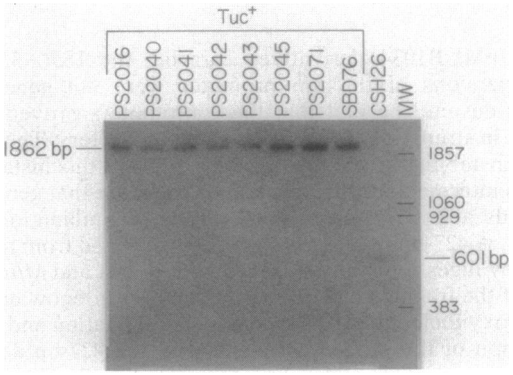

FIG. 3. Southern blot analysis of Tuc$^+$ chromosomal DNA. Chromosomal DNA prepared from eight Tuc$^+$ strains and one Tuc^0 strain was digested with the restriction endonucleases *Eco*RI and *Eco*RV. The resulting fragments were electrophoretically separated on a 1.2% agarose gel and blotted onto a nylon membrane. The immobilized DNA was then hybridized to a ^{32}P-labeled probe corresponding to the upstream *Eco*RI-*Hin*cII fragment (Fig. 2).

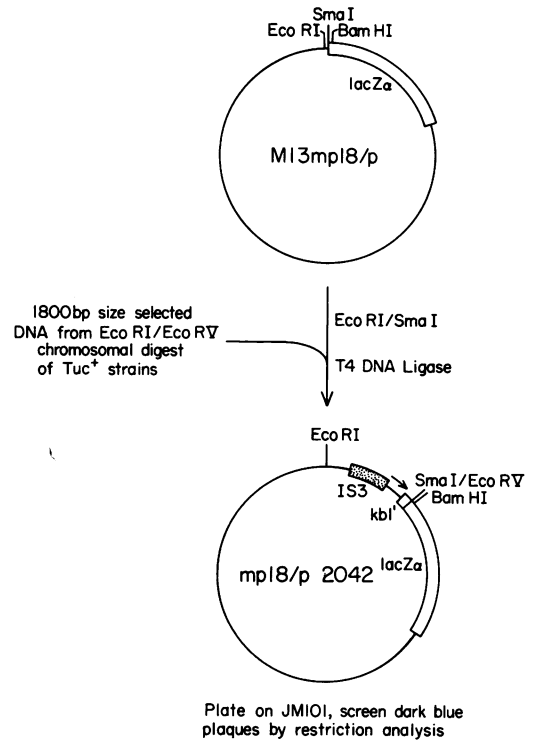

FIG. 4. Cloning of *tdh* promoter regions of Tuc$^+$ strains. The cloning vector utilized was a promoterless derivative of M13mp18 called M13mp18/p (see Materials and Methods). Chromosomal DNA from Tuc$^+$ strains was doubly digested with restriction endonucleases *Eco*RI and *Eco*RV. Fragments of 1,800 bp were selected after electrophoresis through a 1.0% low-melting-point agarose gel. This DNA was inserted into M13mp18/p digested with restriction endonucleases *Eco*RI and *Sma*I. The *tdh* promoter fragment creates an in-frame fusion of *kbl* with *lacZ*α. These clones therefore have a Lac$^+$ phenotype when plated on *E. coli* JM101. Lac$^+$ plaques were screened for the presence of an 1,800-bp insert and for the presence of an *Mlu*I site. DNA sequencing verified that the insert carried DNA from the *tdh* operon. The corresponding wild-type *tdh* promoter fragment from pDR121 (41) was also cloned into M13mp18/p. This clone is designated as M13mp18/p p-121.

transcription start site; (ii) identically oriented IS*3* elements (orientation II by convention); and (iii) a 3-bp duplication of host DNA at the insertion site, typical of all previously characterized IS*3* insertions (11, 48, 55). The 1,258-bp IS*3* element accounts in full for the increased sizes of the Tuc$^+$ promoter fragments revealed by genomic Southern blotting.

There is a minor difference at the nucleotide level in one of the six Tuc$^+$ *tdh* promoters, mp18/p 971 (Fig. 5). This difference resides within the 3 bp that are duplicated concomitantly with the movement of IS*3*. It is presumed that this difference reflects either a replication or repair error, which took place during the process of insertion of the IS*3* element, or a spontaneously arising mutation that occurred as this strain was serially transferred. This difference may have a slight effect on promoter activity (Table 3).

The two Tuc$^+$ strains whose *tdh* promoters have not been cloned also contained IS*3* elements in the same orientations as the cloned Tuc$^+$ *tdh* promoters, based on Southern hybridization experiments (data not shown). Probing of chromosomal DNA with labeled IS*3*-specific DNA showed that one additional IS*3* element was present in the chromosomes of all Tuc$^+$ strains relative to the Tuc^0 parent. We conclude that in all eight Tuc$^+$ strains examined, an IS*3* element had become inserted, in an orientation-specific manner, into the promoter region of the *tdh* operon.

Activation of *tdh* operon in Tuc$^+$ strains occurs as a result of hybrid promoter formation. The correlation between the Tuc$^+$ phenotype and the presence of the IS*3* element in the *tdh* promoter suggested that the presence of the IS*3* element is the underlying basis for the elevated levels of threonine dehydrogenase. IS*3* has been shown (11, 20, 57) to activate a crippled operon by transcription from an outward-facing promoter that lies entirely within IS*3*. The IS*3* element in Tuc$^+$ strains, however, is in the opposite orientation from this previously reported case. Thus the internal promoter of IS*3* cannot be directly responsible for the elevation of threonine dehydrogenase activity.

IS*1* and IS*2* have been shown to activate genes by the creation of hybrid promoters (22, 29, 38, 46). In such cases, the −10 hexamer is derived from the target DNA into which the IS element inserts, while the −35 hexamer is part of the inverted repeat sequence at the terminus of the IS element. In the present set of isolates, the IS*3* element was situated between the −10 and the −35 hexamer recognition elements of the *tdh* promoter. If the same transcription start were utilized both in the Tuc$^+$ *tdh* operon and in the Tuc^0 *tdh* operon, then the −35 sequence of the mutant promoters must lie within the IS*3* element. In the Tuc$^+$ strains, the IS*3* terminus proximal to the −10 hexamer of *tdh* can provide a −35 sequence that is a better match to the consensus than the sequence present in the native *tdh* promoter. In essence, the highly conserved −35 sequence TTG replaces the TCG of the wild-type promoter. The IS3::*tdh* hybrid promoter, according to one computer algorithm, was predicted to be a more efficient promoter than the wild-type *tdh* promoter (35; Fig. 5), consistent with the notion that IS*3* had activated the *tdh* operon at least in part by the formation of a hybrid promoter of enhanced signal strength.

The transcription start site of the *tdh* mRNA of the wild type was compared with the start site in Tuc$^+$ strains (Fig. 6). If a hybrid promoter is functional in Tuc$^+$ strains the transcription start site should be unchanged from that of the wild type. A 17-residue oligodeoxynucleotide complementary to the *tdh* mRNA was annealed and extended using reverse transcriptase in the presence of [α-^{32}P]dATP. Analysis of the resulting products by electrophoresis and autoradiography showed that the 5′ ends of the *tdh* mRNA were identical in both a Tuc$^+$ strain and a strain carrying the wild-type *tdh* operon. Thus, the transcription of *tdh* mRNA in Tuc$^+$ strains initiates from a hybrid promoter.

Quantitation of transcriptional activation of Tuc$^+$ *tdh* promoters. The level of transcriptional activation of the *tdh* operon in Tuc$^+$ strains was quantitatively evaluated by constructing *tdh*::*lacZ* fusions (Fig. 4 and 7). These fusions were inserted into Δ*lac* host strains in single copy in the form of lambda lysogens. The β-galactosidase assays of these lysogens, grown in either minimal glycerol or leucine-glycerol medium, are presented in Table 3. Comparison of the enzyme levels of the wild-type *tdh-lacZ* fusion with β-galactosidase levels of a *tdh-lacZ* fusion expressed from an IS*3*::*tdh* hybrid promoter illustrates two points. First, the wild-type promoter is induced during growth in the presence of leucine. This is consistent with previous observations (36, 37) that this growth condition leads to a five- to sevenfold increase in the levels of threonine dehydrogenase. Second, the IS*3*-activated *tdh* promoter is not only stronger than that of wild type, but is also constitutive. There is no alteration in β-galactosidase activity by leucine.

Demonstration that constitutivity is a result of the displace-

	-30 -20 -10 +1	Promoter Homology Score (35)
mp18/p p-121	TCTCGTCGCGACCTATAAGTTTGGGTAATATGTGCTGGA	(46.2)
mp18/p 2042	TCTTGCTGGGTAAGATCAGTTTGGGTAATATGTGCTGGA	(53.8)
mp18/p 971	TCTTGCTGGGTAAGATCAGATTGGGTAATATGTGCTGGA	(53.8)
mp18/p IS3(-35)	TCTTGCTGCGACCTATAAGTTTGGGTAATATGTGCTGGA	(53.8)
mp18/p N4-2	TCTTGTCGCGACCTATAAGTTTGGGTAATATGTGCTGGA	(56.2)
Consensus	tcTTGacat t t tg TAtaaT	(100)

FIG. 5. Alignment of the *tdh* promoter regions of wild-type and Tuc$^+$ clones. The six Tuc$^+$ *tdh* promoters that were cloned and sequenced have an IS*3* element inserted between nucleotides −19 and −20 relative to the transcription start site. Five of the six sequences are identical; mp18/p 971, however, has a single-base-pair difference at position −18. The −35 hexamer sequence, donated by the IS*3* element, is predicted to be a strong promoter compared with the wild-type *tdh* promoter. The increase in homology score for Tuc$^+$ promoters represents a predicted increase of in vitro promoter activity of approximately 10-fold. The single-nucleotide difference at −18 of the SP971 Tuc$^+$ promoter is predicted to have only a small effect on promoter strength. The *tdh* promoter derivatives IS*3*(−35) and N4-2 are also shown. Underlined sequences are specific to IS*3*.

ment of an operator site. The loss of regulation of Tuc$^+$ *tdh* promoters may reflect either the formation of a strong promoter unresponsive to leucine-specific regulatory signals or the relocation of *cis*-acting *tdh* regulatory sequences to a position 1,258 bp upstream, where control of the resulting promoter becomes insignificant. Two constructs were made to test whether the IS*3* −35 hexamer alone is sufficient to account for the increase in expression in Tuc$^+$ strains. The first of these, λRZ11 IS*3*(−35), contains the wild-type *tdh* promoter sequence except for the −35 hexamer, which is identical to the −35 hexamer of Tuc$^+$ *tdh* promoters. The second construct is a promoter-up point mutation at coordinate −34 that is the result of a C→T change (Fig. 5).

If the promoter strength alone leads to high-level constitutive expression, then cells having these constructs should have β-galactosidase activities similar to those with Tuc$^+$ *tdh-lacZ* fusions (Table 3). The two modified promoter constructs gave rise to elevated β-galactosidase activities on minimal media, confirming the notion that they are promoter-up mutants. The reporter enzyme activities on minimal media were 20-fold higher than those of the wild type. When compared with Tuc$^+$ *tdh-lacZ* fusions, however, the activities were about eightfold lower. On leucine medium the β-galactosidase values of the two promoter mutants were similar to those of the Tuc$^+$ *tdh-lacZ* fusions. This means that the promoters of λRZ11 IS3(−35) and λRZ11 N4-2, like the wild type, are inducible by leucine. Thus, these data

TABLE 3. β-Galactosidase activities under two growth conditions[a]

tdh fusion	β-Galactosidase activity with: Minimal glycerol	Leucine glycerol	Induction ratio
λRZ11 p-121	8	69	8.6
λRZ11 2042	1,477	1,490	1.0
λRZ11 971	1,840	1,765	0.96
λRZ11 IS3(−35)	160	1,325	8.3
λRZ11 N4-2	221	1,271	5.8

[a] β-Galactosidase values are in Miller units and are the means of quadruplicate assays (σ < 10% of x). BW3912 (*phoA5* Δ*lacIZYA*) was used as the host strain and has β-galactosidase values of less than 1 under either growth condition.

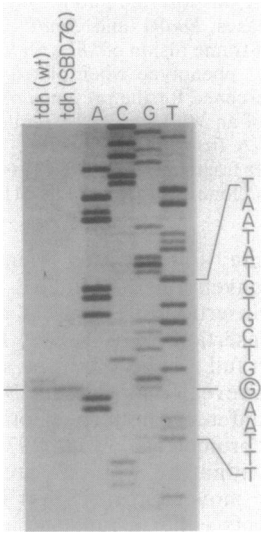

FIG. 6. Mapping the 5′ end of *tdh* mRNA from Tuc$^+$ and wild-type *tdh* operons. RNA was isolated from the Tuc$^+$ strain SBD76 and from a strain carrying a wild-type plasmid-borne *tdh* operon. A 17-residue oligodeoxynucleotide was annealed to either 20 μg (pDR121) or 40 μg (SBD76) of RNA and extended using reverse transcriptase in the presence of dCTP, dGTP, dTTP, and [^{32}P]dATP. The extension products were run on a 6% sequencing gel next to a dideoxy sequencing ladder generated using the same oligodeoxynucleotide primer. The circled nucleotide represents the predominant 5′ end of the *tdh* mRNA in both wild-type and Tuc$^+$ operons. The second, less abundant product in the *tdh* (wt) lane (corresponding to the G at position −1) is believed to be an alternate transcription start site from the same promoter.

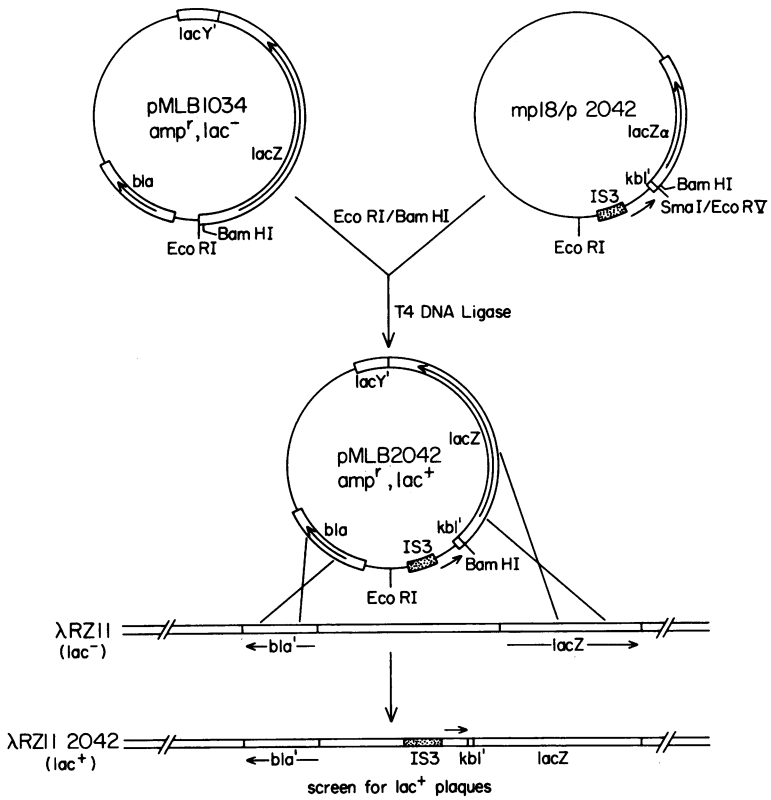

FIG. 7. Construction of λ *tdh-lacZ* fusions. *Eco*RI-*Bam*HI fragments were subcloned from M13mp18/p into pMLB1034. The plasmids from the resulting Lac$^+$ colonies were used for in vivo recombination with λRZ11. Phage from Lac$^+$ plaques were then used to lysogenize strain BW3912.

show that the effect of the IS*3* element is twofold. First, the insertion event creates a hybrid promoter from which transcription can be initiated at a higher rate than the wild-type *tdh* promoter. Second, by displacing *tdh* promoter DNA that includes the wild-type −35 region 1,258 bp upstream, IS*3* disengages the *tdh* promoter from its normal regulation, thereby rendering expression from the hybrid promoter constitutive.

On minimal media, transcription from λRZ11 IS3(−35) appears to be less efficient than transcription from *tdh*::IS*3* promoters (λRZ11 971 and λRZ11 2042) despite the fact that these promoters have equal efficiencies on minimal media in the presence of leucine. This implies that induction of transcription by leucine at the *tdh* promoter is not an activation event but involves the loss of repression.

DISCUSSION

In procaryotes there are a limited number of mechanisms by which transposable elements can activate gene expression. First, transposable elements may carry promoter sequences near their ends that mediate outwardly directed transcription. Gene activation can take place when such elements become positioned upstream of, and in the appropriate orientation with respect to, the target gene. In such cases, the transposable element serves as a mobile promoter. Tn*10*, Tn*5*, and IS*3* have been shown to activate operons by such a mechanism (2, 11, 20, 57). Charlier et al. (11) have shown that transcription of the activated *argECBH* operon initiates within the IS*3* element.

A second mechanism of gene activation by transposable elements involves the formation of hybrid promoters. This occurs (as in the present set of examples) when a transposable element inserts between the −10 and −35 hexamers of a preexisting promoter. If the terminal sequences of the transposable element contain a sequence similar to that of a consensus −35 hexamer, and if this sequence becomes situated at an appropriate distance from one that can function as a −10 hexamer, new promoters are formed. Such promoters are hybrid in that they are composed of sequences from a transposable element and the target DNA. In such cases, the transposable element is acting as a mobile −35 hexamer. IS*1* and IS2 have been shown to activate genes in this fashion (1, 22, 29, 38, 46). In this paper we have shown that IS*3* activates the *tdh* operon of *E. coli* by this mechanism.

To date, IS*3* is the only mobile element shown to activate genes by both mechanisms. Examination of the published sequences of the terminal 25 bp of IS elements led Prentki et al. (38) to predict which ones would be able to form hybrid promoters. They based their prediction on a four-of-six match to the consensus −35 hexamer. IS*3* has no such match. In the activation of the *tdh* operon it is the TTG sequence that seems to be most important for promoter activity. If TTG is the minimum sequence required for formation of hybrid promoters by IS elements, then IS*3*, IS*103*, and IS*26* (21, 33, 46) should be added to the list of Prentki et al. (38). The recently described mobile element IS*30* also has several four-of-six matches to the −35 hexamer in both the left and right ends (16). In addition, IS*21* has a perfect match to the consensus −35 hexamer in the right end and a five-of-six match to the consensus in the left end (42).

Recent work concerning promoter structure has shown that in some cases sequences bearing little structural similarity to the consensus −35 hexamer can function in the initiation of transcription (24, 25). Thus the potential of transposable elements to activate genes through the formation of hybrid promoters may be greater than previously supposed. Furthermore, activation of the *bgl* operon (43) and the *nar* operon (7) demonstrates that transposable elements can activate genes by mechanisms other than by acting as a mobile promoter or by formation of hybrid promoters.

Activation of a target gene by a promoter internal to an IS element is predicted to require less specificity at the insertion site than the formation of a hybrid promoter. Whereas a transposable element functioning as a mobile promoter can activate a gene by inserting anywhere upstream of the coding region of the target gene, hybrid promoter formation requires the insertion at one particular site upstream of the gene being activated. Thus one would expect the frequency of activation of the *tdh* operon by IS*3* to be low. Although a precise measurement of the frequency of the $Tuc^0 \rightarrow Tuc^+$ event has not been made, we estimate that it is less than 10^{-7}.

The selective conditions that gave rise to the strains in this study involved the same insertion event in eight independent isolates. We believe the specificity of the insertion site and orientation of IS*3* reflect the stringency of selection for the Tuc^+ phenotype. Presumably threonine dehydrogenase activity must increase greater than eightfold to observe the Tuc^+ phenotype, since wild-type cells cannot utilize threonine as a carbon source, even in the presence of leucine. Therefore an event in which IS*3* insertion acted only to disrupt leucine regulation would be predicted to retain the Tuc^0 phenotype. This would limit Tuc^+ IS*3* insertion events to regions where both promoter and regulatory functions are affected. IS*3* transposition would allow both formation of an efficient promoter and disruption of leucine regulation only by inserting between nucleotides −19 and −20. This is consistent with the observed insertion site specificity. Analysis of the right end of IS*3* shows that there is no sequence that is similar to the consensus −35 hexamer. Therefore, IS*3* insertion in the opposite orientation at any site in the *tdh* promoter region should not form an efficient hybrid promoter. In addition, the outward-facing promoter at the right end of IS*3* is predicted to be of weak transcriptional activity. This accounts for the orientation bias of IS*3* observed in this study. It is unclear why the Tuc^+ selection failed to yield hybrid promoters involving IS*1*, IS*2*, or other transposable elements. Presumably the transposition frequency of IS*3* into the *tdh* locus exceeds that of the other transposable elements capable of forming hybrid promoters. Perhaps the screening of a larger sample of Tuc^+ isolates would yield cases of hybrid *tdh* promoters formed by other transposable elements. We are currently characterizing Tuc^+ mutants of *Salmonella typhimurium*, a bacterial strain harboring no IS*3* elements.

There are two reported sequence discrepancies in IS*3* (11, 48, 50). Our sequencing data agree with those of Timmerman and Tu (50) at positions 1237 and 1190. It is possible that the sequence differences are due to microheterogeneity among the resident chromosomal copies of IS*3* (18, 26). If such microheterogeneity exists, particularly at the ends of IS*3* elements, this could potentially limit the number of copies of IS*3* that would be capable of activating expression of the *tdh* operon.

The insertion of IS*3* into the *tdh* promoter during the emergence of Tuc^+ strains both activates and deregulates the expression of the *tdh* operon (Tables 2 and 3). It has been shown by others (36, 37) that threonine dehydrogenase activity increases about sevenfold when wild-type cells are grown in the presence of leucine. Through the properties of *tdh-lacZ* fusions, we have shown that this activation is at the transcriptional level. In cells where the *tdh* promoter is activated by IS*3*, mRNA is formed that is identical to that found in wild type, but expression from the *tdh*::IS*3* promoter is unresponsive to leucine. Presumably a *cis*-acting target for the leucine effect is situated upstream of coordinates −19/−20. The putative target would be relocated 1,258 bp upstream by the IS*3* insertion event, thus disengaging leucine regulation. Our results suggest that regulation by leucine involves the relief of a repression event, rather than the positive activation of the *tdh* promoter by leucine.

When one compares the upstream noncoding sequence of the *tdh* operon with the upstream noncoding sequence of *lsd* (23), another gene regulated by leucine, an 11-bp region of similarity is evident. Preliminary experiments (B. D. Aronson, unpublished data) have shown that when these 11-bp sequences are disrupted, there is a loss of leucine regulation. This short sequence may thus (at least in part) be a target of the leucine-mediated activation of the *tdh* promoter.

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