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Isoleucine Synthesis in *Corynebacterium glutamicum*: Molecular Analysis of the *ilvB-ilvN-ilvC* Operon

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Acetohydroxy acid synthase (AHAS) and isomeroreductase (IR) catalyze subsequent reactions in the flux of metabolites towards isoleucine, valine, leucine, and pantothenate. A 4,705-bp DNA fragment from *Corynebacterium glutamicum* known to code for AHAS and IR was sequenced and analyzed by Northern (RNA blot) analysis. As in other bacteria, the AHAS of this gram-positive organism is encoded by two genes, *ilvB* and *ilvN*. Gene disruption verified that these genes encode the single AHAS activity in *C. glutamicum*. The start of *ilvB* was determined by amino-terminal sequencing of a fusion peptide. By Northern analysis of the *ilvBNC* cluster, three in vivo transcripts of 3.9, 2.3, and 1.1 kb were identified, corresponding to *ilvBNC*, *ilvNC*, and *ilvC* messages, respectively. The *ilvC* transcript (encoding IR) was by far the most abundant one. With a clone from which the *ilvB* upstream regions had been deleted, only the *ilvNC* and *ilvC* transcripts were synthesized, and with a clone from which the *ilvN* upstream regions had been deleted, only the smallest *ilvC* transcript was formed. It is therefore concluded that in the *ilv* operon of *C. glutamicum*, three promoters are active. The amounts of the *ilvBNC* and *ilvNC* transcripts increased in response to the addition of α-ketobutyrate to the growth medium. This was correlated to an increase in specific AHAS activity, whereas IR activity was not increased because of the relatively large amount of the *ilvC* transcript present under all conditions assayed. Therefore, the steady-state level of the *ilvBNC* and *ilvNC* messages contributes significantly to the total activity of the single AHAS. The *ilvC* transcript of this operon, however, is regulated independently and present in a large excess, which is in accord with the constant IR activities determined.

Within the highly branched pathway of isoleucine synthesis, acetohydroxy acid synthase (AHAS) is unique because it naturally uses different substrates, allowing the ultimate synthesis of isoleucine (Ile), valine (Val), leucine (Leu), and pantothenate in parallel reactions. Consequently, AHAS activity is complexly regulated at various levels. Up to five isoenzymes exist in enterobacteria, of which three have been extensively studied. AHAS I (*ilvBN*) is feedback sensitive to Val; its expression is controlled by Val and Leu but also by cyclic AMP (8). AHAS II (*ilvGM*) is controlled by Val-, Leu-, and Ile-mediated attenuation. *ilvGM* is embedded in the *ilvGMEDA* operon, which, together with the downstream *ilvYC* genes, codes for all enzymes needed for Ile synthesis (28). AHAS III (*ilvIH*) is feedback sensitive to Val, and its expression is controlled by interaction of the Leu-Lrp protein complex with regulatory regions (32). In addition to these mechanisms for regulating total AHAS activity, each enzyme has a different specificity towards its two possible substrate mixtures, again influencing the total flux through this one reaction (1).

In contrast to this plurality of sophisticated mechanisms for enterobacteria, only limited information is available for gram-positive organisms, mostly focusing on gene structure. Thus, the sequence of the clustered *leu-ilv* genes of *Lactococcus lactis* has recently been established (9). A similar arrangement of *ilv* and *leu* genes is also known for *Bacillus subtilis* but in the inverse order, *ilv-leu* (14). In the latter organism, transcription attenuation is involved in control of the *ilv-leu* operon (10). The other gram-positive organism for which information on AHAS control is available is *Corynebacterium glutamicum*. Physiological studies showed that AHAS activity in *C. glutamicum* is inhibited by valine and that addition of α-ketobutyrate (which is one AHAS substrate) to cultures results in an up to 20-fold increase in specific AHAS activity (7). AHAS, together with isomeroreductase (IR), catalyzing the subsequent reaction in metabolite flow, resides in *C. glutamicum* on a 7-kb DNA fragment (4), suggesting proximity of the corresponding genes, as in *B. subtilis* and *L. lactis*. However, evidence obtained from functional studies with subcloned fragments points to independent expression of AHAS and IR. To analyze the transcriptional control of this *ilv* locus of *C. glutamicum*, we here present its structure together with in vivo transcript formation and response to α-ketobutyrate addition to cultures.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. All strains and plasmids used are listed in Table 1. *Escherichia coli* was grown on LB medium (19). *C. glutamicum* was grown on the complex medium CGIII (15) or TY (for RNA isolation) or on the minimal medium CGXII, consisting of (per liter) 20 g of $(NH_4)_2SO_4$, 5 g of urea, 1 g of KH_2PO_4, 1 g of K_2HPO_4, 0.25 g of $MgSO_4 \cdot 7H_2O$, 42 g of 3-morpholinopropanesulfonic acid, 10 mg of $CaCl_2$, 10 mg of $FeSO_4 \cdot 7H_2O$, 10 mg of $MnSO_4 \cdot H_2O$, 1 mg of $ZnSO_4 \cdot 7H_2O$, 0.2 mg of $CuSO_4$, 0.02 mg of $NiCl_2 \cdot 6H_2O$, 0.2 mg of biotin (pH 7), 40 g of glucose, and 0.03 mg of protocatechuic acid. When appropriate, kanamycin (50 μg/ml) or ampicillin (40 μg/ml) was added.

Genetic engineering. DNA was isolated from *C. glutamicum* by a modified alkaline extraction procedure with lysozyme (23). In vitro procedures and the analysis of plasmid and chromosomal DNA were done by standard procedures (19). The enzymes used for this purpose were supplied by Boehringer (Mannheim, Germany), as was the

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant characteristics	Source or reference
E. coli		
DH5	F⁻ *supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	11
S17-1	Mobilizing donor strain	26
C. glutamicum		
ATCC 13032	Type strain	American Type Culture Collection
CK21-1	ATCC 13032 *ilvB*::pCK21-1; AHAS⁻	This work
Plasmids		
pUC18	Cloning vector; Ap^r	29
pEM1	Mobilizable cloning vector; Km^r *oriT*^{RP4}	23
pGEM3z	Transcription vector; Ap^r	Promega
pRIT21	IgG-binding domain of *spa* (protein A)	18
pCC2-42	pUC18::*ilvBNC* (7.5-kb chromosomal fragment)	4
pKK5	pJC4::*ilvBNC* (5.9-kb chromosomal fragment)	4
pKK5-2	Exonuclease III deletion of pKK5	4
pKK5-5	Exonuclease III deletion of pKK5	4
pCK54	1.6-kb *Pvu*II-*Eco*RI fragment from pCC2-42, blunted and ligated into *Hin*dII site of pUC18	This work
pCK21-1	pEM1::*ilvB* 1,555-bp internal *Xho*I fragment	This work
pCKHyl-1	Fusion of amino-terminal end of *ilvB* in pKK5 with *Eco*RV fragment of *spa* from pRIT21	This work

nonradioactive DNA labeling and detection kit for localizing hybridized DNA and RNA fragments. Plasmids were introduced into *C. glutamicum* via electroporation or by conjugation with *E. coli* S17-1 as the mobilizing donor strain (21). The latter method was used to integrate the nonreplicative plasmid pCK21-1 into the chromosome (24). Plasmid pCK21-1 was constructed by integrating the 1,555-bp *Xho*I fragment of pCC2-42, carrying an internal fragment of *ilvB* (Fig. 1), into the *Sal*I site of the mobilizable vector pEM1 (23).

Protein analysis. To synthesize a fusion protein of the amino-terminal end of the *ilvB* polypeptide with the immunoglobulin G (IgG)-binding domain of the *spa* gene product, a 1,099-bp sequence of *spa* in pRIT21 (18) was amplified by the polymerase chain reaction with the synthetic oligonucleotides 5'-AAGATATCCCTGCTGCCAATGCTGCGC-3' and 5'-TAAATTCATGCCGGAGAGGG-3'. An *Eco*RV site was thus generated at the 5' end of *spa* to allow subsequent ligation with the 9.0-kb *Eco*RV fragment of pKK5 (4), providing the amino-terminal end of *ilvB*. The resulting plasmid was designated pCKHyl-1, and the correct reading frame of the chimeric gene was verified by sequencing with synthetic primers.

The wild type of *C. glutamicum* was transformed with pCKHyl-1. The resulting strain was grown to the exponential growth phase on CGXII, harvested by centrifugation, and incubated for 10 min at 95°C to inactivate proteases. After sonification, the synthesized fusion protein was isolated from the cell extract by affinity chromatography with Fast-Flow-IgG-Sepharose (Pharmacia, Piscataway, N.J.). Specific detection of the chimeric protein was achieved by Western immunoblot analysis with rabbit IgG and horseradish peroxidase-linked anti-rabbit IgG antibody. The amino acid sequence of blotted protein was determined by automatic Edman degradation with the Applied Biosystems 477A protein sequencer.

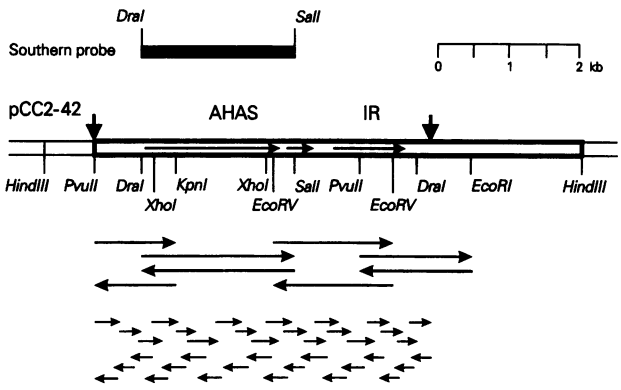

FIG. 1. Overview of Southern analysis and sequencing strategy of the *ilvBNC* cluster (AHAS and IR) of *C. glutamicum*. The *Dra*I-*Sal*I probe was used to verify the integrity of the 7-kb *Pvu*II-*Hin*dIII chromosomal fragment. Also shown are the clones and subclones used for sequencing. The sequence presented in Fig. 2 is bounded by the large vertical arrows.

DNA sequencing and analysis. Subclones were made in pUC18 in both orientations, and deletion clones were prepared by exonuclease III treatment (Promega, Madison, Wis.). DNA sequence determination was performed by the dideoxy chain termination method (20) with Klenow enzyme from Boehringer. Sequence data were compiled and analyzed with the HUSAR program package, release 2.0 (EMBL, Heidelberg, Germany). Multiple alignments were carried out with the algorithm of Myers and Miller (17).

RNA isolation and Northern (RNA blot) analysis. RNA analysis was done by the method of Börmann et al. (2) with several modifications. *C. glutamicum* was grown on complex medium TY or minimal medium to the early exponential growth phase. The cultures were chilled on ice for 10 min, harvested at 4°C by centrifugation, and washed twice with 10 ml of ice-cold 50 mM Tris–50 mM NaCl, pH 6.6. The resulting pellet was resuspended in 5 ml of 10 mM sodium acetate–1% sodium dodecyl sulfate (SDS) at pH 5 (65°C), and 5 ml of water-saturated phenol at pH 5.5 (65°C) was added. After incubation for 10 min at 65°C, the aqueous phase was separated by centrifugation, and phenol extraction was repeated two further times. RNA was precipitated with absolute ethanol (−20°C), washed with 70% ethanol, dried, and resuspended in 50 μl of diethylpyrocarbonate-treated water containing 80 U of RNasin (Promega). Quantification by absorbance readings at 260 nm yielded about 300 μg of RNA per 50-ml culture.

For Northern hybridization, about 20 μg of RNA was treated with 1 U of DNase RQ for 10 min at 37°C. Subsequently, 10 μl of loading dye (19) was added, and the sample was heated for 5 min at 95°C and loaded on an agarose gel containing 17% formaldehyde. The separated RNA was transferred to a nylon membrane, and hybridization was performed with digoxygenin-labeled RNA probes.

Four antisense mRNA probes were prepared (see Fig. 3).

As the *ilvB* probe, the 972-bp *Nco*I fragment was cloned into the *Sma*I site of the transcription vector pGEM3z (Promega). After linearization with *Hin*dIII, digoxygenin-labeled antisense mRNA was synthesized in vitro by using T7 or SP6 polymerase and the RNA labeling kit from Boehringer. Likewise, the *ilvN*-specific probe was generated from the 541-bp *Aat*II-*Spe*I fragment, and the *ilvC*-specific probe was generated from the 804-bp *Nco*I-*Cla*I fragment. The fourth probe was prepared from a 1-kb fragment 20 nucleotides downstream of the presumed terminator of the operon. The fragment was isolated as an *Sno*I-*Bam*HI fragment from plasmid pCK54. Hybridization and detection were performed with the nucleic acid detection kit from Boehringer.

Nucleotide sequence accession number. The GenBank accession number of the nucleotide sequence shown in Fig. 2 is L09232.

RESULTS

Primary structure of the *ilv* operon. An overview of the gene arrangement and the sequencing strategy for the cluster are shown in Fig. 1. Within the sequence determined, comprising 4,705 bp, a large coding region is predicted with the stop codon at nucleotide (nt) 2627 and starting either from nt 824, with ATG as the initiation codon, or at nt 749, with the rarer codon GTG (Fig. 2). In order to determine the translational start actually used in vivo, plasmid pCKHyl-1 was constructed, carrying an amino-terminal fusion at nt 1165 with the coding region for the IgG-binding domains of protein A of *Staphylococcus aureus* (27). The plasmid was introduced into *C. glutamicum*, the fusion protein was isolated by a one-step procedure, and its amino acid sequence was determined. The sequence Asn-Val-Ala-Ala-Ser-Gln-Gln-Pro- was obtained, which is in perfect agreement with GTG (nt 749) as the start codon but with the initiator amino acid methionine removed. The second open reading frame, separated by only 13 nt from the first, starts at nt 2643 and ends at nt 3069. In front of this second putative coding region is an appropriate ribosome-binding site, 5'-AAGGAG-3', which overlaps the stop codon of the first coding region. Since both of the assigned coding regions are located in those parts of DNA fragments functionally identified as resulting in high AHAS activity (4), the genes were named *ilvB* and *ilvN*, in accordance with the *E. coli* and *B. subtilis* nomenclature. The deduced gene products have molecular masses of 66,813 and 15,417 Da, respectively.

The sequence determined was used to apply gene-directed mutation and to disrupt *ilvB* in the chromosome of wild-type *C. glutamicum*. The resulting strain, CK21-1, was devoid of AHAS activity (not shown) and required all three branched-chain amino acids for growth on minimal medium, with somewhat improved growth when pantothenate was also added to the medium. This is proof that, in contrast to the members of the family *Enterobacteriacae* (28), no isoenzymes are present in *C. glutamicum*, but only one single AHAS activity.

Downstream (269 nt) of *ilvN* is the third open reading frame. It corresponds to that DNA region which resulted in high plasmid-encoded IR activity (4) and was therefore designated *ilvC*. It extends from the ATG at nt 3342 (with the ribosome-binding site [5'-GAAAGGCG-3']) to the TAA at nt 4355. The deduced *ilvC* gene product therefore consists of 338 amino acids, with a molecular mass of 36,159 Da. *ilvC* is followed by a rho-independent terminator-like structure at nt 4394 to 4428, with a ΔG^0 of −23 kcal/mol.

Transcripts of the *ilv* operon. Northern hybridization was used to determine the chromosomally derived mRNA species encoded by the *ilvBNC* operon. As probes, three antisense mRNAs specific to *ilvB*, *ilvN*, and *ilvC* messages were made (Fig. 3). In addition, a fourth probe from the sequence directly downstream of the predicted terminator was synthesized. The *ilvB* gene-specific probe hybridized to a 3.9-kb transcript (Fig. 4, lane 1), which is the size predicted for the full-length *ilvBNC* operon transcript. The *ilvN* and *ilvC* gene-specific probes also hybridized to this transcript (Fig. 4, lanes 2 and 3). Since the *ilvBNC* genes span 3.6 kb of DNA, the 3.9-kb transcript was positioned as shown in Fig. 3, additionally assuming that its 3' end is located at the predicted terminator downstream of *ilvC*. This is appropriate, since no hybridization was found with the fourth antisense mRNA (Fig. 4, lane 7), which would probe for mRNA directly downstream of the terminator. (Still more evidence comes from deletion clone pKK5-5, described further below.) A shorter transcript of 2.3 kb was detected with either the *ilvN* or *ilvC* gene-specific probe (Fig. 4, lanes 2 and 3). This unexpected transcript is of sufficient length to code for the entire *ilvN* and *ilvC* products and was positioned accordingly, assuming the same termination site as for the 3.9-kb transcript. The third transcript of the cluster detected was 1.1 kb long and hybridized to the *ilvC* gene-specific probe only (Fig. 4, lane 3). The size of this transcript is only slightly larger than that of *ilvC* (which is 1,016 kb) and is once again in very good agreement with termination at the proposed terminator.

We also analyzed the origin of the transcripts, since degradation products (either natural or artificial) are frequently observed during RNA analysis. For this purpose, we took advantage of two subclones of the operon, pKK5-2 and pKK5-5, with *ilvB* or *ilvN* 5' upstream regions, respectively, removed (Fig. 3). Plasmid pKK5 (with the entire operon) yielded the expected three transcripts (Fig. 4, lane 4), whereas pKK5-2, with the 5' upstream region of *ilvB* removed (Fig. 3), abolished synthesis of the *ilvBNC* transcript (Fig. 4, lane 5). However, it did not affect synthesis of the two smaller transcripts. This is proof that the *ilvNC* transcript is due to separate transcription initiation and that it does not arise from the large transcript by posttranscriptional events. Moreover, due to the deletion in pKK5-2, the region of the transcription start of the *ilvBNC* message can also be restricted. It is probably located upstream of the *Nar*I site. Plasmid pKK5-5 was used to investigate the origin of the most abundant 1.1-kb transcript. This plasmid still yielded the smallest transcript but not the two larger ones (Fig. 4, lane 6), confirming that its own promoter drives *ilvC* expression, as already suggested from functional studies (4).

In *C. glutamicum*, the AHAS enzyme level is increased by α-ketobutyrate addition because of the imbalance of the two possible substrates of the enzyme (7). We assayed whether this effect is related to the steady-state levels of the operon-derived transcripts. For this purpose, cells were grown on minimal medium with and without α-ketobutyrate, and transcripts and enzyme activities were determined. By comparing lanes 1 and 2 in Fig. 5, it is apparent that the intensity of the hybridization signal of the 3.9-kb transcript was strongly increased in response to α-ketobutyrate addition. This also holds for the 2.3-kb transcript, since lane 2 received only about one-third as much RNA because otherwise dark signals overlapped on the filter. In parallel, the specific AHAS activity was increased about sevenfold (Fig. 5). Therefore, α-ketobutyrate addition results in increased steady-state levels of the *ilvBNC* and *ilvNC* messages, with concomitant increased translation. The IR specific activity

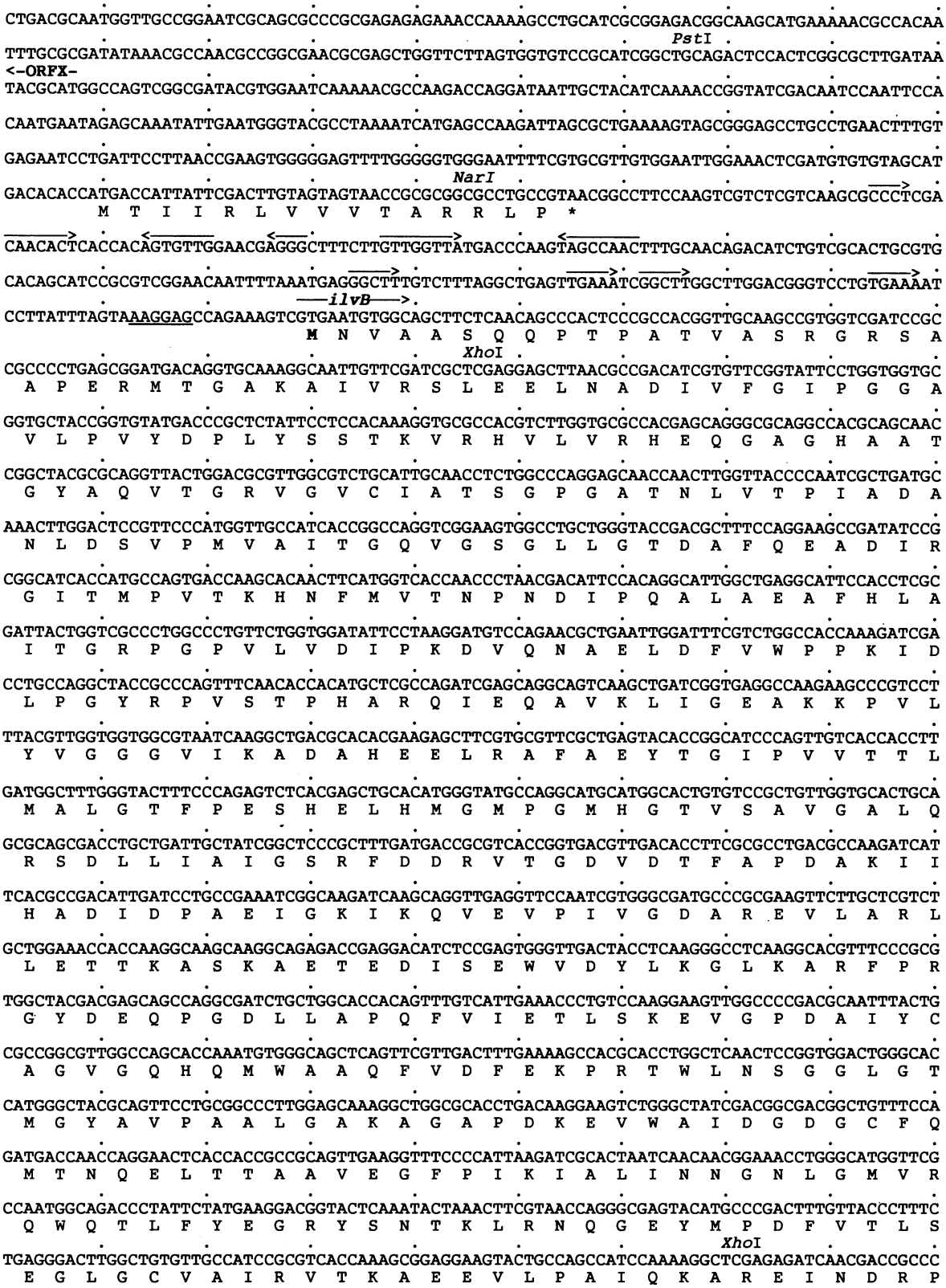

FIG. 2. Sequence of the *ilvBNC* cluster, with deduced polypeptide sequences and selected restriction sites. In front of *ilvB*, a possible leader peptide is given. The three genes of the cluster and the start of an unknown open reading frame (ORFX) are indicated. Stops are indicated by asterisks, the putative Shine-Dalgarno sequences are underlined, and inverted repeats are indicated by arrows.

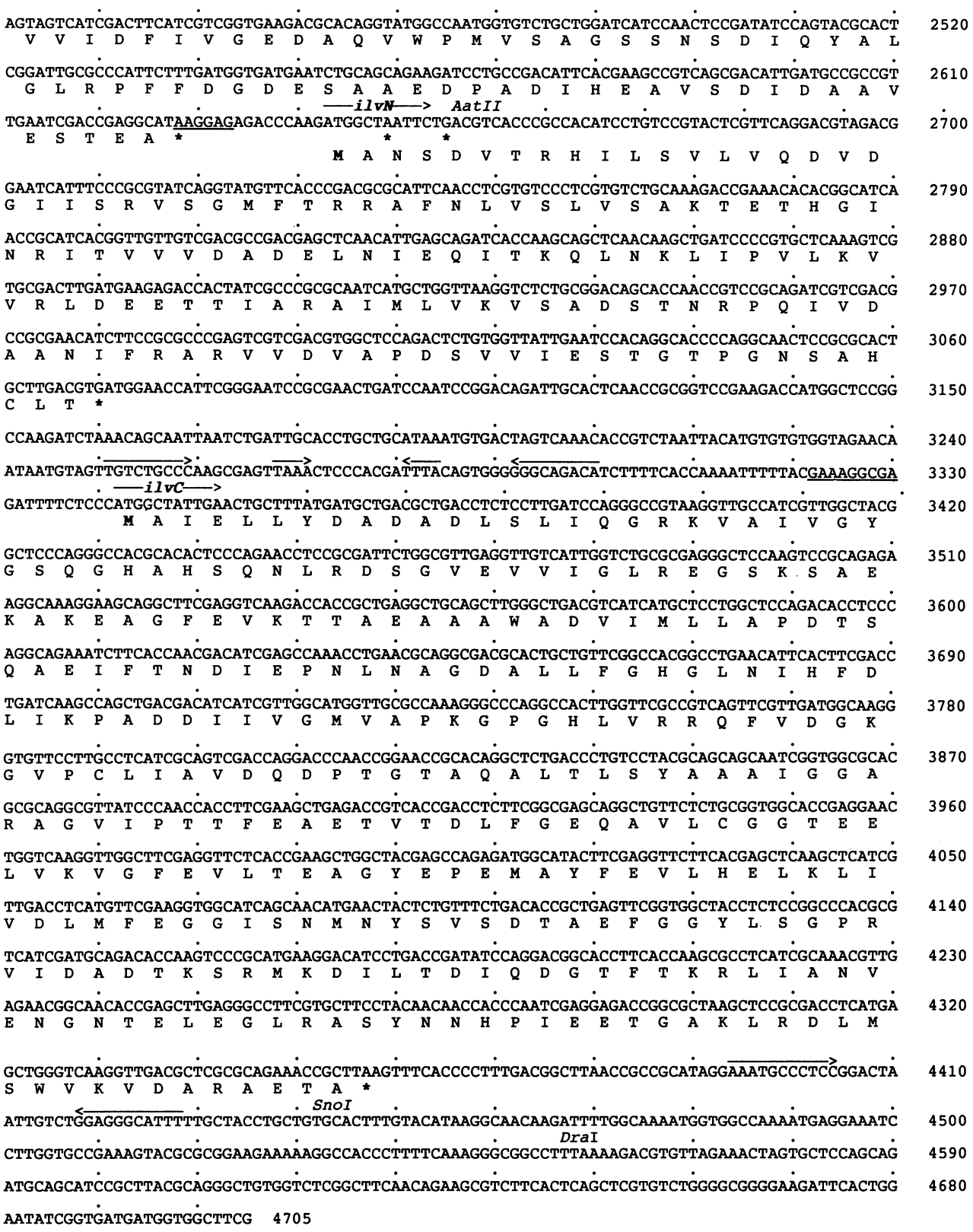

FIG. 2—*Continued.*

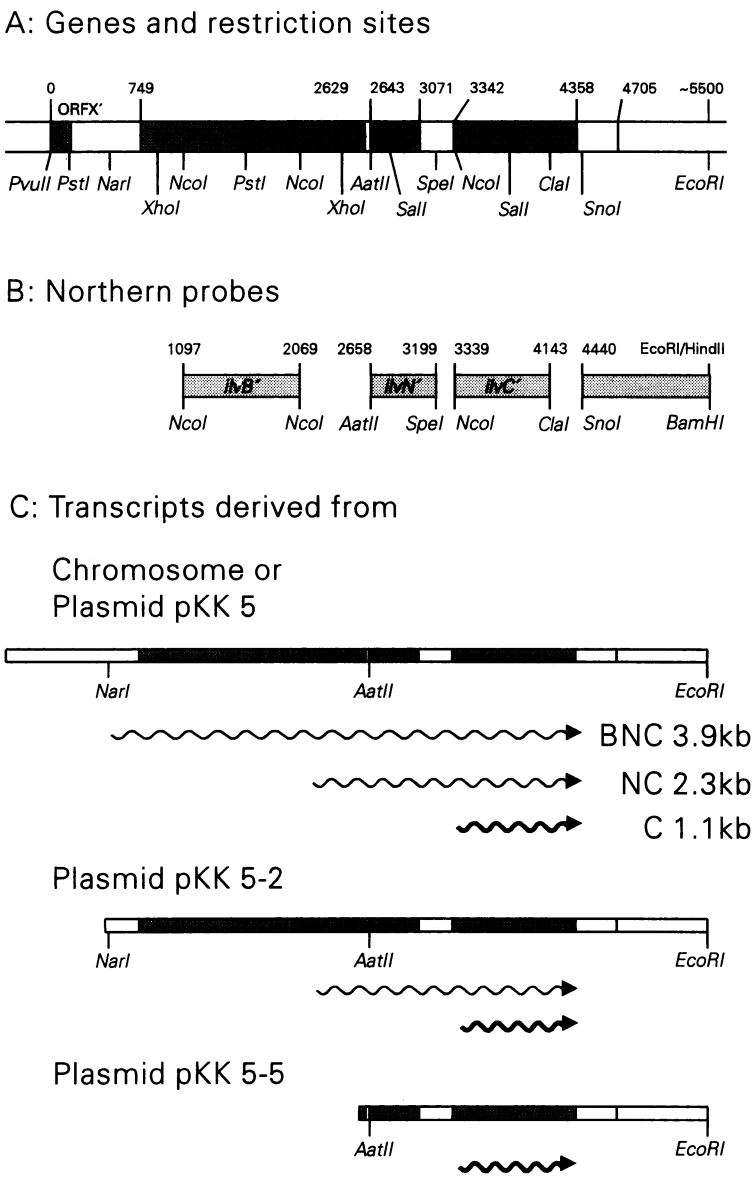

FIG. 3. (A) Locations of the coding regions of the *ilvBNC* cluster and selected restriction enzyme sites. (B) Probes used in Northern blots, which were used as antisense mRNA. (C) In vivo transcripts detected in wild-type *C. glutamicum* and transcripts from plasmids with various 5′ upstream regions of the operon removed. The relative abundances of the three mRNAs mapped by Northern blotting (wavy lines) are indicated by the thickness of the line.

was not increased by α-ketobutyrate addition, reflecting the facts that the *ilvC* transcript is by far the most abundant one of the operon and that the level of this transcript is independent of the presence of α-ketobutyrate. In complex medium, the effect of α-ketobutyrate on AHAS activity was always less pronounced (not shown). Nevertheless, at least a significant effect on the level of the 2.3-kb transcript can be seen (Fig. 5, lane 4). Also, the specific activity of the AHAS was increased, but not that of IR.

DISCUSSION

The established nucleotide sequence of the *ilv* operon of *C. glutamicum* shows that the single AHAS of this organism is encoded by two genes (*ilvB* and *ilvN*), as found for all other bacterial biosynthetic AHAS enzymes (28). It is also apparent on the protein level that AHAS from *C. glutamicum* is remarkably homologous to the group of the biosynthetic AHAS enzymes (also to those of plants with only one subunit), which constitute a structurally related group with pyruvate decarboxylase and pyruvate oxidase (3). In contrast to this group of enzymes, the IR proteins are apparently less uniform in structure. It can be seen from homology comparisons (not shown) that in the *E. coli* (and spinach) polypeptides (6, 13), a stretch of about 135 amino acid residues is inserted which has no counterpart in the *C. glutamicum* IR polypeptide. A specific function could not be attributed either to this additional sequence of amino acids in the *E. coli* polypeptide or to any other part of the IR. We here propose specific domains of the *C. glutamicum* IR polypeptide.

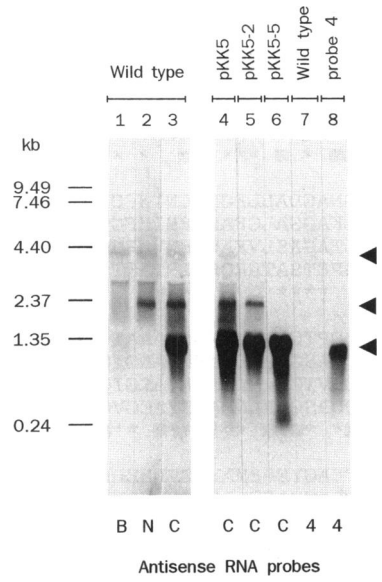

FIG. 4. Northern analysis of the *ilv* operon of *C. glutamicum* ATCC 13032. The *ilvB* probe (lane 1) hybridizes to the 3.9-kb message of the chromosome. The *ilvN* probe (lane 2) hybridizes to the 3.9-kb and 2.3-kb messages. The *ilvC* probe (lane 3) hybridizes to the 3.9-kb, 2.3-kb, and 1.1-kb messages. Recombinants containing plasmids with the entire operon (pKK5, lane 4) or 5′ deletions of the operon (lanes 5 and 6) yielded all three transcripts, the two smaller transcripts, and the smallest transcript, respectively. About 20 μg of RNA was applied to lanes 1 to 3 and 7, and 4 μg was applied to lanes 4 to 6. In lane 7, the terminator downstream probe was used, which did not hybridize to mRNA, although the probe itself (lane 8) was labeled comparably to the operon-derived probes used in lanes 1 to 6. At the left, the positions of RNA standards (GIBCO/BRL, Eggenstein, Germany) are given. Arrowheads indicate the locations of the three transcripts of the cluster.

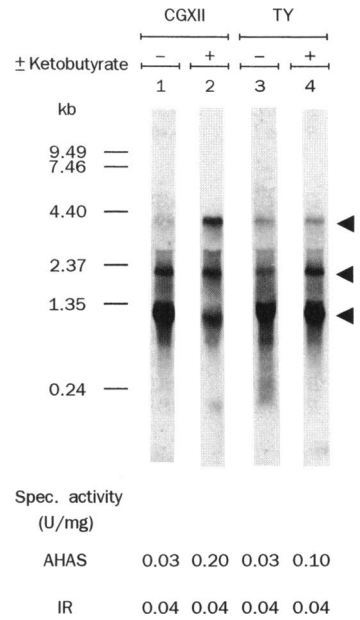

FIG. 5. Northern analysis of the *ilv* operon in response to α-ketobutyrate addition. RNA was extracted from wild-type *C. glutamicum* grown on minimal medium (CGXII) or complex medium (TY) with and without α-ketobutyrate, as indicated. The three different transcripts were monitored with the *ilvC* probe. About 20 μg of RNA was applied to lanes 1, 3, and 4, whereas lane 2 received only 7 μg. Extract was prepared from the same cells for AHAS (*ilvBN*) and IR (*ilvC*) activity determinations.

Within the *C. glutamicum* IR, an almost perfect fit to the ADP fingerprint of NAD-binding domains (31) can be identified at residues 20 to 51. Therefore, this highly conserved sequence is predicted to constitute the β1-αA-β2 structure of the NAD-binding domain, where the glycines at positions 25, 27, and 30 (Fig. 6) mark the tight turn at the end of the first β-strand and the beginning of the succeeding α-helix. The NAD-binding domain in dehydrogenases is about 140 amino acids long and is frequently located at the amino terminus, as is the case for the *C. glutamicum* IR protein. Figure 6 shows a comparison of the IR peptides from *C. glutamicum*, *Lactococcus lactis*, *B. subtilis*, and *Rhizobium meliloti*. A block of high homology (about 40% identical amino acids) is located at residues 1 to 191 of the *C. glutamicum* sequence, the majority of which would therefore represent the NAD-binding domain. An extremely highly conserved block follows the designated NAD-binding domain. It is most conspicuous that within its first 50 amino acid residues, negatively charged and aromatic amino acids are greatly overrepresented. Conserved cysteine and histidine residues are also located in this region. This region is therefore predicted to represent the microenvironment required for the complex two-step reaction of intramolecular alkyl transfer and reduction to the diol of the IR substrate. The F-E-X-L-X-E motif, which occurs twice (starting at positions 212 and 227), is probably directly involved in alkyl migration, since various acid-base mechanisms for its catalysis have been discussed recently (6, 16).

Northern analysis gives the actual sizes and distributions of the *ilvBNC* operon-derived mRNA species. In *C. glutamicum*, we found three transcripts encoded by the *ilvBNC* operon, which are the *ilvBNC*, *ilvNC*, and *ilvC* transcripts. The most abundant *ilvC* transcript was expected from the physiological and genetic investigations, since noncoordinate IR and AHAS expression was found (22), and IR oversynthesis with cloned fragments was unaffected by *ilvB* deletions, as in pKK5-5 (4). Even if the *ilvBNC* and *ilvNC* transcripts are present at higher levels after α-ketobutyrate addition, this is apparently of no significance for the overall IR activity, since because of their relatively small quantity, these large transcripts can make only a marginal contribution to IR synthesis.

The *ilvNC* transcript was unexpected. This is the first case of a transcript also coding for the small subunit of the AHAS. Only recently has a Northern analysis of the *ilvGMEDA* operon of *E. coli* been carried out (12). However, only the full-length operon transcript was found, which codes for both subunits of AHAS. In *C. glutamicum*, however, both the *ilvBNC* and *ilvNC* transcripts might contribute to the synthesis of the small subunit of the AHAS. In this respect, it is interesting that the small subunit is necessary for allosteric control (30), and under in vitro conditions for AHAS III of *E. coli* (*ilvIH*), an excess of the small subunit was shown to be necessary for maximal activity. The additional *ilvNC* transcript in *C. glutamicum* may contribute to the fidelity of the allosteric regulation of AHAS activity.

The three transcripts of the *ilv* operon of *C. glutamicum* are the result of their own transcription initiation, as is evident from plasmids with progressive 5′ deletions. Their localization with respect to the physical map is fairly unambiguous. However, sequence analysis does not allow the

FIG. 6. Alignment of the deduced IR polypeptide sequences from *C. glutamicum* (C.glu), *Lactococcus lactis* (L.lac), *B. subtilis* (B.sub), and *Rhizobium meliloti* (R.mel). Identical (■) and similar (*) amino acids are marked.

corresponding promoters to be assigned, since knowledge about promoter consensus sequences of this gram-positive bacterium is still minimal (25). The length and localization of the *ilvBNC* transcripts, however, permit the conclusion that the *ilvB* promoter is not far upstream of the *Nar*I site. Consistent with this is the fact that in the large untranslated region in front of *ilvB*, several stem-loop structures are present, which are likely involved in controlling *ilvBNC* transcription. Within the *ilv* operon, two prominent stem-loop structures of the corresponding mRNA are present. One is the predicted terminator directly downstream of *ilvC*, which has a ΔG^0 (25°C) of −23 kcal/mol. The second stem-loop is comparably favored (ΔG^0 of −21.4 kcal/mol) but is obviously not a terminator, since no *ilvBN* or *ilvN* transcript was formed. This stem-loop is therefore predicted to stabilize the 5′ end of the *ilvC* transcript.

On comparing the steady-state levels of the various transcripts of the *ilvBNC* operon of *C. glutamicum* and the *ilvGMEDA* operon of *E. coli* (12), it is obvious that the full-length operon transcripts coding for AHAS are present in comparably small amounts. The AHAS key reaction important for the flux to Ile, Val, Leu, and pantothenate is thus more strongly controlled than the downstream gene(s) not only on the biochemical level (28) but also genetically. A close correlation of the full-length operon transcripts with the flux is therefore conceivable. It has been reported that the addition of α-ketobutyrate (7) or hydroxybutyrate (22) to *C. glutamicum* results in a high flux of precursors towards Ile. It is speculated that this is a result of a Val and Leu shortage in the cells (5, 9). It is now known that this α-ketobutyrate effect is related to an increased steady-state level of the *ilvBNC* transcript, and the present investigation will enable the molecular effects of AHAS expression to be studied in detail.

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