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Loss of *Hin*dIII cleavage sites in the D-amino acid oxidase gene in some inbred strains of mice

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Summary. D-Amino acid oxidase cDNA was amplified by a polymerase chain reaction using RNA extracted from the mouse kidney. When digested with *Hin*dIII, the cDNAs of the BALB/c and ddY/DAO$^-$ mice were cleaved into two fragments whereas the cDNA of the ddY/DAO$^+$ mice was not. Sequencing revealed that nucleotide-471 of the cDNAs was G in the BALB/c and ddY/DAO$^-$ mice whereas it was substituted for C in the ddY/DAO$^+$ mice. This base substitution was the cause of the failure of the cleavage of the cDNA of the ddY/DAO$^+$ mice.

Examination of other strains of inbred mice showed that D-amino-acid oxidase cDNAs of A/J, AKR, C57BL/6, CD-1, CF#1, ICR, DBA/2, NZB and NZW mice were cleaved with *Hin*dIII into two fragments whereas those of C3H/He, CBA/J and NC mice were not. Genomic DNAs extracted from the mice of these 15 strains were digested with *Hin*dIII and hybridized with D-amino-acid oxidase cDNA. A 18.2-kb fragment hybridized with the probe in the C3H/He, CBA/J, ddY/DAO$^+$ and NC mice whereas two fragments of 12 kb and 6.2 kb hybridized in the other mice. These results are consistent with those of the cDNAs, confirming the loss of the *Hin*dIII cleavage site in the C3H/He, CBA/J, ddY/DAO$^+$ and NC mice. The Southern hybridization revealed a loss of a different *Hin*dIII cleavage site in the A/J, AKR, C57BL/6, DBA/2, ICR and NZB mice.

The substitution at nucleotide-471 should cause a substitution of an amino acid residue. However, this substitution did not affect catalytic activity of D-amino acid oxidase.

Keywords: Amino acids – D-Amino acid oxidase – *Hin*dIII – RFLP – Mouse – PCR

Introduction

D-Amino-acid oxidase (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 (Krebs, 1935). It has a wide range of substrate specificity. Many D-amino acids are oxidized but L-amino acids are not oxidized at a measurable rate. Almost all higher animals have this enzyme in their kidneys, livers, and brains. However, the physiological role of this enzyme has been enigmatic because D-amino acids are rare in higher animals (Meiser, 1965). Recent investigations have shown that one of the functions of D-amino acid oxidase is the metabolism of D-amino acids derived from intestinal bacteria (Konno and Yasumura, 1992; Konno et al., 1993). The high enzyme activity and its more than adequate existence to metabolize bacterial D-amino acids suggest that this enzyme has some other functions.

D-Amino acid oxidase has been conserved through evolution: it exists in fungi, invertebrates and vertebrates (Meister, 1965). The amino acid sequences of this enzyme were determined in the pig by Ronchi et al. (1982). Since then, the nucleotide sequences of D-amino acid oxidase cDNAs have been determined in several species. This enzyme consists of 347 amino acid residues in the human (Momoi et al., 1990), pig (Fukui et al., 1987) and rabbit (Momoi et al., 1988). Strangely, the mouse enzyme consists of 345 amino acid residues. The 25th and 173rd amino acid residues existing in the other three mammals are missing in the mouse (Tada et al., 1990). However, the homology of the amino acid sequences is high and about 80% of the amino acid residues are the same among these mammals.

The nucleotide sequences of cDNA encoding D-amino acid oxidase were determined in BALB/c mice. The cDNA has a coding region of 1,035 nucleotides between a 5′-untranslated region of 68 nucleotides and a 3′-untranslated region of 544 nucleotides. A cleavage site of the restriction endonuclease *Hin*dIII is present in the middle of the coding region. The D-amino acid oxidase gene is expressed in the kidney and brain but not in the heart, liver, lung, pancreas, spleen, submandibular gland, testis or thymus of the mouse (Tada et al., 1990).

Mutant ddY/DAO$^-$ mice lacking D-amino acid oxidase activity had D-amino-acid oxidase mRNA carrying a single-base substitution at nucleotide-451. This substitution leads to a change of an amino acid residue glycine-181 to arginine. The substitution was demonstrated to be the cause of the loss of enzyme activity (Sasaki et al., 1992).

While cloning D-amino acid oxidase cDNA of normal ddY/DAO$^+$ mice, we noticed that their cDNA was not cleaved with *Hin*dIII in contrast to the cDNAs of the BALB/c and ddY/DAO$^-$ mice. Therefore, in this study, we examined the validity of this observation and whether the loss of the *Hin*dIII cleavage site was unique to the ddY/DAO$^+$ mice.

Materials and methods

Mice

A/J Slc, AKR/N SLC and Slc: ICR mice were obtained from Japan SLC (Hamamatsu). C3H/HeN Jcl, C57BL/6N Jcl, CBA/J Jcl, DBA/2J Jcl and NC/Jic mice were purchased

from Nihon CLEA (Tokyo). Crj: CD-1, NZB/NCrj and NZW/NCrj mice were obtained from Japan Charles River (Yokohama). BALB/c, ddY/DAO$^+$ and ddY/DAO$^-$ mice were maintained in our laboratories. The kidneys of CF#1 mice were kindly supplied by Dr. T. Mizutani (The Institute of Physical and Chemical Research (RIKEN), Wako).

The mice were anesthetized with chloroform and their kidneys and livers were removed. These organs were either used immediately or kept frozen at −80°C until used.

Amplification of D-amino acid oxidase cDNA and HindIII digestion

Total RNA was extracted from the kidneys of mice of 15 strains according to the procedure of Chomczynski and Sacchi (1987). The first strand of cDNA was synthesized with a reverse transcriptase and an oligo (dT) primer using the Superscript Preamplification System (BRL, Gaithersburg, MD). The D-amino acid oxidase cDNA was selectively amplified by a polymerase chain reaction (PCR) in a 100 μl reaction mixture containing 4 μl of the first strand of cDNA solution, 0.2 μM each of the F1 and R1 primers (Fig. 1), and 2.5 units of *AmpliTaq* DNA polymerase using a Gene Amp PCR Reagent Kit (Perkin-Elmer Cetus, Norwalk, CT). The positions of these primers in the D-amino acid oxidase cDNA sequences are shown in Fig. 1 and described in detail in our previous paper (Sasaki et al., 1992). After an initial melting at 94°C for 1 min, 40 cycles of PCR (melting: 94°C, 1 min; annealing; 55°C, 1 min; extension: 72°C, 2 min) were carried out using a Thermal Sequencer (TRS-300, Iwaki Glass, Tokyo). A final extension was done at 72°C for 10 min.

Ten μl of the PCR product were mixed with 0.3 μl of *Hin*dIII (4.4 units) (Nippon Gene, Tokyo) and the mixture was incubated at 37°C for 2 h. The digested DNA fragments were electrophoresed through 1% agarose gel.

Molecular cloning and sequencing

Recombinant plasmid carrying an insert of D-amino-acid oxidase cDNA from ddY/DAO$^+$ mice was constructed according to the procedure used before in the construction of the plasmids carrying inserts of D-amino-acid oxidase cDNAs from BALB/c and ddY/DAO$^-$ mice (Sasaki et al., 1992). In brief, total RNA was extracted from the kidney of ddY/DAO$^+$ mice. The first strand of cDNA was synthesized using reverse transcriptase and an oligo (dT) primer. D-Amino-acid oxidase cDNA was selectively amplified by a nested PCR using the F1 and R1 primers for the first reaction and the F2 and R2 primers for the second reaction (Fig. 1). The amplified cDNA was digested with *Bam*HI (Nippon Gene) and *Xho*I (Nippon Gene), and inserted in the *Bam*HI/*Xho*I site of a pBluescript plasmid (Stratagene, La Jolla, CA). The recombinant plasmid (pnDAO (ddY+)) was grown in *Esherichia coli* strain XL1-Blue and the plasmid DNA was purified by standard methods (Sambrook et al., 1989). The nucleotide sequences of the insert were determined by the dideoxy chain termination method (Sanger et al., 1977) using Sequenase (US Biochemicals, Cleveland, OH), a Dye Terminator Sequencing Kit, and a DNA Sequencer (SQ 3000, Hitachi, Tokyo).

Southern hybridization

Genomic DNA was extracted from the livers or kidneys of mice according to the standard method (Sambrook et al., 1989). The DNAs (10 μg) were digested with 20 units of *Hin*dIII and electrophoresed through 0.9% agarose gel. The DNA fragments were transferred to nylon membranes (GeneScreen Plus, NEN Research Products, Boston, MA) according to the method of Southern (1975). The membranes were incubated at 45°C in a prehybridization buffer (1% SDS/2× SSC/10% dextran sulphate/50% formamide). 1× SSC is a solution of 0.15 M sodium chloride/0.015 M sodium citrate.

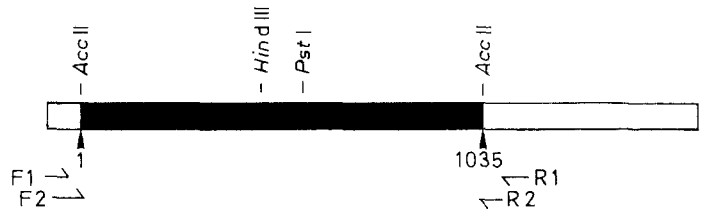

Fig. 1. Structure of cDNA encoding mouse D-amino acid oxidase and positions of primers used for PCR amplification. The open boxes show the 5'- and 3'-noncoding regions. The solid box shows the coding region for the D-amino acid oxidase protein. The arrows show primers used for PCR amplification. Base A of the initiation codon is numbered 1 and the bases are numbered downstream. This structure is based on the nucleotide sequence determined in the BALB/c mice by Tada et al. (1990)

The plasmid pnDAO (ddY/DAO+) was cleaved with *Bam*HI and *Xho*I, and the digested fragments were electrophoresed through a low-melting-temperature agarose gel. A gel slice containing the D-amino acid oxidase cDNA insert was cut out under ultraviolet illumination. The DNA was purified by the standard method (Sambrook et al., 1989).

The D-amino-acid oxidase cDNA (25 ng) was labeled with [α-^{32}P]dCTP (~110 TBq/mmol, Amersham, Backinghamshire) by the method of Feinberg and Vogelstein (1983) using a Random Primer DNA Labeling Kit (Takara, Kyoto). The labeled probe was mixed with carrier salmon sperm DNA, boiled for 10 min, cooled on ice, and added to the prehybridization buffer. The hybridization was carried out in a roller bottle at 45°C for about 16 h. The membranes were washed with 2× SSC for 10 min, twice with 2× SSC/1% SDS for 20 min each, and twice with 0.2× SSC/1% SDS at 45°C for 20 min each. The membranes were subjected to autoradiography at −80°C with intensifying screens.

Assay of D-amino acid oxidase activity

D-Amino acid oxidase activity was measured in kidney homogenates as described in our previous paper (Konno and Yasumura, 1983). Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard. The enzyme activity is expressed as the amount of D-alanine oxidized per min per milligram of protein.

Results

Figure 1 shows the structure of D-amino acid oxidase cDNA determined in BALB/c mice. It contains a coding region of 1,035 nucleotides between a 5′- and 3′-untranslated region.

The total RNA was extracted from the kidneys of BALB/c, ddY/DAO$^-$ and ddY/DAO$^+$ mice. The first strand of cDNA was synthesized using an oligo(dT) primer. Then, D-amino acid oxidase cDNA was amplified by PCR using the F1 and R1 primers flanking the coding region of the oxidase cDNA (Fig. 1). After the amplification, DNA fragments of about 1,100 base pairs (bp) were observed in agarose gel electrophoresis in all three strains (Fig. 2). This was the expected size for the coding region of D-amino acid oxidase cDNA. Digestion of the amplified DNAs with *Hin*dIII produced two fragments of about 610 bp and 500 bp in the BALB/c and ddY/DAO$^-$ mice (Fig. 2). This was expected because a *Hin*dIII cleavage site is present in the middle

of the coding region of D-amino acid oxidase cDNA (Fig. 1). However, the cDNA of the ddY/DAO$^+$ mice was not cleaved with *Hin*dIII (Fig. 2).

To examine why the D-amino acid oxidase cDNA of the ddY/DAO$^+$ mice was not cleaved with *Hin*dIII, we cloned the cDNA into a plasmid and determined its nucleotide sequences. Sequencing revealed that nucleotide-471 of the cDNA was G in the BALB/c and ddY/DAO$^-$ mice whereas it was substituted for C in the cDNA of the ddY/DAO$^+$ mice (Fig. 3). This G-to-C substitution abolished the *Hin*dIII recognition sequence (AAGCTT).

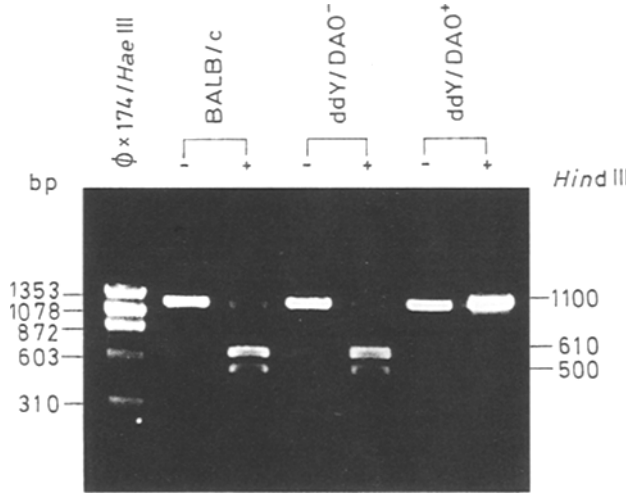

Fig. 2. PCR amplification of D-amino acid oxidase cDNA and digestion of the amplified cDNA with *Hin*dIII. D-Amino acid oxidase cDNA was amplified by PCR in BALB/c, ddY/DAO$^-$ and ddY/DAO$^+$ mice. The amplified cDNAs were digested with *Hin*dIII and electrophoresed through agarose gel together with the control, undigested cDNAs

We examined if the loss of the *Hin*dIII cleavage site was unique to the ddY/DAO$^+$ mice. D-Amino acid oxidase cDNAs were amplified in 12 inbred strains of mice and the amplified cDNAs were digested with *Hin*dIII. Figure 4 shows that the cDNAs of A/J, AKR, C57BL/6, CD-1, CF#1, DBA/2, ICR, NZB and NZW mice were cleaved into two fragments as observed in the BALB/c and ddY/DAO$^-$ mice whereas the cDNAs of the C3H/He, CBA/J and NC mice were not. These results suggest that the *Hin*dIII cleavage site is also lost in the C3H/He, CBA/J and NC mice.

Genomic DNA was extracted from the livers or kidneys of mice of the 15 strains. The DNAs were digested with *Hin*dIII and electrophoresed through agarose gel. The DNA fragments were transferred to membranes and hybridized with a D-amino acid oxidase cDNA probe containing the entire coding region. Figure 5 shows that the probe hybridized with three fragments in the A/J, AKR, C3H/He, C57BL/6, CBA/J, DBA/2, ddY/DAO$^+$, ICR, NC and NZB mice, four fragments in the BALB/c, CF#1, ddY/DAO$^-$ and NZW mice, and five fragments in the CD-1 mouse. The largest 18.2-kb fragment was observed only in the C3H/He, CBA/J, ddY/DAO$^+$ and NC mice. Instead of

this fragment, two hybridizing fragments of 12.0 and 6.2 kb were observed in the other 11 strains of mice (Fig. 5). These results indicate that the 18.2-kb fragment in the C3H/He, CBA/J, ddY/DAO$^+$ and NC mice was cleaved into 12.0- and 6.2-kb fragments in the other mice. These results are, therefore, consistent with the result that the *Hin*dIII cleavage site is lost in the cDNAs of the C3H/He, CBA/J, ddY/DAO$^+$ and NC mice.

The Southern hybridization revealed the loss of a different *Hin*dIII site in the genomic DNAs of six strains (Fig. 5). A 9.9-kb hybridizing fragment was observed in the A/J, AKR, C57BL/6, DBA/2, ICR and NZB mice. However, instead of this fragment, faint 8.2-kb and 1.7-kb fragments were observed in

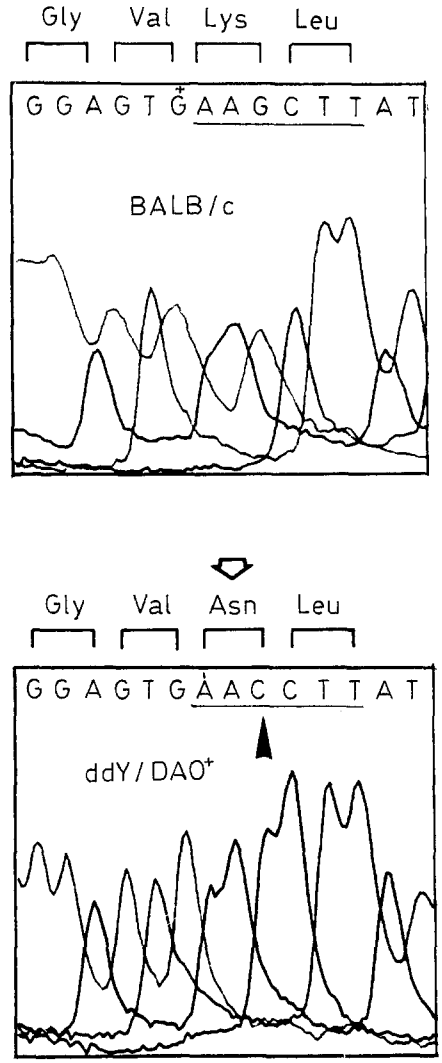

Fig. 3. Nucleotide sequence around the *Hin*dIII recognition site in D-amino acid oxidase cDNA. Upper panel: a BALB/c mouse. Lower panel: a ddY/DAO$^+$ mouse. The underline shows nucleotide sequence recognized with *Hin*dIII. The closed arrow head shows the substituted nucleotide in the ddY/DAO$^+$ mouse. The open arrow shows the resultant, substituted amino acid residue

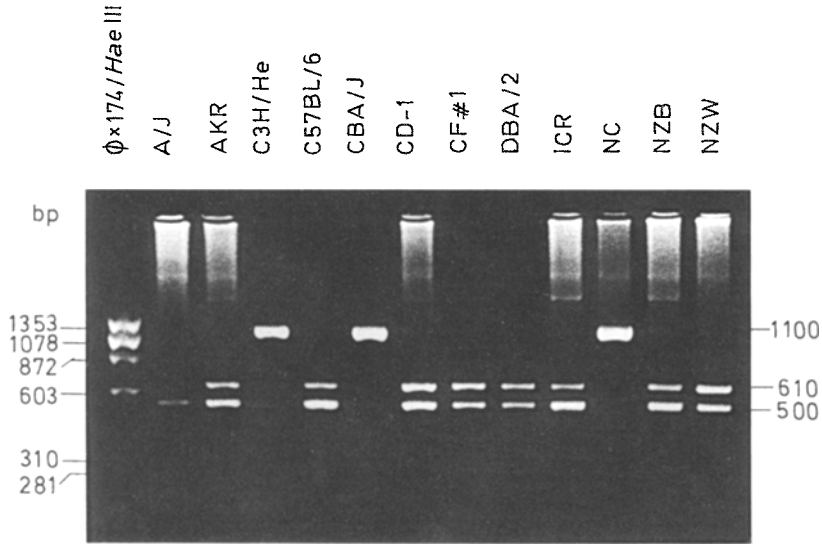

Fig. 4. Digestion of D-amino-acid oxidase cDNA with *Hin*dIII. D-Amino-acid oxidase cDNA was amplified by PCR in 12 mouse strains. The cDNAs were digested with *Hin*dIII and electrophoresed through agarose gel. The 610-bp fragment of the A/J mouse is not clearly shown in the picture, but did occur as 310-and 218-bp fragments of *Hae*III-digested φ (phi) X174 DNA

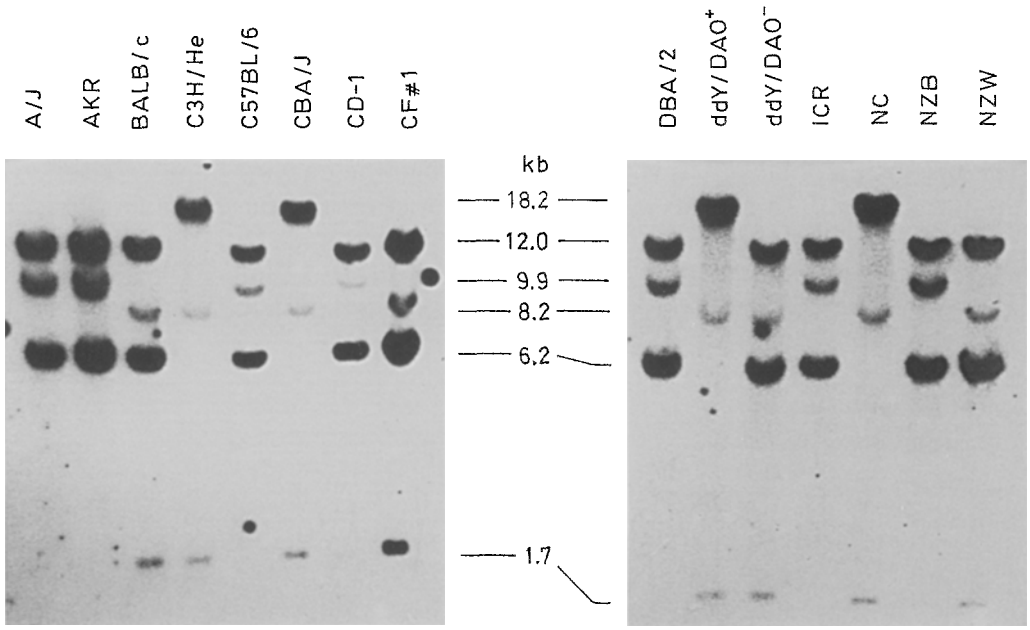

Fig. 5. Southern hybridization of *Hin*dIII-digested genomic DNA with a D-amino acid oxidase cDNA probe. Mouse genomic DNAs were digested with *Hin*dIII and electrophoresed through agarose gel. The DNA fragments were transferred to nylon membranes and hybridized with a D-amino acid oxidase cDNA probe prepared from a ddY/DAO$^+$ mouse. Some hybridizing fragments of the CD-1 strain were not clearly reproduced in the picture because they were vary faint

Table 1. D-Amino acid oxidase activity in various mouse strains

Strain	Activity* (nmol/min·mg protein)
BALB/c	10.6 ± 1.2 (6)
C3H/He	11.1 ± 1.4 (6)
C57BL/6	7.8 (1)
CBA/J	11.6 (1)
DBA/2	13.9 (1)
ddY/DAO$^+$	8.3 ± 0.9 (6)
NC	9.0 (1)

* The values shown in the BALB/c, C3H/He and ddY/DAO$^+$ mice represent the mean and standard deviation. The numbers in parentheses indicate the number of mice used. Other values show enzyme activity in a single mouse.

the BALB/c, C3H/He, CBA/J, CF#1, ddY/DAO$^+$, ddY/DAO$^-$, NC and NZW mice. This result is explained by the loss of the other *Hin*dIII cleavage site in the A/J, AKR, C57BL/6, DBA/2, ICR and NZB mice. In the CD-1 mouse, faint fragments of 9.9 kb, 8.2 kb and 1.7 kb were observed. This was most likely a result of incomplete digestion of the DNA with the restriction enzyme.

In contrast to the *Hin*dIII-digested DNAs, restriction fragment length polymorphism (RFLP) was not observed in the *Bam*HI-digested DNAs of these 15 strains (data not shown).

The substitution of G for C at nucleotide-471 should cause a substitution of the amino acid residue lysine-157 for asparagine in the D-amino acid oxidase molecule (Fig. 3). Since the substitution of the basic amino acid residue for the polar one was thought to change the enzyme configuration, D-amino acid oxidase activity was examined in several mouse strains. Table 1 shows that the C3H/He, CBA/J, ddY/DAO$^+$ and NC mice had similar levels of henzyme activity as the BALB/c, C57BL/6 and DBA/2 mice. Therefore, the substitution of lysine-157 for asparagine was concluded to have little effect on catalytic activity of D-amino acid oxidase.

Discussion

The D-amino acid oxidase gene was cleaved with *Hin*dIII into four fragments of 12.0, 8.2, 6.2 and 1.7 kb in the BALB/c, CF#1, ddY/DAO$^-$ and NZW mice (Fig. 5). Three cleavage sites of *Hin*dIII are therefore present inside the gene. In the C3H/He, CBA/J, ddY/DAO$^+$ and NC mice, the digestion of the genomic DNAs produced a 18.2-kb fragment instead of two fragments of 12.0 and 6.2 kb, indicating the loss of one of the *Hin*dIII cleavage sites. This *Hin*dIII cleavage site should be in an exon, because the D-amino acid oxidase cDNAs of these mice, different from those of the other strains of mice, were not cleaved with *Hin*dIII either (Figs. 2 and 4). The other two *Hin*dIII cleav-

age sites should be present in the introns. One of these sites are lost in the A/J, AKR, C57BL/6, DBA/2, ICR and NZB mice, because the digestion of their genomic DNAs with the restriction enzyme produced a 9.9-kb fragment instead of two fragments of 8.2 and 1.7 kb (Fig. 5).

The Southern hybridization indicated that the mouse D-amino acid oxidase gene which encodes the entire coding region is confined within a total of 28.1-kb *Hin*dIII fragments. The size of this gene is not different from that reported in the human. The human D-amino acid oxidase gene spans 20 kb (Fukui and Miyake, 1992).

One of the *Hin*dIII cleavage sites present in the D-amino acid oxidase gene is lost in the C3H/He, CBA/J, ddY/DAO$^+$ and NC mice. Among these mouse strains, the C3H/He and CBA/J strains have a common origin. They have originated from a cross between female Bagg albino mice and male DBA mice (Graff and Snell, 1969). Therefore, the loss of the *Hin*dIII cleavage site might have occurred before the C3H and CBA strains diverted. However, it is important to note that both BALB/c and DBA/2 strains which are descendants of the original Bagg albino and DBA strains, respectively, do have this *Hin*dIII cleavage site (Figs. 4 and 5). The ddY/DAO$^+$ and NC strains do not have the common origin of the C3H/He or CBA/J strains. The ddY mice were derived from dd mice, which had been imported to Japan from Germany before 1920 (Heston et al., 1964) and subsequently maintained in a closed colony. The ddY/DAO$^+$ and ddY/DAO$^-$ strains are inbred strains established from the ddY mice in 1983 (Konno and Yasumura, 1983). The NC mice were derived from Japanese fancy mice (Staats, 1980). It is presently unknown why the loss of the *Hin*dIII cleavage site has occurred commonly in these divergent strains.

The G-to-C substitution at nucleotide-471 should cause the substitution of lysine-157 for asparagine. This substitution did not change the catalytic activity of D-amino acid oxidase, because enzyme activity in the C3H/He, CBA/J, ddY/DAO$^+$ and NC mice was not different from that in the BALB/c, C57BL/6 and DBA/2 mice (Table 1). Our previous experiments also showed that the C3H/He, ddY/DAO$^+$ and NC mice had similar levels of D-amino acid oxidase activity as the BALB/c, C57BL/6 and DBA/2 mice (Konno and Yasumura, 1983, 1984). Amino acid residues from arginine-150 through lysine-157 and those from arginine-161 through serine-165 are conserved in the D-amino acid oxidases of mouse (Tada et al., 1990), rabbit (Momoi et al., 1990), pig (Fukui et al., 1987) and human (Momoi et al., 1988). Between these conserved sequences, three amino acid residues (158th–160th) vary among these mammals. Therefore, the change of lysine-157 to asparagine may be tolerable for the catalytic function. (Since the 25th amino acid residue of D-amino acid oxidase of rabbit, pig and human is missing in the mouse enzyme, the nth amino acid residue in the mouse enzyme corresponds to the $(n + 1)$th amino acid residue in the other mammalian enzymes.) However, since D-amino acid oxidase activity was only determined under a single set of assay conditions, the enzymes of the C3H/He, CBA/J, ddY/DAO$^+$ and NC mice may show different catalytic activity from the enzymes of the other strains of mice under other assay conditions.

The *Dao-1* locus for D-amino acid oxidase is mapped 19.8 cM distal to the *Pgm-1* locus for phosphoglucomutase-1 which is the most widely used biochemical-genetic marker on mouse chromosome 5. (Hilgers and Arends, 1985; Konno et al., 1989). The *Hin*dIII cleavage sites within this gene are lost in some of the widely used inbred strains of mice. The resultant RFLP may be used as a useful marker for gene mapping, linkage analysis, RFLP analysis and genome analysis.

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