Amino Acids (2001) 20: 175–185

Amino
Acids

Establishment of a rat hepatoma-derived cell line proliferating in D-phenylalanine medium and expressing D-amino-acid oxidase

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Accepted August 4, 2000

Summary. A cell line (R-Y121B·DF) has been established from a cell line (R-Y121B) derived from a rat hepatoma line (H4-II-E). The R-Y121B·DF cells have been continuously cultured in a serum-free modified Eagle's minimum essential medium in which L-phenylalanine was replaced by D-phenylalanine. They had D-amino-acid oxidase (DAO) activity which is essential for the growth in the medium containing D-amino acids. The enzyme activity of the R-Y121B·DF cells was approximately one-fourth of that of the rat liver. Northern hybridization using a DAO cDNA probe detected a hybridizing signal in the R-Y121B·DF cells and the rat liver but not in the parental R-Y121B and H4-II-E cells. Reverse transcription-polymerase chain reaction using DAO-specific primers amplified a DNA fragment of the expected size in the R-Y121B·DF cells but not in the R-Y121B and H4-II-E cells. This fragment was confirmed to be DAO cDNA by nucleotide sequencing. Western blotting showed that DAO protein was present in the R-Y121B·DF cells and the rat liver but not in the R-Y121B and H4-II-E cells. Southern hybridization showed that the DAO gene structure was not different among the R-Y121B·DF cells, R-Y121B cells, H4-II-E cells, and the rat liver. These results indicate that the R-Y121B·DF is a unique cell line which proliferates in the medium containing D-phenylalanine and explicitly expresses DAO. This line is useful for the study of DAO *in vitro*.

Keywords: Amino acids – Hepatoma cell line – D-Amino-acid oxidase – Rat – D-Phenylalanine – Gene expression

Introduction

D-Amino-acid oxidase (DAO, EC 1.4.3.3) catalyzes oxidative deamination of D-amino acids (stereoisomers of naturally occurring L-amino acids) to the corresponding 2-oxo acids, producing ammonia and hydrogen peroxide in the course of the reaction (Krebs, 1935). This enzyme is present in a wide variety of organisms: mammals, birds, reptiles, amphibians, fish, insects, mollusks, fungi, and yeast (Meister, 1965). It exists in the kidney, liver and brain in

higher animals. DAO metabolizes D-amino acids of internal and external origin (Konno et al., 1993; D'Aniello et al., 1993). However, the physiological function of this enzyme is unclear.

In vitro cell culture systems offer a good model for the elucidation of the cellular and molecular mechanisms of enzyme regulation. However, there are no cell lines of the hepatic origin which retain DAO. Therefore, we have tried to establish a cell line which has DAO. A rat hepatoma-derived cell line, H4-II-E, is a unique cell line which retains many enzymes present in the liver (Pitot et al., 1964). From the H4-II-E cells, Niwa et al. (1979) have established a subline (R-Y121B) which can be propagated in a serum-free modified Eagle's minimum essential medium which lacks L-arginine, L-glutamine, and L-tyrosine but is supplemented with L-ornithine. We have cultured these R-Y121B cells in the medium in which L-phenylalanine has been gradually reduced but D-phenylalanine was supplemented. L-Phenylalanine is such an essential amino acid that the cells can not survive without this amino acid. Therefore, under these conditions, the cells had to express DAO to utilize the D-phenylalanine for the production of L-phenylalanine. D-Phenylalanine is considered to be oxidatively deaminated to phenylpyruvate by DAO, and then the phenylpyruvate is re-aminated by ubiquitous transaminase to form L-phenylalanine. At the end, we have established a new cell line (R-Y121B·DF) which is continuously propagated in the medium in which L-phenylalanine is completely replaced by D-phenylalanine. We report here the growth of the R-Y121B·DF cells in this medium and the expression of DAO.

Materials and methods

Cells and culture media

The original cell line used in this study was a rat hepatoma line H4-II-E (Pitot et al., 1964) which was established from Reuber hepatoma H-35 (Reuber, 1961). The H4-II-E cells have been cultured in Eagle's minimum essential medium (MEM) supplemented with 6% serum. R-Y121B is a subline established from H4-II-E (Niwa et al., 1979). The R-Y121B cells have been cultured in the EM#Y121B medium which is a serum-free modified MEM which lacks L-arginine, L-glutamine and L-tyrosine but is supplemented with L-ornithine (0.6 mM), biotin (25 μg/ml), and 5-bromodeoxyuridine (50 μg/ml). This medium contains L-phenylalanine and is hereafter referred to LF medium. R-Y121B·DF is a subline established from the parental R-Y121B line. The R-Y121B·DF cells have been cultured in EM#Y121DF·B medium in which L-phenylalanine in the EM#121B medium is replaced with the equal amount of D-phenylalanine. This medium contains D-phenylalanine and is hereafter referred to DF medium. The LF medium and DF medium have identical composition except for the stereoisomers of phenylalanine.

All media were prepared in our laboratory and sterilized by autoclaving at 121°C for 20 min. Streptomycin and penicillin were added to the media at a concentration of 100 μg/ml and 100 units/ml, respectively. Culture media were renewed every 3 or 4 days. The cells were subcultured at approximately two-week intervals.

Growth curves of cells

The R-Y121B and R-Y121B·DF cells were harvested by scraping a monolayer with a silicon-rubber policeman and pipetting the cell suspension gently. The cells were collected

by centrifugation and the cell pellets were suspended in the LF medium or DF medium. Triplicate cultures were initiated by inoculation of 5 ml of the cell suspension per two-ounce screw-capped glass bottle. After seeding, the bottles were kept at 37°C. At intervals, three cultures were harvested and the media of the remaining cultures were changed. The growth rate was monitored by measuring cell protein. The cell protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Measurement of DAO activity

The H4-II-E, R-Y121B, and R-Y121B·DF cells proliferating in glass bottles were harvested with the policeman and washed with phosphate-buffered saline by centrifugation. The cell pellet was stored at −20°C until assayed. The cell pellet was thawed by the addition of a small volume of 7 mM pyrophophate buffer (pH8.3) and homogenized using a Physcotron homogenizer (Niti-On, Chiba, Japan). The liver was removed from Sprague-Dawley rats and homogenized similarly. The rat liver homogenate was centrifuged at 2,000 rpm for 5 min to remove insoluble materials. DAO activity in the homogenates was measured by the method of Watanabe et al. (1978). The protein contents of the homogenates were measured by the method of Lowry et al. (1951). DAO activity is expressed as the amount of D-alanine oxidized/min per mg protein.

Northern hybridization

Total RNA was extracted from H4-II-E cells, R-Y121B cells, R-Y121B·DF cells and the rat liver by the method of Chomcyzynski and Sacchi (1987) using the Isogen solution (Nippon Gene, Tokyo, Japan). Poly(A)$^+$ RNA was isolated from the total RNA using Fast Track 2.0 Kit (Invitrogen, San Diego, CA, USA). The poly(A)$^+$ RNA (5 μg) was electrophoresed on a 1.2% denaturing agarose gel. The RNA was tranferred to a nylon membrane (Hybond N, Amersham, Buckinghamshire, UK). The membrane was hybridized with a [^{32}P]-labeled DAO cDNA probe. Hybridization and signal detection were carried out as previously described (Konno et al., 1997). After the DAO cDNA probe was removed from the membrane, a [^{32}P]-labeled human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA probe (Clontech, Palo Alto, CA, USA) was hybridized.

Amplification of DAO cDNA by PCR

A primer pair (5′-TTC GAA GAG GTG GTG AGA GGA-3′, 5′-AGT GAA TCG TGA GCC CGT AAC-3′) was designed from the sequence of rat DAO cDNA (Konno, 1998). Total RNA was extracted from H4-II-E, R-Y121B, and R-Y121B·DF cells. The first strand of cDNA was synthesized as previously described (Shinohara et al., 1997). Using the primer pair and the first strand cDNA, DAO cDNA was amplified by PCR. The PCR conditions were described before (Konno et al., 1997). Five μl of the PCR product was electrophoresed on a 1% agarose gel and photographed under ultraviolet illumination.

Sequencing of the PCR product

The nucleotide sequence of the PCR product above was determined by use of Dye Terminator Cycle Sequencing Kit and Model 377 DNA Sequencer (Applied Biosystems, Chiba, Japan) as previously described (Shinohara et al., 1997).

Western blotting

Western blotting was performed as previously described (Konno et al., 1997). In brief, the H4-II-E cells, R-Y121B cells, R-Y121B·DF cells, and the rat liver were homogenized in distilled water. The rat liver homogenate was centrifuged at 2,000 rpm for 5 min to remove insoluble materials. The homogenates were mixed with sample buffer [63 mM Tris–HCI (pH 6.8), 2% SDS, 5% 2-mercaptoethanol, 10% glycerol and 0.002% bromophenol blue] and were heated in a boiling water for 5 min. The samples were electrophoresed on a 12% polyacrylamide gel (TEFCO, Tokyo, Japan). Proteins were transferred to a PVDF membrane (Immobilon-P, Millipore, Bedford, MA, USA). The membrane was blotted with rabbit anti-hog DAO IgG, followed by donkey anti-rabbit IgG labeled with horseradish peroxidase. The immune complexes were detected by peroxidase-mediated chemiluminescence production (ECL Western Blotting Analysis System, Amersham).

Southern hybridization

High molecular weight DNA was extracted from the H4-II-E cells, R-Y121B cells, R-Y121B·DF cells, and the rat liver according to the standard method (Sambrook et al., 1989). The DNAs (10μg) were digested with 20 units of *Pvu*II or *Xba*I (Nippon Gene, Tokyo, Japan) and electrophoresed on a 0.9% agarose gel. The DNA fragments were transferred to a nylon membrane (GeneScreen Plus, NEN Research Products, Boston, MA, USA). The membrane was hybridized with a [^{32}P]-labeled DAO cDNA probe. Hybridization and signal detection were performed as previously described (Konno et al., 1997).

Results

R-Y121B cells were maintained in a serum-free modified Eagle's MEM which lacked L-arginine, L-glutamine, and L-tyrosine but was supplemented with L-ornithine and 5-bromodeoxyuridine. This medium contained 0.2 mM L-phenylalanine and is hereafter referred to LF medium. To obtain the cells which can grow in the medium containing D-phenylalanine, the R-Y121B cells were cultured in the medium in which D-phenylalanine was supplemented but L-phenylalanine was gradually reduced. For the first step, they were seeded in a 60-ml glass bottle with the above modified Eagle's MEM which contained both 0.2 mM D-phenylalanine and 0.02 mM L-phenylalanine. In this medium, they started to proliferate slowly and were subcultured after one month. Thereafter, they were passaged four times in this medium. Over the next eight passages, the L-phenylalanine concentration in the medium was decreased stepwise to 0.002 mM. At seven months, they were subcultured in the medium which contained only 0.2 mM D-phenylalanine (the medium is referred to DF medium). They grew in this medium and were constantly subcultured every 2 weeks. This cell line was named R-Y121B·DF.

Figure 1 shows the growth curve of the R-Y121B·DF cells and the parental R-Y121B cells in the DF and LF medium. The R-Y121B·DF cells grew in the DF medium at the same rate as in the LF medium. The parental R-Y121B cells grew in the LF medium but could not grow in the DF medium.

DAO activity was examined in the R-Y121B·DF cells, R-Y121B cells, H4-II-E cells, and rat liver (Table 1). The R-Y121B·DF cells had DAO activity of

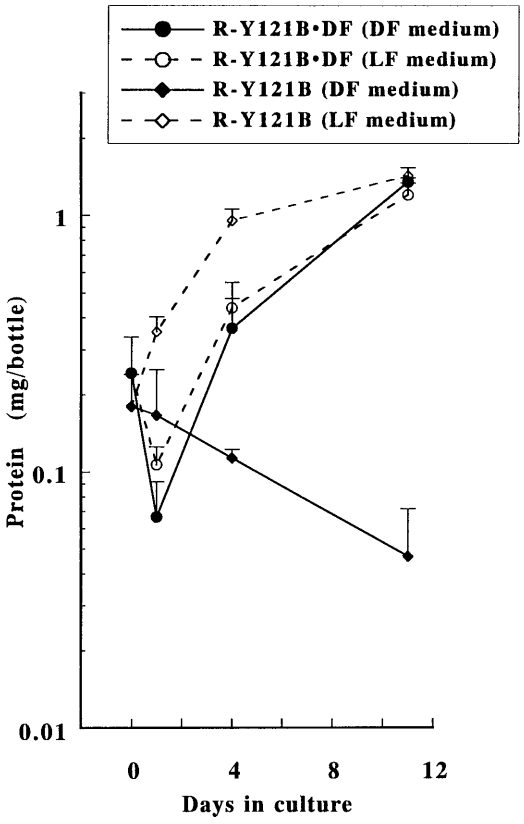

Fig. 1. Growth curve of the R-Y121B·DF cells and parental R-Y121B cells. The R-Y121B·DF cells (○ · ●) and parental R-Y121B cells (◇ · ◆) were plated in glass bottles with LF medium (○ · ◇, L-phenylalanine-containing medium) and DF medium (● · ◆, D-phenylalanine-containing medium). At indicated times after plating, the cells were harvested and their protein was measured. Each point represents the average and standard deviation (vertical bar) of three bottles

Table 1. DAO activity in cell lines and rat liver

	Activity (nmol/min per mg protein)*
H4-II-E	<0.1 (3)
R-Y121B	<0.1 (3)
R-Y121B·DF	1.1 ± 0.3 (3)
Rat liver	4.2 ± 0.5 (3)

*The enzyme activity is expressed as the mean ± standard deviation. The number of the samples used for the assay is shown in parentheses.

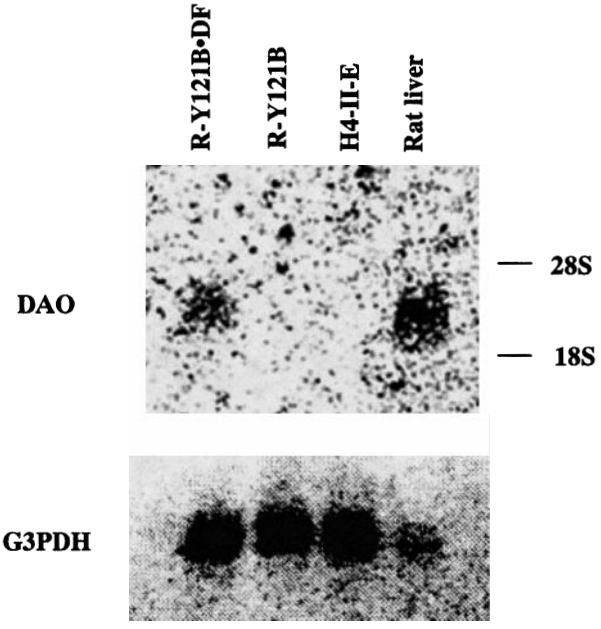

Fig. 2. Northern hybridization for detection of DAO mRNA in cell lines. Poly(A)$^+$ RNA was extracted from the R-Y121B·DF cells, R-Y121B cells, H4-II-E cells, and the rat liver. Five μg of the poly(A)$^+$ RNA was electrophoresed, transferred to a nylon membrane, and hybridized with a DAO probe (the upper panel). After the DAO probe was removed from the membrane, the membrane was rehybridized with a control G3PDH probe (the lower panel)

approximately one-fourth of that of the rat liver. Parental R-Y121B cells and H4-II-E cells did not have DAO activity.

To determine whether DAO mRNA was present in the R-Y121B·DF cells, parental R-Y121B cells, and H4-II-E cells, Northern hybridization was carried out. Poly(A)$^+$ RNA extracted from these cells was hybridized with a DAO cDNA probe. A hybridizing band was detected in the R-Y121B·DF cells and the rat liver which was used as a control (Fig. 2). The size of the band was approximately 2 kb, which was the expected size for rat DAO mRNA (Konno, 1998). The hybridizing band was not detected in the parental R-Y121B and H4-II-E cells.

The presence of DAO mRNA was further examined using reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from the R-Y121B·DF, R-Y121B, and H4-II-E cells and was reverse transcribed into cDNA. Using a primer pair specific for DAO cDNA, PCR was carried out. Figure 3 shows that a DNA fragment of the expected size (460 bp) was amplified in the R-Y121B·DF cells but not in the parental R-Y121B and H4-II-E cells. Sequencing of the amplified fragment indicated the presence of sequence corresponding to the nucleotides 602-1,051 of the rat DAO cDNA (Fig. 4).

To determine whether the DAO protein was present in the R-Y121B·DF, R-Y121B, and H4-II-E cells, Western blotting was performed. The protein

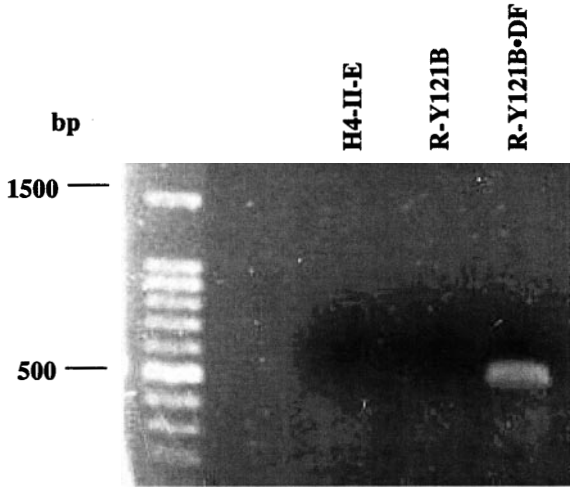

Fig. 3. PCR amplification of DAO cDNA. Total RNA was extracted from the R-Y121B·DF cells, R-Y121B cells and H4-II-E cells. The first strand of cDNA was synthesized using reverse transcriptase and an oligo(dT) primer. The resultant cDNA was used as a template for PCR. The PCR products were electrophoresed on an agarose gel together with a size marker (100 bp DNA ladder)

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R-Y121B•DF  CCGGGGCTCTGCAAGCAGACGCCTCCCTGCAGCCAGGCCGGGGCCAGATC
            ||||||||||||||||||||||||||||||||||||||||||||||||||
Rat liver   CCGGGGCTCTGCAAGCAGACGCCTCCCTGCAGCCAGGCCGGGGCCAGATC

            ATCCAGGTGGAGGCCCCTTGGATAAAGCACTTCATCCTCAGCCATGATCC
            |||||||||||||||||||||||||||||||||||||| |||||||||
            ATCCAGGTGGAGGCCCCTTGGATAAAGCACTTCATCCTCACCCATGATCC

            CAGCCTTGGCATCTACAACTCTCCATACATCATCCCAGGTTCCAAGACAG
            ||||||||||||||||||||||||||||||||||||||||||||||||||
            CAGCCTTGGCATCTACAACTCTCCATACATCATCCCAGGTTCCAAGACAG
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Fig. 4. Nucleotide sequence of the DNA fragment amplified by PCR. Total RNA extracted from the R-Y121B·DF cells was reverse transcribed. DAO cDNA was amplified by PCR and the amplified fragment was sequenced. Nucleotide sequence was compared with rat DAO cDNA sequence. Vertical bars indicate identical nucleotides

reactive with anti-DAO IgG was present in the R-Y121B·DF cells and the rat liver, but not in the parental R-Y121B and H4-II-E cells (Fig. 5). The size of the protein is in good agreement with the predicted molecular mass of rat DAO (38,818 Da) (Konno, 1998).

Genomic DNA was extracted from the R-Y121B·DF cells, R-Y121B cells, H4-II-E cells, and the rat liver, and was digested with *Pvu*II or *Xba*I. The DNA fragments were electrophoresed, transferred to a membrane, and hybridized with the DAO cDNA probe. Figure 6 shows that the hybridization patterns were almost the same among the R-Y121B·DF cells, R-Y121B cells, H4-II-E cells, and the rat liver. These results indicate that the structure of the DAO gene in these cells is not significantly different from that of the rat liver cells.

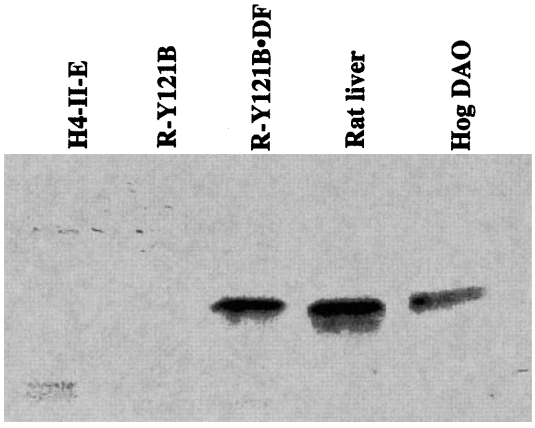

Fig. 5. Western blotting for detection of DAO protein in cell lines and the rat liver. Homogenates were prepared from the R-Y121B·DF cells, R-Y121B cells, H4-II-E cells, and the rat liver. They were electrophoresed together with control hog DAO on a SDS-polyacrylamide gel. Proteins were transferred to a PVDF membrane. The membrane was blotted with rabbit anti-hog DAO IgG, followed by donkey anti-rabbit IgG labeled with horseradish peroxidase. The immune complexes were detected by the peroxidase-mediated chemiluminescence production

The present results indicate that the R-Y121B·DF cells proliferate in the D-phenylalanine medium and explicitly express DAO. Therefore, this cell line is useful for the study of DAO *in vitro*.

Discussion

Although DAO is present in a variety of organisms, the physiological role of this enzyme is not clear. DAO expression and repression at the cellular and molecular levels are not known. These problems may be elucidated by the use of the appropriate cell lines which possess DAO. Yasumura et al. (1978) reported that VERO cells derived from the kidney of an African green monkey could be passaged in the media in which some L-amino acids were replaced by the corresponding D-amino acids. Fukui et al. (1986) and Miyake et al. (1987) reported that a pig kidney cell line had DAO. However, DAO in these cells have not been examined in detail. To our best knowledge, there is no cell line of the hepatic origin which possesses DAO. Since the liver is one of the major organs for DAO, we thought it would be important to establish a hepatic cell line for the study of DAO.

We have established the R-Y121B·DF cell line which proliferates in the medium containing D-phenylalanine in place of essential L-phenylalanine. The method used to establish this line is a variation of the method of Gilbert and Migeon (1975) who used D-valine-containing medium to select epithelial cells having DAO by preventing overgrowth of fibroblasts in primary culture. The parental R-Y121B cells were initiated to grow in the medium which contained 0.2 mM D-phenylalanine and 0.02 mM L-phenylalanine. The content

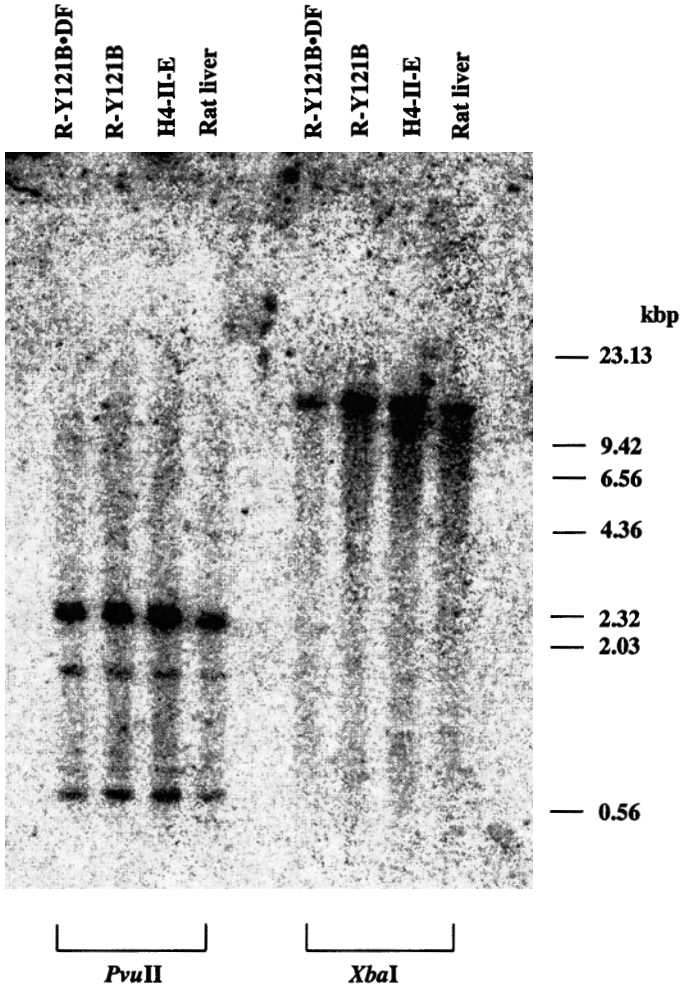

Fig. 6. Southern hybridization for DAO gene in cell lines and the rat liver. DNA was extracted from the R-Y121B·DF cells, R-Y121B cells, H4-II-E cells, and the rat liver, and was digested with *Pvu*II or *Xba*I. The DNA fragments were electrophoresed, transferred to a membrane, and hybridized with the DAO cDNA probe. Size marker was λ DNA digested with *Hin*dIII

of L-phenylalanine in the medium was reduced step by step. Under these conditions, the cells were forced to express DAO and convert the D-phenylalanine to phenylpyruvate to make L-phenylalanine for their survival and growth. Finally, the cells became to grow in the medium containing only D-phenylalanine at the similar rate as the parental R-Y121B cells growing in the medium containing L-phenylalanine (Fig. 1). The DAO expression was confirmed in the R-Y121B·DF cells in every respect: mRNA for DAO, DAO protein, and DAO activity.

DAO activity, DAO mRNA, and the DAO protein were not detected in the R-Y121B and H4-II-E cells (Table 1, Figs. 2 and 5). RT-PCR amplified a DAO cDNA fragment in the R-Y121B·DF cells but not in the R-Y121B and

H4-II-E cells (Fig. 3). However, when the cycle of PCR was increased ten times more to 35 cycles, a faint band of the amplification was observed in the R-Y121B. A much fainter band was observed in the H4-II-E cells (data not shown). Therefore, a very few cells in the population of these cells seem to have DAO mRNA. These cells may have become founders of the R-Y121B·DF line.

DAO activity determined in the rat liver was in accordance with that reported by other researchers (Hayashi et al., 1973; Ishii et al., 1980). DAO activity of the R-Y121B·DF cells was approximately one-fourth of that of the rat liver (Table 1). The R-Y121B·DF cells have been cultured continuously for more than four years. Therefore, this level of DAO activity may be sufficient to sustain the continuous growth of cells in the medium containing D-phenylalanine. However, by selecting the cells which grow at higher rates, it may be possible to obtain cells possessing higher DAO activity.

RT-PCR amplified a DAO cDNA fragment in the R-Y121B·DF cells (Fig. 3). When this fragment was sequenced, a substitution of the nucleotide was detected. The 740th nucleotide was C in DAO cDNA from the rat liver whereas it was changed to G in the R-Y121B·DF cells (Fig. 4). This nucleotide substitution causes an amino acid substitution (Thr-215 → Ser). It is not clear at present whether the nucleotide substitution was due to an error in RT-PCR or a difference in the rat strain. The R-Y121B·DF cells have originated from a AxC rat (Reuber, 1961; Pitot et al., 1964; Niwa et al., 1979) and DAO cDNA sequence was determined from Sprague-Dawley rats (Konno, 1998).

The present experiments have shown that the R-Y121B·DF cells proliferate in the D-phenylalanine medium and explicitly express DAO. Their medium does not contain serum or other growth-promoting substances. This would allow us to examine the regulation of DAO expression even by a small quantity of biologically active substances.

Acknowledgements

The authors thank Ms. S. Yachuda and Y. Shinozaki for preparing the culture media. They are grateful to Professor A. Niwa, Department of Microbiology, Dokkyo University School of Medicine, for his advice and encouragement throughout this research.

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Received March 15, 2000