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Cloning and Characterization of the *Escherichia coli* K-12 Alanine-Valine Transaminase (*avtA*) Gene

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***avtA*, which encodes the alanine-valine transaminase, transaminase C, was cloned in vivo with high- and low-copy-number mini-Mu cloning vectors. The phenotype conferred by the cloned *avtA*$^+$ gene usually depended upon the plasmid copy number; most high-copy-number *avtA*$^+$ plasmids permitted isoleucine-requiring *ilvE* strains to grow in the absence of isoleucine (multicopy suppression), while low-copy-number *avtA*$^+$ plasmids did not. *avtA* was mapped to a 1.25-kilobase segment by comparison of the restriction maps of 24 independent mini-Mu plasmids and then by gamma-delta (Tn*1000*) mutagenesis of a pBR322-*avtA*$^+$ plasmid. The direction of transcription of *avtA* on the cloned fragment was determined with fusions to a promoterless *lac* gene.**

In *Escherichia coli*, the alanine-valine transaminase (transaminase C), encoded by *avtA*, normally catalyzes the transamination of alanine, valine, and 2-aminobutyrate with their respective 2-keto acids. Transaminase C is repressed by alanine (9, 21), suggesting that its primary role is in the biosynthesis of alanine, rather than valine (21). *avtA* is also repressed by leucine and structurally related amino acids (22). *avtA* is at 84 min on the *E. coli* map, 55% cotransduced with the *ilv* operon (9, 21).

We report here our use of transposable elements to clone *avtA*$^+$ on both high- and low-copy-number plasmids, to delimit the gene in the cloned fragment, to determine its direction of transcription, and to isolate mutations that reduce *avtA* expression.

MATERIALS AND METHODS

Chemicals and media. Specialized chemicals were purchased from Sigma Chemical Co., St. Louis, Mo. Restriction endonucleases and the DNA size standards were purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md., and agarose was purchased from FMC Corp., Rockland, Maine.

Lennox (L) complex medium and Vogel-Bonner glucose-medium E salts were used and supplemented as described previously (2, 6). When required, the medium was supplemented with ampicillin (Amp) (50 μg/ml), chloramphenicol (Cam) (20 μg/ml), kanamycin (Kan) (30 μg/ml), or streptomycin (Str) (25 μg/ml). Difco MacConkey agar plus thymine (0.079 mM) was used as the lactose indicator medium.

Bacterial strains, plasmids, and bacteriophage. Table 1 shows the strains of *E. coli* K-12, phage, and plasmids used. Cultures containing mini-Mu or Mu *cts* were grown at 30°C. Mu lysates were prepared as described elsewhere (20). Generalized transduction with phage P1 was performed as described previously (6, 21).

Enzyme assays. For most assays, cells were grown overnight at 30°C in appropriately supplemented medium E and crude extracts were prepared as described previously (21), except that the appropriate antibiotic was added to the medium for strains containing a plasmid. Transaminase B activity was determined by measuring the production of 2-ketomethylvalerate from isoleucine (2). Transaminase C activity was determined by measuring the production of pyruvate from alanine (21). β-Galactosidase activity was determined by measuring the production of *o*-nitrophenol from *o*-nitrophenyl-β-D-galactoside (17). To determine the specific activity of chloramphenicol acetyltransferase, cells were grown to a density of about 5×10^8/ml in L broth plus chloramphenicol. Enzyme activity was assayed by measuring the production of 5-thio-2-nitrobenzoate from 5,5′-dithiobis-2-nitrobenzoic acid and expressed as micromoles of 5,5′-dithiobis-2-nitrobenzoic acid reduced per minute per milligram of total protein (18).

In vivo cloning. Mu dII4042, the mini-Mu plasmid used for most cloning of *avtA*$^+$, is a short (16.7-kilobase [kb]) derivative of phage Mu (37.5 kb) which has Mu genes *A* and *B*, the two Mu ends needed for replication and transposition, a chloramphenicol resistance gene, a high-copy-number plasmid origin of replication, and a promoterless *lac* operon (12). Mu dII5117, an analogous 21.7-kb mini-Mu plasmid which has a low-copy-number plasmid origin of replication and kanamycin and streptomycin-spectinomycin resistance genes (11), was used in some cloning experiments. Mini-Mu plasmid formation (Fig. 1) is described in detail elsewhere (12, 20).

Mini-Mu phage was prepared in Mu dII4042-Mu *cts* double lysogens of XPh43 or CBK801 and in a Mu dII5117-Mu *cts* double lysogen of CBK812 by thermal induction. Cultures containing about 10^7 cells per ml growing at 30°C in L broth plus 10 mM $MgSO_4$ with aeration were shifted to 42°C for 20 min and then to 37°C for 1 to 2 h until clearing or foaming occurred.

Mu dII4042-*avtA*$^+$ plasmids were isolated in CBK741 (Ile$^-$ Val$^-$) by selecting Val$^+$ Cam^r transductants, in CBK760 (Ile$^-$ Ala$^-$) by selecting Ala$^+$ Cam^r transductants, and in CBK717 (Ile$^-$ Leu$^-$) by selecting Leu$^+$ Cam^r transductants. The isolation of *avtA*$^+$ plasmids by suppression of an alanine or leucine requirement is described elsewhere (M.-D. Wang, L. Buckley, and C. M. Berg, J. Bacteriol., in press). Mu

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TABLE 1. *E. coli* K-12 strains, plasmids, and bacteriophage used[a]

Strain, phage, or plasmid	Genotype	Relevant phenotype or traits conferred	Derivation or reference
Strains			
CBK319	Δ(*proB-lac*) *thyA* Δ*ilvGEDA724*::Tn*5*-131(*tet*)[b] *recA200*	Ile$^-$ Leu$^-$ Val$^-$[c] Tet^r	1
CBK699	Δ(*proB-lac*) *thyA*		21
CBK700	Δ(*proB-lac*) *thyA ilvE720*::Tn*5*	Ile$^-$ Kan^r	21
CBK703	Δ(*proB-lac*) *thyA avtA21*::Mu dl	Amp^r	21
CBK717	CBK700 *tyrB17*::Mu dl	Ile$^-$ Leu$^-$[d] Amp^r Kan^r	Mu dl mutagenesis of CBK700 to Leu$^-$[e]
CBK740	Δ(*proB-lac*) *thyA ilvE12*	Ile$^-$	22
CBK741	Δ(*proB-lac*) *thyA ilvE12 avtA23*::Tn*5*	Ile$^-$ Val$^-$ Kan^r	22
CBK748	CBK740 *avtA21*::Mu dl	Ile$^-$ Val$^-$ Amp^r	22
CBK759	CBK700 *alaA20*::Mu dl	Ile$^-$ Ala/Val$^-$[f] Amp^r Kan^r	Mu dl mutagenesis of CBK700 to Ala$^-$[e]
CBK760	Δ(*proB-lac*) *thyA ilvE12 alaA20*::Mu dl	Ile$^-$ Ala/Val$^-$ Amp^r	Transduction of CBK740 to Amp^r with P1 · CBK759
CBK799	CBK748 *avtA21*::Mu dl(*lac*::Tn*10*)	Ile$^-$ Val$^-$ Lac$^-$ Amp^r Tet^r	Tn*10* mutagenesis of CBK748 to Lac$^-$[g]
CBK800	CBK799 *avtA21*::Mu dlΔ(*tet-amp*)	Ile$^-$ Val$^-$ Lac$^-$ Amp^s Tet^s	Deletion derivative of CBK799[h]
CBK801	XPh43 *ilvE720*::Tn*5*	Ile$^-$ Kan^r	Transduction of XPh43 to Kan^r with P1 · CBK748
CBK802	CBK717 *aspC25* Δ(*zbc2011*::Tn*10*)*275*	Ile$^-$ Leu$^-$ Asp$^-$ Phe$^-$ Tyr$^-$[i] Amp^r Kan^r	Tn*10* mutagenesis of CBK717 to Asp$^-$ Phe$^-$ Tyr$^-$ and deletion of *tet*[j]
CBK803	MC1061 *leu*$^+$	Leu$^+$ Str^r	Transduction of MC1061 to Leu$^+$ with P1 · CBK699
CBK804	CBK803 Δ*ilvGEDA724*::Tn*5*-131(*tet*)	Ile$^-$ Val$^-$ Tet^r Str^r	Transduction of CBK803 to Tet^r with P1 · CBK319[c]
CBK805	CBK804 *ilvE12*	Ile$^-$	Transduction of CBK804 to Leu$^+$ Val$^+$ with P1 · CBK740
CBK807	CBK805 *avtA23*::Tn*5*	Ile$^-$ Val$^-$ Kan^r	Transduction of CBK805 to Kan^r with P1 · CBK741
CBK811	XPh43 Δ*ilvGEDA724*::Tn*5*-131(*tet*)	Ile$^-$ Val$^-$ Tet^r	Transduction of XPh43 to Tet^r with P1 · CBK319[c]
CBK812	XPh43 *ilvE12*	Ile$^-$ Tet^s	Transduction of CBK811 to Val$^+$ with P1 · CBK740
MC1061	*araD139* Δ(*ara-leu*)*7696 lacX74 galU galK hrs hsm rpsL*	Leu$^-$ Str^r	7
MG1063	F$^+$ *recA56*		13
POI1681TR	*ara*::(Mu cts)*3* Δ(*proAB-argF-lacIPOZYA*)*XIII rpsL recA56 srl*::Tn*10* Mu dI1681	Tet^r Str^r	8
XPh43	Δ(*argF lacIPOZYA*)*U169 trp* Δ(*brnO-phoA-proC-phoB-phoR*)*24*	Trp$^-$	5
Phages			
Mu *cts*62	*cts*62	Temperature-sensitive repressor	15
Mu dII4042	*cts*62 *A*$^+$ *B*$^+$ *cam rep*P15A *lac*('*ZYA*)*931*	Cam^r	12
Mu dII5117	*cts*62 *A*$^+$ *B*$^+$ *kan spe*/*str rep*pSa *lac*('*ZYA*)*931*	Kan^r Spe/Str^r	11
Mu dI1681	*cts*62 *A*$^+$ *B*$^+$ *kan trp'B A'-W209-lac*('*ZYA*)	Kan^r	8
Plasmids			
pBR322	*tet amp*	Tet^r Amp^r	4
pEG109	Mu dII4042-*phoA*$^+$-*proC*$^+$	Cam^r	12
pEG5117	Mu dII5117-*tet-rep*	Kan^r Spe/Str^r	11
pIF001 to pIF003	Mu dII4042-*avtA*$^+$	Cam^r[k]	Transduction of CBK741 to Val$^+$ with XPh43Mu *cts*(pEG109) lysate
pIF004	pBR322-*avtA*$^+$	Amp^r	Present study (Fig. 3)
pIF005	pBR322-*avtA*$^+$	Amp^r	Present study (Fig. 3)
pIF005-1 to pIF005-49	pIF005::γδ	Amp^r	Gamma-delta mutagenesis of pIF005
pIF009 to pIF035	Mu dII4042-*avtA*$^+$	Cam^r[k]	Transduction of CBK760 to BCA^r, Ala/Val$^+$ with CBK801Mu *cts*(pEG109) lysate
pIF036 to pIF038	Mu dII4042-*avtA*$^+$	Cam^r	Transduction of CBK760 to Ala/Val$^+$ with CBK801Mu *cts*(pEG109) lysate
pIF065 to pIF067	Mu dII4042-*avtA*$^+$	Cam^r	Transduction of CBK741 to Val$^+$ with CBK801Mu *cts*(pEG109) lysate

Continued on following page

TABLE 1—*Continued*

Strain, phage, or plasmid	Genotype	Relevant phenotype or traits conferred	Derivation or reference
pIF104 to pIF105	Mu dII5117-*avtA*$^+$	Kan^r	Transduction of CBK741 to Val$^+$ with CBK812Mu *c*ts(pEG5117) lysate
pIF235 to pIF237	pIF005 *avtA*::Mu dI1681	Amp^r Kan^r Lac$^+$	Mu dI1681 mutagenesis of pIF005
pIF240, pIF241	Mu dII4042-*avtA*$^+$	Cam^r	Transduction of CBK717 to Leu$^+$ with CBK801Mu *c*ts(pEG109) lysate

[a] Abbreviations: Ala, alanine; Asp, aspartate; Ile, isoleucine; Lac, lactose; Leu, leucine; Phe, phenylalanine; Trp, tryptophan; Tyr, tyrosine; Val, valine; Amp^r, ampicillin resistant; BCA^r, 3-chloro-L-alanine resistant; Cam^r, chloramphenicol resistant; Kan^r, kanamycin resistant; Spe^r, spectinomycin resistant; Str^r, streptomycin resistant; Tet^r, tetracycline resistant.

[b] Tn*5*-131 is a transposase-deficient derivative of Tn*5* in which *kan* and the other antibiotic resistance determinants of Tn*5* have been replaced by the *tet* gene of Tn*10*.

[c] CBK319 requires isoleucine and valine because most of the *ilv* operon is deleted. In addition, it requires leucine because *avtA* is poorly expressed in a *recA* background and hence, valine is not efficiently deaminated to form 2-ketoisovalerate, the precursor of leucine (W. A. Whalen, C. M. Berg, and L. Bersche, Genetics **100s**:74, 1982). Since transaminase C is expressed better in *rec*$^+$ strains, CBK804 and CBK811 do not require leucine.

[d] In an *ilvE* genetic background, *tyrB* mutants require leucine because *ilvE* and *tyrB* encode the only two transaminases (B and D) that catalyze the final step in leucine biosynthesis (10).

[e] W. A. Whalen, Ph.D. thesis, University of Connecticut, Storrs, 1984.

[f] In an *ilvE* genetic background, *alaA* mutants require alanine or valine. The role of the *alaA* product in amino acid metabolism is not yet understood, but *alaA* is most likely a regulatory gene.

[g] Tn*10* inserted into the *lac* gene of Mu dI1.

[h] Isolated as a Tet^s Amp^s derivative by the procedure of Bochner et al. (3).

[i] In an *ilvE tyrB* genetic background, *aspC* mutants require aspartate, phenylalanine, and tyrosine because *tyrB* and *aspC* encode the only two transaminases (D and A) that catalyze the final steps in tyrosine and aspartate biosynthesis, while *ilvE*, *tyrB*, and *aspC* encode the three transaminases that catalyze the final step in phenylalanine biosynthesis (10).

[j] The Tn*10* insertion was found to be about 20% linked to *aspC* by P1 transduction. A Tet^s derivative was isolated by the procedure of Bochner et al. (3).

[k] Also makes the strain isoleucine independent.

dII5117-*avtA*$^+$ plasmids were isolated in CBK741 by selecting Val$^+$ Str^r transductants. Plasmid transductants were distinguished from generalized transductants by antibiotic resistance and the cosegregation of antibiotic sensitivity and auxotrophy (12, 20).

In vitro DNA manipulations. Plasmid-containing cells were grown overnight in L broth plus an appropriate antibiotic (chloramphenicol to maintain Mu dII4042, kanamycin to maintain Mu dI1681-containing plasmids, streptomycin to maintain Mu dII5117, and ampicillin to maintain pBR322 derivatives). Plasmid DNAs were extracted by an alkaline lysis procedure (16) and digested with restriction endonucleases according to the instructions of the supplier. The digests were electrophoresed in 0.7% agarose, and fragment sizes were estimated by comparison with fragments of the DNA size standard. Subcloning was accomplished by general procedures (16, 19).

Transposable element mutagenesis of *avtA*. The *recA* F$^+$ donor strain, MG1063, was transformed with pIF005, selecting for ampicillin resistance, and a monomeric transformant was chosen. pIF005 was mutagenized with gamma-delta (Tn*1000*) by selecting for conjugative transfer of this plasmid from MG1063 to the F$^-$ recipient strain, CBK741. Before mating, both parents were grown at 37°C, without shaking, to 5×10^7 to 1×10^8 cells per ml in L broth. One milliliter of donor cells and 0.5 ml of recipient cells were mixed in 20 ml of prewarmed L broth and incubated without shaking at 37°C for 2 h before adding kanamycin to kill the donor cells. After 2 h of further incubation, the surviving cells were collected and plated on L agar plus ampicillin and kanamycin to select for CBK741(pIF005) exconjugants. Under these conditions, essentially all of the transfer of a nonconjugative plasmid, such as pIF005, is due to F mobilization resulting from transient gamma-delta catalyzed cointegrate formation (13, 14).

pIF005 was also mutagenized with Mu dI1681, a Mu-derived transposon possessing a kanamycin resistance determinant and a promoterless *lac* gene but not an origin of replication (8). POI1681TR, a *recA* Mu *c*ts-Mu dI1681 (*kan*

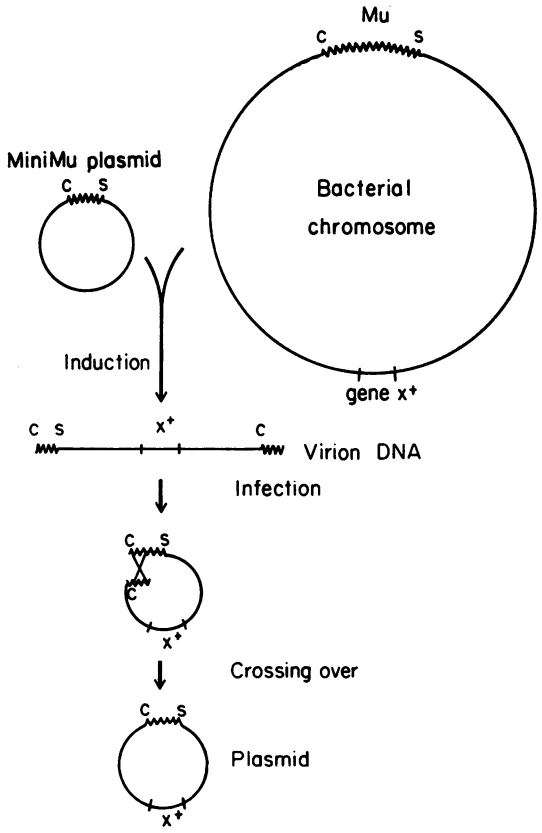

FIG. 1. Production of mini-Mu (MiniMu) plasmids by transducing phage from Mu–mini-Mu double lysogens. Zigzag line indicates Mu or mini-Mu DNA. Packaging is initiated at the left (c) end of mini-Mu. In some phage particles, one complete mini-Mu genome

TABLE 2. Transaminase activities and phenotypes of representative strains[a]

Strain and plasmid	Relevant genotype	Phenotype			Sp act[b]		
					Transaminase C		Transaminase B
		Ile	Val	Leu	Without Ala	With Ala	
High-copy-number Mu dII4042 plasmids isolated by complementation of *avtA*							
XPh43(pEG109)	*ilvE*$^+$ *avtA*$^+$	+	+	+	29.1	16.6	34.8
CBK741	*ilvE avtA*	−	−	+	<0.5	ND	<1.8
CBK741(pIF001)	*ilvE avtA/avtA*$^+$	+	+	+	903.7	ND	<1.8
CBK741(pIF002)	*ilvE avtA/avtA*$^+$	+	+	+	835.6	530.0	<1.8
CBK741(pIF003)	*ilvE avtA/avtA*$^+$	+	+	+	885.7	471.8	<1.8
High-copy-number Mu dII4042 plasmids isolated by suppression of *alaA* or *tyrB*							
CBK801(pEG109)	*ilvE avtA*$^+$	−	+	+	25.3	ND	<1.8
CBK760	*ilvE alaA*	−	−[c]	+	20.3	6.8	<1.8
CBK760(pIF011)	*ilvE alaA/avtA*$^+$	+	+	+	616.0	251.6	ND
CBK760(pIF012)	*ilvE alaA/avtA*$^+$	+	+	+	584.0	302.0	ND
CBK717	*ilvE tyrB*	−	+	−	19.0	ND	<1.8
CBK717(pIF240)	*ilvE tyrB/avtA*$^+$	+	+	+	441.6	ND	ND
CBK717(pIF241)	*ilvE tyrB/avtA*$^+$	+	+	+	589.2	ND	ND
Mu dII4042 plasmids isolated by complementation of *avtA* which confer an aberrant phenotype							
CBK741(pIF065)	*ilvE avtA/avtA*$^+$	−	+	+	52.2	17.7	ND
CBK741(pIF066)	*ilvE avtA/avtA*$^+$	−	+	+	187.3	131.1	ND
CBK741(pIF067)	*ilvE avtA/avtA*$^+$	−	+	+	46.8	20.4	ND
Low-copy-number Mu dII5117 plasmids isolated by complementation of *avtA*							
CBK741(pIF104)	*ilvE avtA/avtA*$^+$	−	+	+	45.6	32.2	ND
CBK741(pIF105)	*ilvE avtA/avtA*$^+$	−	+	+	32.8	26.0	ND
pBR322-*avtA* plasmids with gamma-delta insertions							
CBK741(pIF005)	*ilvE avtA/avtA*$^+$	+	+	+	771.1	439.6	ND
CBK741(pIF005-44)	*ilvE avtA/avtA*$^+$	+	+	+	367.8[d]	293.4[d]	ND
CBK741(pIF005-49)	*ilvE avtA/avtA*$^+$	+	+	+	407.1	317.9	ND
CBK741(pIF005-35)	*ilvE avtA/avtA*$^+$	−	+	+	177.5	96.8	ND
CBK741(pIF005-20)	*ilvE avtA/avtA*	−	−	+	5.7	5.8	ND
CBK741(pIF005-4)	*ilvE avtA/avtA*$^+$	−	+	+	75.8	83.9	ND
CBK741(pIF005-34)	*ilvE avtA/avtA*$^+$	−	+	+	152.7	166.6	ND
CBK741(pIF005-43)	*ilvE avtA/avtA*$^+$	+	+	+	346.1	220.2	ND

[a] Cells were grown overnight in glucose-limited medium E containing the required supplements plus isoleucine and valine (even if not required) and 1.0 mM alanine to repress *avtA*. Cells were harvested by centrifugation, washed once in medium E salt solution, and suspended in medium E with and without 1.0 mM alanine to an A_{600} of about 0.15. All strains are Mu *cts* lysogens, except for those with pBR322-*avtA* plasmids with gamma-delta insertions.

[b] Transaminase C activity is expressed as nanomoles of pyruvate produced per minute per milligram of protein, and transaminase B (the branched-chain amino acid transaminase, encoded by *ilvE*) activity is expressed as nanomoles of 2-keto-3-methylvalerate produced per minute per milligram of protein. ND, Not determined.

[c] Requires valine or alanine.

[d] Gamma-delta insertion, per se, reduces gene expression.

lac) double lysogen, was transformed with pIF005, selecting for ampicillin resistance, and a monomeric transformant was chosen. Mu lysates prepared on POI1681TR(pIF005) were used to infect CBK800 (*ilvE avtA*), and Kan^r Amp^r Lac$^+$ transductants were selected on MacConkey lactose indicator plates.

RESULTS

In vivo cloning and characterization of *avtA*$^+$ plasmids. With the high-copy-number plasmid element Mu dII4042 as the cloning vector (Fig. 1), *avtA*$^+$ was cloned in vivo from XPh43(pEG109) into CBK741 (*ilvE avtA*), which requires isoleucine and valine. Each of three valine-independent plasmid transductants grew in the absence of isoleucine, even though they were *ilvE* and lacked transaminase B (Table 2), which is the enzyme normally responsible for isoleucine synthesis in haploid strains of *E. coli*. Each plasmid-containing strain had about 30-fold-elevated transaminase C activity, and this activity was still repressible by alanine. Segregants that lost the unstable mini-Mu plasmid regained requirements for both isoleucine and valine and simultaneously lost transaminase C activity (data not shown), indicating that the gene responsible for isoleucine independence is plasmid-borne. Additional Mu dII4042-*avtA*$^+$ plasmids, obtained by cloning *avtA*$^+$ from CBK801 (pEG109) into CBK717 (*ilvE tyrB*) and CBK760 (*ilvE alaA*), were also found to confer isoleucine independence and to have elevated transaminase C activity (Table 2).

Restriction maps of 21 Mu dII4042-*avtA*$^+$ plasmids (Fig. 2B) were analyzed. The cloned fragments in these plasmids range in size from 6.6 to 19.8 kb and encompass about 27 kb

plus bacterial DNA and at least a portion of Mu or mini-Mu in the same orientation are packaged. Upon introduction into the recipient, homologous Mu segments can pair, cross over, and circularize to form a plasmid. Subsequent recombination with the chromosome and loss of the unstable plasmid can yield a generalized transductant.

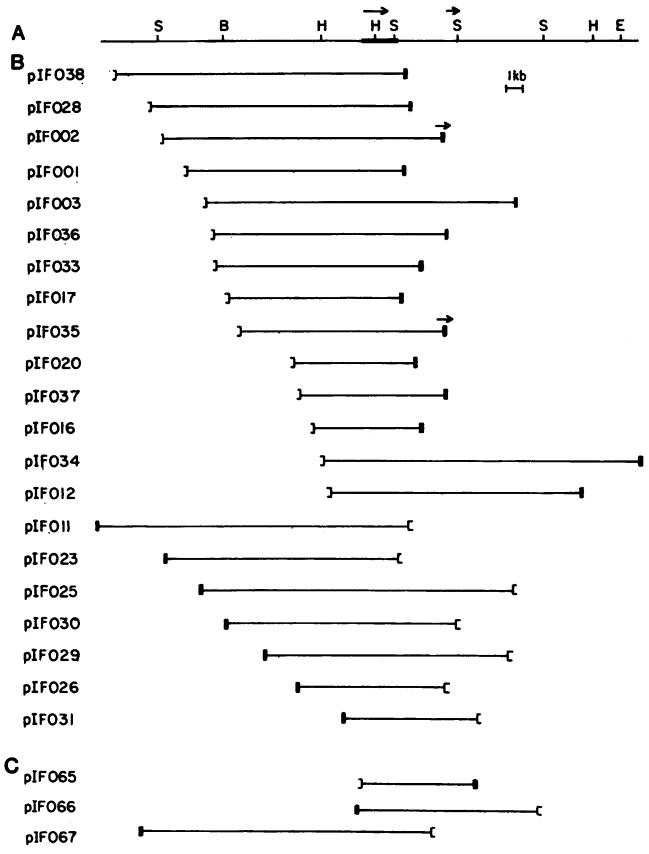

FIG. 2. Restriction map of the chromosomal portion of Mu dII4042-*avtA*$^+$ plasmids. (A) Summary restriction map of 27 kb encompassing *avtA*. The left end of the map was defined by pIF011, and the right end was defined by pIF034. The critical plasmids for localizing *avtA* to a 2.3-kb segment (heavy line) are pIF065 for the left end and pIF023 for the right end. The arrow over the heavy line depicts the direction of transcription, as determined by Mu dI1681 mutagenesis. The other arrow indicates another gene that was detected by insertion of the promoterless *lac* gene of Mu dII4042. (B) Plasmids that confer an isoleucine-independent phenotype. The *Hin*dIII site 1 kb from the *c* end and the *Bam*HI site 0.1 kb from the *S* end of Mu dII4042 (10) were used to orient the fragments. (C) Plasmids that confer an isoleucine-requiring phenotype. Restriction enzyme abbreviations: B, *Bam*HI; H, *Hin*dIII; S, *Sal*I; E, *Eco*RI. Symbols:), the left (*c*) end of mini-Mu; ▮, the right (*S*) end of mini-Mu. The promoterless *lacZYA* operon is located at the *S* end (12). The arrows over the *S* ends of pIF002 and pIF035 show that these plasmids confer a Lac$^+$ phenotype upon the Lac$^-$ host strain. Since the *S* end of Mu dII4042 contains a promoterless *lac* gene, the *S*-end insertion site is in a structural gene in the correct orientation and reading frame in these plasmids.

of the *avtA* region. The overlap among these plasmids localized *avtA* within a 3.4-kb region.

In another experiment, three exceptional isoleucine-requiring Mu dII4042-*avtA*$^+$ plasmid strains were found among 1,200 independent plasmid transductants of CBK801 that did not require valine (20). Each transductant had relatively low levels of transaminase C activity (Table 2). Plasmid copy number was assessed by measuring Mu dII4042-encoded chloramphenicol acetyltransferase. The specific activity found in the strain carrying pIF067 was 1.5, about 10% of the activity found in other Mu dII4042-*avtA*$^+$ strains, including those carrying pIF065 and pIF066 (activity ranged from 13.5 to 17). Thus, a low-copy-number mutation caused the reduced level of transaminase C in one of the three strains. The plasmids from the other two aberrant transductants were high copy number and had one Mu dII4042-chromosome juncture within the previously defined 3.4-kb *avtA* region near the *avtA* promoter (Fig. 2C). Therefore, their aberrant phenotype is probably due to interference with transcription. By this interpretation, *avtA* was further localized to an approximately 2.3-kb region of the cloned fragment (Fig. 2A).

avtA$^+$ was also cloned from CBK812(pEG5117) into CBK741 by using the low-copy-number mini-Mu element Mu dII5117. Over 400 independent valine-independent transductants were isolated, and all required isoleucine. Transaminase C was elevated just twofold above the haploid *avtA*$^+$ level in the two transductants tested (Table 2).

Mu dII4042 forms *lac* protein fusions if the right end of mini-Mu is inserted into a transcribed and translated gene in the correct orientation and reading frame (12). Of 24 plasmids tested, two, pIF002 and pIF035, conferred a Lac$^+$ phenotype, indicating that there is at least one gene in the cloned fragment 3 to 4 kb to the right of *avtA*, which is transcribed from left to right, as shown in Fig. 2. The β-galactosidase activity in both Lac$^+$ strains was insensitive to alanine repression (data not shown), suggesting that this gene is not in the *avtA* operon. Because of the reading frame requirements for protein fusions, only about one-third of the properly oriented insertions in genes will produce β-galactosidase. Therefore, the number of plasmids studied is too small to indicate if there are additional rightward-transcribed genes in the cloned region to the right of *avtA* or leftward-transcribed genes to the left of *avtA* (oppositely transcribed genes could not be detected by *lac* fusions in Mu dII4042-*avtA* plasmids).

In vitro subcloning of *avtA*. The *avtA*$^+$ gene was subcloned on a 5.3-kb *Hin*dIII-*Bam*HI fragment from pIF001 into the *tet* gene of pBR322 (Fig. 3) (the *Bam*HI restriction site was from Mu dII4042 [Fig. 2B]). Removal of the 0.7-kb *Sal*I fragment from pIF004 did not affect transaminase C activity (Table 2). This plasmid, pIF005, which has a 4.6-kb cloned *avtA*$^+$ fragment (Fig. 3), was used in further studies.

Determination of the direction of transcription of *avtA*. To determine the direction of *avtA* transcription, pIF005 was mutagenized with the promoter-probe transposon Mu dI1681 (*kan lac*) (8). Three Kan^r Amp^r Lac$^+$ isoleucine- and valine-requiring derivatives were obtained which lacked transaminase C activity (Table 3) and had Mu dI1681 inserted into different sites in *avtA* (Fig. 4). Each plasmid had Mu dI1681 inserted in the same orientation, with *lac*, and hence *avtA*, transcribed from left to right as shown in Fig. 4.

Localization of *avtA* by gamma-delta mutagenesis. After mating between MG1063(pIF005) and CBK741, 1,200 Amp^r Kan^r exconjugants were screened for phenotype; 336 required valine, and 864 did not. Of 51 plasmids from isoleucine- and valine-independent exconjugants, 17 had gamma-delta insertions scattered within the cloned *Hin*dIII or *Hin*dIII-*Sal*I chromosomal fragments. The plasmids of the valine-requiring exconjugants had insertions that were clustered within a 1.25-kb segment of this region (Fig. 4).

Because the Ile$^-$ Val$^+$ phenotypes of the low-activity plasmids (pIF065 and pIF066) suggested that insertions near an end of *avtA* might affect *avtA* expression, the 864 valine-independent exconjugants were screened for growth in the absence of isoleucine. Four were found that required isoleucine but not valine and had low transaminase C activity (Table 2). In one (pIF005-35), gamma-delta was inserted upstream, near the position of the pIF065 breakpoint, while

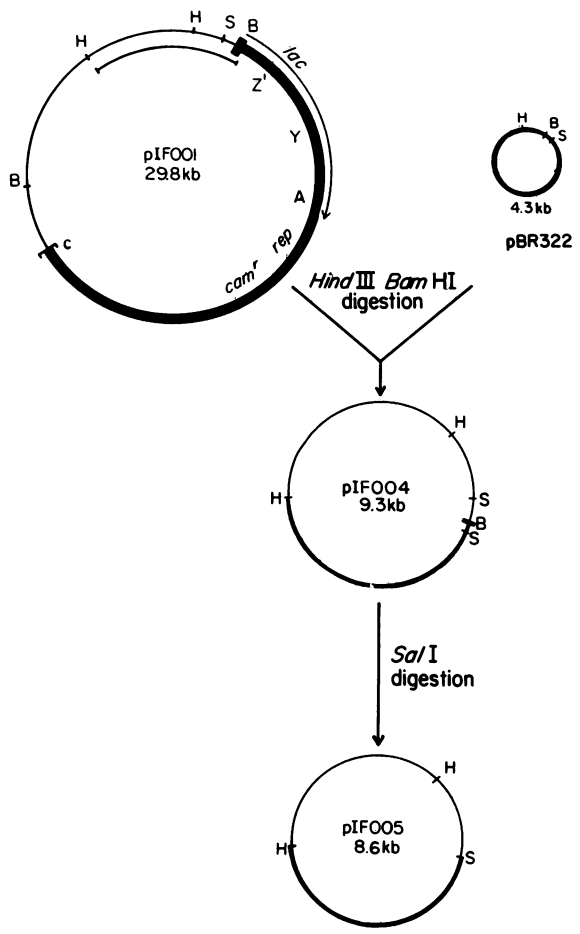

FIG. 3. Subcloning *avtA*$^+$. *Hin*dIII-*Bam*HI partial digestion fragments from pIF001 were subcloned into the *tet* gene of pBR322, replacing the *Hin*dIII-*Bam*HI fragment. A 5.3-kb fragment with an internal *Hin*dIII site yielded an *avtA*$^+$ plasmid, pIF004. A 0.7-kb *Sal*I fragment was removed to give *avtA* on a 4.6-kb *Hin*dIII-*Sal*I fragment in pIF005. Abbreviations: B, *Bam*HI; H, *Hin*dIII; S, *Sal*I; E, *Eco*RI.

TABLE 3. Transaminase C and β-galactosidase activities of *avtA*::*lac* strains[a]

Strain and plasmid	Relevant genotype	Transaminase C activity[b]	β-Galactosidase activity[c]
CBK703	*avtA*::Mu dl	<0.5	107.4
POI1681TR(pIF005)	*avtA*$^+$/*avtA*$^+$	211.2	11.1
CBK800(pIF235)[d]	*avtA*/*avtA*::Mu dI1681	<0.5	439.4
CBK800(pIF236)[d]	*avtA*/*avtA*::Mu dI1681	<0.5	783.0
CBK800(pIF237)[d]	*avtA*/*avtA*::Mu dI1681	<0.5	376.0

[a] Cells were grown overnight at 30°C in glucose-limited medium E containing isoleucine and valine plus 1.0 mM alanine to repress *avtA*::Mu dl or *avtA*::Mu dI1681, harvested by centrifugation, and washed once in medium E salt solution. Cells were suspended in same medium without alanine to an A_{600} of about 0.15 and grown for 2.5 h before harvesting.

[b] Expressed as nanomoles of pyruvate produced per minute per milligram of protein with alanine as the amino donor.

[c] Measured as described by Miller (17), with the activity expressed as units of β-galactosidase.

[d] The chromosomal Tn*10* *tet* and Mu dI1 *lac* genes have been deleted in CBK800 (Table 1).

in the others (pIF005-4, pIF005-25, and pIF005-34), gamma-delta was inserted in the cloned fragment 1.2 and 1.4 kb downstream of the pIF065 juncture (Fig. 4). Surprisingly, partial and null activity insertions were found interspersed in this region.

DISCUSSION

We have cloned *avtA*, the gene encoding transaminase C (alanine-valine transaminase), in vivo with high- (Mu dII4042) and low- (Mu dII5117) copy-number plasmid vectors. A comparison of the restriction maps of 21 high- and 3 low-expression Mu dII4042 plasmids localized this gene to a 2.3-kb segment of the *E. coli* chromosome.

Gamma-delta mutagenesis was used to further define the limits of the *avtA* gene. Insertions which mapped in a 1.25-kb segment of the *avtA* region resulted in a requirement for both isoleucine and valine. Four insertions (out of 1,200 screened) conferred a requirement for isoleucine but not for valine. One of these mapped within 10 base pairs of the upstream insertion site of a Mu dII4042 plasmid with the same phenotype, while the other three mapped at the downstream (right) end of the gene within the region defined by the null activity insertions (Fig. 4; Table 2).

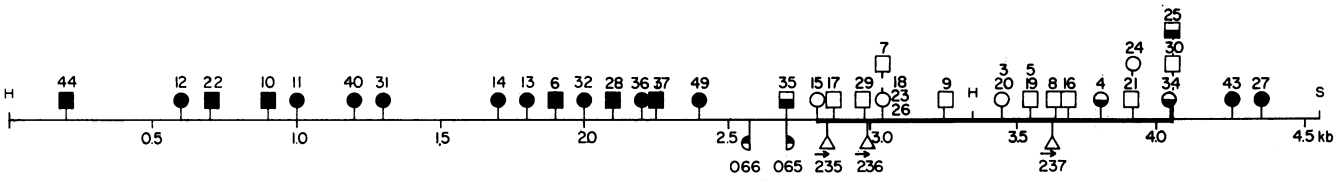

FIG. 4. Transposon insertions in the *avtA* region of pIF005. Gamma-delta insertions with the gamma end on the left (□) or with the delta end on the left (○) are shown above the line. The insertions were first localized to the 3.2-kb *Hin*dIII or the 1.4-kb *Hin*dIII-*Sal*I fragment. Then the *Eco*RI site 0.9 kb from the gamma end and the *Eco*RI site 4.0 kb from the delta end (13) were used to orient gamma-delta insertions. Mu dI1681 insertions (△) and one end of aberrant Mu dII4042 insertions (◖ and ◗) (also shown in Fig. 2C) are shown below the line. The arrows below the triangular Mu dI1681 insertions show the direction of *lac* transcription (*S* end to *c* end). The *Hin*dIII site 1.0 kb from the *c* end and the *Eco*RI site 4.4 kb from the *S* end of Mu dI1681 were used to orient Mu dI1681 insertions. The half circles depict the *c* end of Mu dII4042 in pIF065 and the *S* end of Mu dII4042 in pIF066. Since Mu dII4042 plasmids are derived from two independent Mu insertions (Fig. 1), only the insertion site adjacent to *avtA* is shown here. The second insertion which gave rise to pIF065 is 8.2 kb to the right of the insertion depicted, and the second insertion which gave rise to pIF066 is 14.8 kb to the right of the insertion depicted (Fig. 2C). The positions of all insertions were determined by restriction endonuclease digestion, and the positions of gamma-delta insertions numbered 35, 15, 29, 9, 20, 8, 16, 4, 21, 34, and 30 were corrected by the results of DNA sequencing (L. Liu, W. Whalen, A. Das, and C. M. Berg, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, H93, p. 155). The heavy line indicates the 1.25-kb *avtA* segment defined by null activity insertions. The 2.3-kb *avtA* segment defined by the region of overlap of mini-Mu plasmids (Fig. 2) is from 2.7 to 4.8 kb on this map. The solid symbols indicate that the plasmid confers an isoleucine- and valine-independent phenotype upon the host, half-filled symbols indicate that the plasmid confers a valine-independent phenotype (requires isoleucine), and open symbols indicate that the plasmid does not confer either isoleucine or valine independence.

In each plasmid and insertion mutation studied, the phenotype was correlated with transaminase activity (Table 2), which in turn was generally correlated with plasmid number. When *avtA*$^+$ was present in one or a few copies, transaminase C did not catalyze the synthesis of physiologically significant levels of isoleucine. However, when *avtA*$^+$ was present in many copies, more than 99% of the plasmids permitted an *ilvE avtA* host strain to grow in the absence of both isoleucine and valine (Table 2). Cells containing this major class of Mu dII4042-*avtA*$^+$ plasmids had transaminase C activity elevated 17- to 36-fold (Table 2), while cells containing the rare Mu dII4042-*avtA*$^+$ plasmids that did not permit an *ilvE avtA* strain to grow in the absence of isoleucine had transaminase C activity elevated only two- to sevenfold (Table 2). Some gamma-delta insertions adjacent to the *avtA* coding region also interfered with *avtA* expression, conferring an isoleucine-requiring, valine-independent phenotype upon the host strains, which also exhibited only a small elevation in transaminase C activity (Table 2). These findings show that high levels of transaminase C (17 or more times higher than the haploid level) eliminated the isoleucine requirement of *ilvE* strains, while lower levels (up to 7 times the haploid level) did not (Table 2). This indicates that transaminase C has a low catalytic ability towards 2-ketomethylvalerate, the precursor of isoleucine, so that it is significant in isoleucine synthesis in vivo only when the enzyme concentration is sufficiently elevated.

The low transaminase C activity in the low-copy-number plasmids is clearly due to few gene copies per cell, while in the aberrant high-copy-number plasmids, the low activity is due to a transposon insertion (either mini-Mu or gamma-delta) adjacent to *avtA*. There are probably two different bases for the position effects exerted by insertions upstream and downstream of the *avtA* coding region. Recent DNA sequence analyses using the gamma-delta insertions as mobile primer-specific sites show that *avtA* encodes a 272- or 226-amino-acid protein whose start codon is adjacent to gamma-delta insertion sites no. 15 or 29 and whose stop codon is in the gamma-delta insertion site no. 18 (L. Liu, W. Whalen, A. Das, and C. M. Berg, submitted for publication). The upstream (left) insertions that reduce transaminase C activity did not affect *avtA* regulation, while the downstream (right) insertions that reduce transaminase C activity were nonrepressible (Table 2; L. Liu, M.-D. Wang, B. Wang, and C. M. Berg, manuscript in preparation). Consequently, the upstream (left) low activity insertions probably interfere with message initiation or stability. The most common class of revertants of the downstream insertions no. 21 and 30 had dimeric plasmids (L. Liu and C. M. Berg, unpublished data), suggesting that the downstream low and null activity insertions may interfere with transcription by altering the DNA topology in the vicinity of the RNA polymerase and repressor binding sites. The constitutive expression in some downstream insertion mutations could reflect initiation from another promoter or transcription from the *avtA* promoter that is refractory to repression.

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