The Promoter Activity of Isovaleryl-CoA Dehydrogenase-Encoding Gene (ivdA) from Aspergillus oryzae Is Strictly Repressed by Glutamic Acid

Nobuo Yamashita, Kazutoshi Sakamoto, Osamu Yamada, Osamu Akita, and Akira Nishimura

1 Research & Development Department, Hakutsuru Sake Brewing Co., Ltd., 4-5-5 Sumiyoshiminami-machi, Higashinada-ku, Kobe 658-0041, Japan
2 National Research Institute of Brewing, 3-7-1 Kagamiyama, Higashi-Hiroshima 739-0046, Japan
3 Faculty of Human Life Sciences, Department of Food and Health Sciences, Jissen Women’s University, 4-1-1 Osakaue, Hino, Tokyo 191-8510, Japan

Received December 19, 2006; Accepted March 20, 2007; Online Publication, June 23, 2007
[doi:10.1271/bbb.60712]

We cloned the isovaleryl-CoA dehydrogenase (IVD)-encoding gene from Aspergillus oryzae. The promoter of ivdA was subjected to β-glucuronidase (GUS) reporter assays in which certain amino acids were used as a major carbon source. L-leucine most strongly induced GUS-activity, while in the case of L-glutamate, significantly low activity was found, indicating that ivdA transcription was strongly repressed by glutamic acid.

Key words: isovaleryl-CoA dehydrogenase-encoding gene (ivdA); isovaleryl-CoA dehydrogenase (IVD); Aspergillus oryzae; GUS reporter assay; amino acid

In humans, isovaleryl-CoA dehydrogenase (IVD, EC 1.3.99.10) is a mitochondrial flavoenzyme that catalyzes the conversion of isovaleryl-CoA to 3-methylcrotonyl-CoA in the leucine catabolism pathway. A deficiency of IVD results in the disorder isovaleric acidemia.1) To study the activation characteristics of the amino acid inducible promoter, we cloned the IVD-encoding gene (ivdA) from the koji fungus Aspergillus oryzae.

We cloned a putative ivdA gene from the A. oryzae RIB40 genomic library using an EST clone homologous to the amino acid sequence of human IVD as a probe. The cloned putative ivdA showed 56% amino acid sequence identity to human IVD. To confirm that the cloned sequence encoded IVD, we measured the increased enzyme activity due to the putative ivdA gene under the control of a Taka-amyrase gene promoter (PamyB). After submerged culture of the three transformants (PamyB-putative ivdA fusion gene introduced) at 30°C for 2 d using DPY-broth (2% dextin, 2% Poly-pepton, 1% yeast extract), the IVD activities in the cell-free extracts of the transformants were assayed by the method of Bode et al.2) All three transformants showed 2.5 to 5.0-fold increased IVD activities as compared to the host strain (A. oryzae HL-1036 (sc-')) originating in industrial strain HL-10343), so we concluded that the cloned sequence was the IVD-encoding gene ivdA (GenBank Accession no. AB177764). Analysis of the upstream region of ivdA revealed the putative β-subunit of the 3-methylcrotonyl-CoA carboxylase-encoding gene (mccB), in reverse direction, located 378 bp upstream from the ATG translation start codon of ivdA (Fig. 1A). These findings match the gene map information from DOGAN (http://www.nite.go.jp/ DOGAN). The ivdA and putative mccB genes correspond to AO090020000439 and AO09002000492 in chromosome 6 of A. oryzae RIB40 respectively. Therefore it is strongly suggested that the ivdA and putative mccB genes own the promoter region jointly. To confirm this suggestion, we cloned this 377 bp predicted common promoter region into the Smal site of pNG14) in the same direction to uida (Escherichia coli β-glucuronidase gene using BKL Kit, Takara Bio, Ohtsu, Japan), to construct pNGIVF and pNGIVR respectively (Fig. 1B). A. oryzae niaD300 (niaD)5) was transformed with pNGIVF or pNGIVR. Transformants which have a single copy of each plasmid in the niaD locus were selected by Southern blot analysis. The colonies of transformants with pNGIVF or pNGIVR both turned significantly dark blue on a GUS-assay plate (Czapek-Dox (CD)-Leu, 1% L-leucine, 0.6% NaNO3, 0.052% KCl, 0.152% KH2PO4, 0.052% MgSO4•7H2O, and 2%...
agar) containing 50 μg/ml 5-bromo-4-chloro-3-indolyl glucuronide) after incubation at 30°C for 3 days, while in the case of glucose as a sole carbon source, they turned very light blue. These results indicate that ivdA and putative mccB have a common promoter region and that both are activated by L-leucine. In addition, two and three potential CreA binding motifs (5'-G/C-Y-G-R-G-3'; R, purine; Y, pyrimidine) were found in the promoter region of putative mccB and ivdA respectively.

Since IVD is a key enzyme in the leucine catabolism pathway, it is fit for ivdA to be induced by L-leucine. To clarify the response of ivdA promoter (PivdA) when various amino acids were used as a sole carbon source, we did the GUS-plate assay described above with each amino acid. The results of plate assay suggested that the activation of PivdA was related to the hydrophobicity of amino acid as a carbon source. The colonies of transformants turned dark blue with the use of the hydrophobic amino acids tested (L-leucine, L-isoleucine, L-valine, L-phenylalanine, and L-alanine), while in the case of amino acids whose hydrophobicity was not very high, they turned very light blue (L-glycine, L-serine, L-glutamine, L-asparagine, L-asparatic acid potassium salt, and L-arginine hydrochloride) or did not turn at all (L-glutamic acid sodium salt).

To measure the changes in PivdA activity induced according to each amino acid, strain TF-F1 transformed with pNGIVF was incubated in submerged culture with CD-based broth (1% amino acids, 0.15% glucose, 0.6% NaNO₃, 0.052% KCl, 0.152% KH₂PO₄, 0.052% MgSO₄·7H₂O, pH 6.5) at 30°C for 48 h. Cell-free extracts were prepared by the method of Tada et al.⁶ GUS activity was determined spectroscopically using p-nitrophenyl glucuronide as a substrate, according to Jefferson et al.⁷ As shown in Fig. 1C, GUS activity was highest when L-leucine was used as major carbon source, and was roughly equal to the activity in dextrin-induced PamyB. In addition, t-valine and t-isoleucine also induced relatively high GUS activity. While in the case of the other amino acids tested and glucose, low GUS activities were found, it was especially low with the use of L-glutamate, indicating that PivdA is strictly repressed by glutamic acid rather than glucose. The GUS activity ratio of Glu/Leu was less than 1/300, whereas under the coexistence of L-leucine and L-glutamate, the GUS activity was approximately 1/10 less than that with L-leucine as a major carbon source. These results suggest the coexistence of positive and negative regulatory systems in which L-leucine and L-glutamate participate, respectively in the activation of

---

**Fig. 1.** GUS-Reporter Assay of the ivdA Promoter of Aspergillus oryzae.

A. The location of ivdA gene and putative mccB gene in Aspergillus oryzae: ivdA, isovaleryl-CoA dehydrogenase encoding gene; mccB, putative β-subunit of 3-methylcrotonyl-CoA carboxylase-encoding gene. B. Structure of promoter analysis plasmids, pNGIVF and pNGIVR. C, GUS production of the ivdA promoter under the culture with various amino acids as a major carbon source: A. oryzae niaD300 transformed with pNGIVF were grown in submerged Czapek-Dox-based broth with various amino acids and glucose as a carbon source. Each cell-free extract for assay of GUS activity was prepared after incubation of transformants at 30°C for 48 h. PivdA and PamyB were promoters of the ivdA and amyB genes respectively. Each amino acid concentration was 1% (Leu, L-leucine; Val, L-valine; Ile, L-isoleucine; Arg, L-arginine hydrochloride; Glu, L-glutamic acid sodium salt) with 0.15% glucose except for Leu+Glu, 0.5% L-leucine and 0.5% L-glutamic acid sodium salt with 0.15% glucose. Glc, 1.15% glucose; Dex, 1.15% dextrin.

---

1562 N. YAMASHITA et al.
The ivdA promoter. Nakahara et al. reported that the high IVD activity in koji prevented the development of isovaleric acid and isobutylic acid (the main component of off-flavor) in soy sauce. The mass production of glutamic acid in koji-making process is possibly involved in the development of soy sauce off-flavor through repression of ivdA expression.

References


8) Nakahara, T., Koyama, Y., Matsushima, K., and Takeichi, J., Kokai Tokkyo Koho, 174786 (July 6, 2006).