

Mutant rat strain lacking D-amino-acid oxidase

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Abstract D-Amino-acid oxidase (DAO) is known to be associated with schizophrenia. Since the expression of DAO gene had been reported to be very low in LEA rats, we examined LEA/SENDAI rats in detail. These rats did not have DAO activity, enzyme protein or mRNA encoding this enzyme. Sequencing of the 5'-upstream region of the DAO gene revealed the deletion of one triplet in the 15 TAA repeats approximately 700-bp upstream of the transcription start point. A 1.3-kb upstream fragment containing the TAA repeats and the transcription start point was inserted into a reporter vector and was transfected into COS-1, NRK-52E and CCL-PK1 cells. Although the fragments containing 15 or 14 repeats had high promoter activity, the fragment containing 13 repeats had very weak activity. Electrophoretic mobility-shift assays showed that the nuclear extracts from COS-1 and COS-7 cells had proteins that bound to the oligonucleotides containing the TAA repeats. These results suggest that the TAA repeats

are important for expression of the DAO gene. The LEA/SENDAI rats lacking DAO would be a useful tool for the investigations aimed at the elucidation of the relationships between this flavoenzyme and schizophrenia.

Keywords D-Amino-acid oxidase · Rat mutant · Promoter · Triplet repeats · Schizophrenia

Introduction

D-Amino-acid oxidase (DAO, EC 1.4.3.3) catalyzes the oxidative deamination of D-amino acids, stereoisomers of the naturally occurring L-amino acids. During the reaction, the corresponding 2-oxo acids, hydrogen peroxide and ammonia are produced (Krebs 1935). DAO is present in a wide variety of organisms from yeasts to humans (Meister 1965). In higher animals, it is mainly present in the kidney, liver and brain. However, since D-amino acids have been considered to be rare in eukaryotes, the physiological role of DAO has been enigmatic for a long time (Meister 1965; see Brosnan 2001). However, improvements in analytical methods have led to the discovery that D-amino acids are widely distributed even in mammals. It has also been revealed that some D-amino acids have important physiological functions. Accordingly, the physiological roles of DAO have been gradually clarified.

Hashimoto et al. (1992) and Nagata et al. (1994) found that substantial quantities of D-serine were present in the rat and mouse, respectively. Human brains also contain high levels of D-serine (Hashimoto et al. 1993b; Chouinard et al. 1993). Hashimoto et al. (1993a) noticed that the distribution of D-serine was parallel to that of *N*-methyl-D-aspartate (NMDA)-subtype glutamate receptors. They suggested that

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D-serine was a ligand for the strychnine-insensitive “glycine” site of the NMDA receptors. Using an antibody against D-serine, Schell et al. (1995) clearly demonstrated the distribution of D-serine in the rat brain. The cerebral cortex, hippocampus, anterior olfactory nucleus, olfactory tubercle, and amygdale had high levels of D-serine. This distribution pattern was similar to that of NMDA receptors and inversely correlated with that of DAO. D-Serine was found to be present in protoplasmic astrocytes in the gray matter. In glial cultures of rat cerebral cortex, D-serine was present abundantly in type 2 astrocytes and was released from these cells when they were stimulated by agonists for non-NMDA receptors such as kainate. Later, Schell et al. (1997) showed that the distribution pattern of D-serine closely resembles that of the NR2A/B subunits of NMDA receptors.

It is known that simultaneous binding of glutamate and a co-agonist to the receptor is required for the full activation of NMDA receptor. Using cloned NMDA receptors expressed on the surface of oocytes, Matsui et al. (1995) showed that D-serine was more potent than glycine in the activation of these NMDA receptors. By regulating the levels of D-serine, DAO is considered to modulate NMDA neurotransmission (Mothet et al. 2000).

Hypofunction of NMDA receptors is recognized as one of the most plausible causes of schizophrenia. Chumakov et al. (2002) found that DAO was associated with schizophrenia. DAO itself had a weak association with schizophrenia but the combination of DAO and the product of *G72* (a novel gene associated with schizophrenia) have a synergistic effect on this disease. Since they found the *G72* product activated DAO, they consider that this combination decreases the level of D-serine, resulting in hypofunction of the NMDA receptors. Schumacher et al. (2004) and Liu et al. (2004) confirmed the association of DAO with schizophrenia.

We have established a mutant mouse strain lacking DAO. These mice showed altered responses that were mediated by the NMDA receptors (Wake et al. 2001; Hashimoto et al. 2005; Maekawa et al. 2005; Almond et al. 2006). However, mouse brains are so small that detailed studies in discrete areas of the brain were very difficult. Since rats are also widely used animals for these studies owing to their suitable size, we searched for mutant rats having disorders in DAO. We noticed that the expression of the DAO gene was considerably lower in wild-type Long–Evans agouti (LEA) rats than the Long–Evans cinnamon (LEC) rats, an animal model for Wilson disease characterized by a disorder in copper metabolism (Klein et al. 2003). Therefore, we examined LEA/SENDAI rats in detail, a subline of the LEA strain, to determine whether they had a DAO disorder.

Materials and methods

Assay for DAO activity

Male F344, Wistar and Sprague–Dawley (SD) rats were purchased from Charles River Japan (Yokohama, Japan). LEA/SENDAI rats were maintained at the Institute for Animal Experimentation, Tohoku University Graduate School of Medicine. Animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of Dokkyo Medical University, Mibu, Tochigi, Japan.

D-Amino-acid oxidase activity in kidney homogenates was determined using D-alanine as a substrate by the method of Watanabe et al. (1978) as described before (Konno et al. 1997).

Western blot analysis

Western blot analysis was carried out as described (Konno et al. 1997). Briefly, rat kidney homogenates were separated by electrophoresis on 10% SDS-polyacrylamide gels. Hog kidney DAO (Boehringer Mannheim, Germany) was used as a control. Precision protein standards (Bio-Rad Laboratories, Hercules, CA, USA) were used as a molecular size marker. The proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Immobilon-P, Millipore, Bedford, MA, USA). The membrane was incubated in a solution containing rabbit anti-hog kidney DAO IgG. The membrane was further treated with donkey antibodies against rabbit IgG linked to horseradish peroxidase (Amersham Biosciences, Buckinghamshire, UK). The immune complex was detected using ECL Western blotting reagents (Amersham). This system was able to detect at least 0.5 µg of hog kidney DAO.

Determination of specific mRNAs

Total RNA was extracted from the kidneys of SD and LEA/SENDAI rats using an RNA extraction reagent (Isogen, Nippon Gene, Tokyo, Japan). The first strand of cDNA was synthesized using the total RNA (2 µg), an oligo(dT) primer and reverse transcriptase (Superscript Preamplification System, Invitrogen, Carlsbad, CA, USA). These cDNAs were used as templates to amplify a DAO cDNA fragment and a serine racemase cDNA fragment. A primer pair (5'-CCCCAGGCCATTTTCTCTA-3' and 5'-CCCATCACTCCATCACTACAA-3') and a pair (5'-CTGTCTACAGCCCTCTGCAT-3' and 5'-CCAAAGTTATG GATGACCTCTG-3') were used to amplify a 1,327-bp and a 890-bp fragment of DAO cDNA, respectively. A primer pair (5'-GATAGCGGGACAAGGGACAAT-3' and 5'-AGGAAGATGGAAAACAAACT-3') was used to amplify a

583-bp fragment of serine racemase cDNA. These primers were designed from the cDNA sequence of rat DAO (Konno 1998) and rat serine racemase (Konno 2003). The PCR conditions were denaturation at 95°C for 9 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 80 s, and a final extension at 72°C for 5 min. AmpliTaq Gold (PE Applied Biosystems, Foster City, CA, USA) was used for this reaction. The PCR products were separated by electrophoresis on 1% agarose gels and visualized with ethidium bromide staining.

Genome walking

Genomic DNA was extracted from the kidneys of LEA and SD rats using a DNeasy Tissue Kit (Qiagen, Hilden, Germany). To amplify the 5'-upstream region of the DAO gene, DNA Walking SpeedUp Kit (Seegene, Seoul, South Korea) was used. Nested PCR using an ACP-4 primer supplied by the manufacturer and a DAO-specific primer (5'-CATCTCTCAGGGGGACATTTC-3'), followed by a pair of an ACP-N primer supplied by the manufacturer and a DAO-specific primer (5'-TAGACCTCTAACTGGGACACG-3') amplified an approximately 1-kb fragment. It was ligated to a TA vector (pCR2.1-TOPO, Invitrogen) and cloned in *Escherichia coli*. The plasmid was amplified and purified with a Plasmid Mini Kit (Qiagen). The nucleotide sequence of the insert was determined using a DNA sequencing kit (BigDye Terminator Cycle Sequencing Ready Reaction; Applied Biosystems, Foster City, CA, USA) and a DNA sequencer (ABI PRISM 310 Genetic Analyzer, Applied Biosystems). Using the sequence information obtained, further upstream sequences were determined until approximately 1.8-kb sequences upstream of the transcription start point were determined.

Determination of transcriptional start point

A Cap Site cDNA library (rat kidney) was purchased from Nippon Gene (Tokyo, Japan). The cap site region of the DAO cDNA was amplified by nested PCR following the procedures specified by the supplier. Briefly, the first PCR was carried out using a 1RC primer supplied by the manufacturer and the DAO-specific primer (5'-CCCACACC CCGGTGCAGTTGA-3') and the second PCR was performed using a 2RC primer supplied by the manufacturer and the DAO-specific primer (5'-TTCCAGAAAGGGTC CGGGACT-3'). AmpliTaq Gold was used for this amplification. The amplified fragment (approximately 450 bp) was ligated to the pCR2.1-TOPO vector and cloned in *E. coli*. The insert was sequenced.

Construction of reporter vectors

The 5'-upstream region of the DAO gene was amplified by nested PCR. The first PCR was done using a primer pair (5'-GACCAGGTTTACAGCATCAGG-3' and 5'-CAAAC CCATCAATAGCATAAT-3') and the genomic DNAs from SD and LEA/SENDAI rats as a template. The second PCR was performed using a primer pair (5'-TAAATTT CAACCTCGCTGCTC-3' and 5'-CATCTCTCAGGGGGA CATTTC-3'). Z-Taq DNA polymerase (Takara, Ohtsu, Japan) was used for these reactions. This nested PCR amplified a 1,561-bp fragment (nucleotides -1,332 to +229).

The amplified fragment was ligated to a pCR2.1-TOPO vector and cloned in *E. coli*. The plasmid was amplified and purified using a QIAprep Spin Miniprep Kit (Qiagen). The inserted 5'-upstream region of the DAO gene was excised from the plasmid with *SacI* and *XhoI* (Nippon Gene). It was gel-purified using a Sephaglas BandPrep Kit (Amersham Pharmacia Biotech) and was ligated to the *SacI/XhoI* sites of a pGL3-Basic vector (Promega, Madison, WI, USA) upstream of the firefly luciferase gene. The construct was cloned in *E. coli* and purified using a Plasmid Midi Kit (Qiagen).

Reporter assay

COS-1 cells were obtained from Dr. J. Yanagisawa (University of Tsukuba). A rat kidney epithelial-like cell line (NRK-52E) and a pig kidney epithelial-like cell line (CCL-PK1) were obtained from Health Science Research Resources Bank (Resource Nos. IF050480 and JCRB0060, respectively; <http://www.jhsf.or.jp>).

The cells were propagated in Dulbecco's modified Eagle's medium (GIBCO, Invitrogen) supplemented with 5% newborn calf serum (GIBCO) and antibiotics (penicillin, 100 U/ml, and streptomycin, 100 µg/ml, GIBCO). They were seeded at 1.8×10^5 cells/35-mm dish and were incubated at 37°C under an atmosphere of 5% CO₂ and 95% air. The following day, the pGL3-DAO construct (1.5 µg) and pRL-TK (*Renilla* luciferase expression plasmid, 0.05 µg, Promega) were co-transfected into the cells using the PolyFect Transfection Reagent (Qiagen) according to the procedures specified by the manufacturer. After 2 days, the cells were washed with phosphate-buffered saline (pH 7.4) and lysed with Passive Lysis Buffer (Promega). The lysate was collected and stored at -80°C until the luciferase assay.

The cell lysates were assayed for firefly luciferase activity and then *Renilla* luciferase activity according to the procedures specified by the manufacturer (Dual-Luciferase Reporter Assay System, Promega). A luminometer (Lumat

LB 9501, EG & G Berthold, Bad Wildbad, Germany) was used to measure the luminescence. The ratio (firefly luciferase activity/*Renilla* luciferase activity) was calculated and used for comparison. A Student's *t* test was used to calculate the statistical significance.

Electrophoresis mobility-shift assay

Complementary oligonucleotides, 5'-GGTGTATTAATAA TAATAATAATAGAAAAGGCT-3' (5 TAA repeats) and 5'-AGCCTTTTCTATTATTATTATTATTAATACACC-3', were synthesized at Sigma Genosis (Ishikari, Japan). 1–3 Biotinylated ribonucleotides were incorporated onto the 3'-ends of these oligonucleotides using a Biotin 3'-End DNA Labeling Kit (Pierce, Rockford, IL, USA). Equal amounts of the biotinylated oligonucleotides were annealed to form double-stranded target oligonucleotides.

For the electrophoresis mobility-shift assay, a LightShift Chemiluminescent EMSA Kit (Pierce) was used. The annealed, biotin-labeled oligonucleotides (25 fmol) were incubated with a nuclear protein extract from COS-7 cells (10 µg, Active Motif, Carlsbad, CA, USA) in a 20 µl of binding reaction containing 1 µg poly(dI-dC) and the binding buffer. When necessary, unlabeled oligonucleotides (4 pmol) were added as a competitor. The binding reactions were incubated at room temperature for 20 min. The reaction was terminated by the addition of 5 µl of 5× loading buffer.

The 20 µl binding reactions were separated by electrophoresis on 6% polyacrylamide gels (Tefco, Tokyo, Japan) at 100 V about 1.2 h. The nucleotides were electrophoretically transferred to a membrane (Hybond-N+, Amersham Pharmacia Biotech) at 380 mA for 30 min. The nucleotides were cross-linked at an optimal cross-link condition in a UV-linker (FS-800, Funakoshi, Tokyo, Japan).

The membrane was treated with streptavidin–horseradish peroxidase conjugate, followed by the luminol/peroxide solution. The membrane was exposed to a Polaroid film in an ECL mini-camera (Amersham Pharmacia Biotech).

Results

DAO activity

When Klein et al. (2003) compared gene expression between the LEA and LEC rats using DNA microarrays, they found that the expression of DAO was much lower in the LEA rats than in the LEC rats. Therefore, we examined DAO activity in the LEA/SENDAI rats. Since this enzyme is most abundant in the kidney, kidney homogenates were used. The enzyme assays indicated that SD and F344 rats

Table 1 DAO activity in the rat kidney

Strain	Activity (nmol/min mg protein)
SD	10.6 ± 1.7 (5)
F344	8.2 ± 1.5 (5)
LEA/SENDAI	<0.1 (5)

The enzyme activity of the LEA/SENDAI rats was below the detection limit of our assay. The values represent the mean ± SE. The numbers in the parentheses indicate the number of rats used

had DAO activity whereas LEA/SENDAI rats did not (Table 1).

DAO proteins

It was examined whether DAO proteins were present in the kidneys of the LEA/SENDAI rats. Western blot analysis using anti-hog DAO IgG showed that DAO proteins were present in the kidney homogenate of a SD rat but not in that of a LEA/SENDAI rat (Fig. 1). It is not likely that the LEA/SENDAI rat had an altered structure of DAO molecules which did not react with the primary antibody because the employed antibody could recognize DAO molecules of other species such as the mouse and hog.

DAO mRNA

Then we examined whether mRNA for DAO was present in the kidney of LEA/SENDAI rats. Total RNA extracted from the kidneys of LEA/SENDAI rats and SD rats was

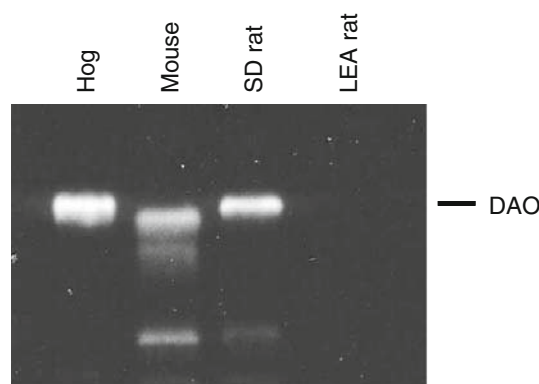


Fig. 1 The absence of DAO proteins in LEA/SENDAI rat. Kidney homogenates from a LEA/SENDAI and a SD rat and of a mouse together with purified hog kidney DAO were separated by electrophoresis. After the proteins were transferred to a membrane, it was treated with rabbit anti-hog DAO IgG, followed by a horseradish peroxidase-linked donkey antibody against rabbit IgG. The immune complex was detected by a chemiluminescence production. Hog DAO (2.5 ng), and kidney homogenates of a mouse (14.8 µg), a SD rat (1.3 µg) and a LEA rat (4.9 µg) were loaded. Precision protein standards were used as a molecular size marker. The molecular weight of hog DAO is 39,335 Da

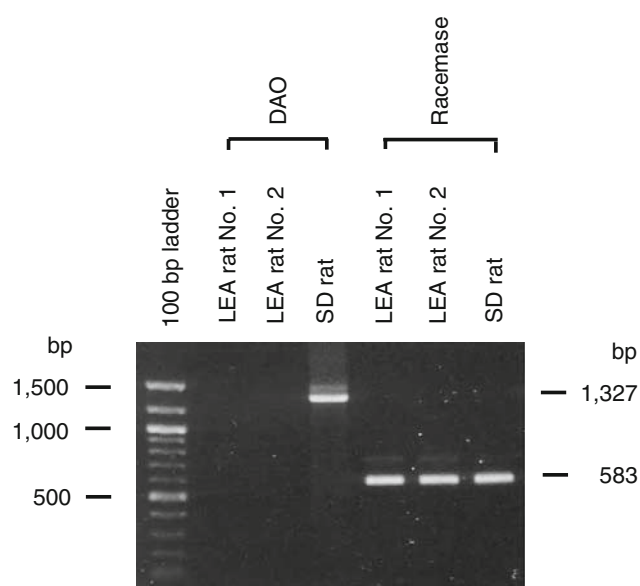


Fig. 2 The absence of DAO mRNA in LEA/SENDAI rats. The first strand of cDNA was synthesized using total RNA extracted from the kidneys of a SD rat and two individuals of LEA/SENDAI rats. These cDNAs were used as templates for PCR-amplification of DAO and serine racemase cDNAs. A 1,327-bp fragment and a 583-bp fragment were expected for the DAO cDNA and serine racemase cDNA, respectively. DAO mRNA was not present in two LEA/SENDAI rats although serine racemase mRNA was present. Both mRNAs were present in a SD rat

reverse transcribed. PCR amplified a DAO cDNA fragment of 1,327 bp in SD rats but not in LEA/SENDAI rats (Fig. 2). This was not due to a defect in the reverse-transcription or PCR amplification because a serine racemase cDNA fragment of the expected size was amplified in both LEA/SENDAI and SD rats (Fig. 2). PCR using a different pair of primers also amplified an expected 890-bp fragment of DAO cDNA in the SD rats but not in the LEA/SENDAI rats (data not shown). Therefore, it was concluded that DAO mRNA was not present in the LEA/SENDAI rats.

Genome walking

The results suggested that transcription of the DAO gene did not occur in the LEA/SENDAI rats. Then, the 5'-upstream sequence of the DAO gene was examined. Genome walking was performed until a 1.8 kb-upstream region of the DAO gene was sequenced in the LEA/SENDAI and SD rats.

TAA triplet repeats were present approximately 700-bp upstream from the transcription start point (Fig. 3). It was found that the TAA triplets were repeated 15 times in the SD rats whereas they were repeated 14 times in the LEA/SENDAI rats. No other difference was detected in the sequence up to 1.8 kb from the transcription start point between these strains of rats.

Determination of transcription start point

Since 700-bp upstream seemed a little far from the ordinary transcription start point to be recognized as the promoter region, we suspected that the real transcription start point might be present further upstream of the initially reported transcriptional start point, which had been previously determined with rapid amplification of the 5'-cDNA end (5'-RACE) (Konno 1998). Then, a Cap Site cDNA library made from the rat kidney, which was advertised to be enriched in transcription start points of genes, was purchased. Nested PCR using anchor primers and DAO-specific primers amplified the fragments containing the expected cap site of the DAO gene. The fragments were sequenced. The real transcription start point was found to be only five nucleotides upstream of the initially reported transcription start point (Fig. 3). Then, the first nucleotide G was numbered +1 and the other nucleotides were numbered accordingly. Consequently, the TAA repeats were determined to be located at -721 to -677.

Reporter assay

Since no difference was detected in the 5'-upstream sequence between the LEA/SENDAI and SD rats except for the deletion of one TAA triplet in the 15 TAA repeats, this deletion was suspected to be associated with the defect in the transcription of the DAO gene in the LEA/SENDAI rats. To examine this possibility, an approximately 1.3-kb fragment containing the TAA repeats and the transcription start point (-1,087 to +229) was amplified from the LEA/SENDAI and SD rats by nested PCR. It was inserted upstream of the firefly luciferase gene in the reporter vector. The constructed reporter vectors were transfected into COS-1 cells together with a control vector containing *Renilla* luciferase. After 2 days, luciferase activity in the transfected cells was determined.

The 1.3-kb fragment containing the normal 15 TAA repeats which was prepared from a SD rat had a high promoter activity (Fig. 4a). Against the expectation, the fragment containing 14 TAA repeats prepared from a LEA/SENDAI rat also had a high promoter activity. However, a fragment containing 13 TAA repeats, which was accidentally produced during the process of the PCR amplification and the construction of the reporter vector, was found to have very weak promoter activity (Fig. 4a).

The fragment containing 14 TAA repeats which derived from the LEA/SENDAI rat had promoter activity. We suspected that this might be due to species difference because the DNA fragment was derived from the rat while the COS-1 cells were from the African green monkey. Then, the reporter vectors were transfected to NRK-52E cells which were derived from the rat kidney. However, the

Fig. 3 Nucleotide sequence of the 5'-upstream region of the DAO gene. The sequence was determined by DNA walking. The first untranslated exon is boxed. The transcriptional start point was revealed by the CapSite Hunting and is numbered +1. The other nucleotides are numbered accordingly. The TAA repeats located at -721 to -677 are also boxed. The TAA sequence was repeated 15 times in the SD rat whereas it was repeated 14 times in the LEA/SENDAI rat

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-1100 TTTTTTCAGAGCTGGGGACCGAACCCAGGGCCTTGCGCTTCCTAGGTAAG
-1050 TGCTCTACCACTGAGCTAAATCCCCAGCCCCCAACAATAAGTTTTTAAGA
-1000 AAAACCTGTGTAATCTATAACATGGCGAGCAGAAGGACGCTGCAGGGAGG
-950 CTTTTATGTATTCAATTAATGGTAGAACTCTAAAGGGGAAAAATGCAGA
-900 TTAATAATAATTTTGAGGGCTGGAGAGATGGCTCAGCAGTTAAGAGCACT
-850 GACTGCTCTTCCAGAGGTCCTGAGTTTAATCCCAGCACCATGGTGGC
-800 TCACAGACATCTGTGATGAGATCTGGCGCCCTCTTCTGGCCTGCAGGCAT
-750 ACATTTCAGGGAGAAAGCTGTATGGTGTATTAATAATAATAATAATAATA
-700 TAATAATAATAATAATAATAATAATAGAAAAGGCTATGGCTGTCCCTCAG
-650 AGTCACAGTGCCTGCCTAGTGTGCTTGTGGCCCTGGGCTCTCTCCCTGT
-600 TACACGTGCTTCCTTCATTGTGGTGGATGTTGCTGAGGTAGAAGACTACA
-550 CCATCACAGTATTCTCCTCTGTTTCAGCAGATGTGTTTTGGTCTCACCGTG
-500 TCCCAGTTAGAGGTCTAGCCTTGGCAGAAGAAGGACCTGGAGTCCTGCTG
-450 CTTTCGTCTCTGGGATACTGAAGCAAAGACTACACATCCTCCCAGCCCAG
-400 GCAGGCGTCCCTTCTCAGCCTCATCTGTCTAGCGTCCCAGAACAGCAGTG
-350 AGCTAGCTGGCTGGCCGGAAGGGCCTGTCCTCTGCCTTTGGCCACAGT
-300 GGCCAGGGAATGGTCCTCCCCTCTGCTGGGGTGCAGCAATGGGGGGGA
-250 GGCTCATCTCCAGCTGGCTTGGGCCTTAGAAACCCCTCCTTCCCCACCC
-200 CCCAGGCCTCTTCGCAGGGGCTTGGGATCAGCCAGAAAAGTCAGGCAGCT
-150 CACTAGAGGGAGGAAACAGAAGGTCCCGTGGCCAGACTGAGTCCTGCTCC
-100 TGGAGAAGTGCAGTGCCAGCCCCACCCCTTGCCCTGTGCCCCCTCCACT
-50  GCCGAAGACTTTGACCTGGCGTTCCAGTCCTAGCTGGTGGGCAGAGGGCT
  1  GAAGTCAACGCAGCCCAGAATCAGGAGCAATCCTGCTGGAGACAGGTTCC
  51  TGCACCCCCAGGCCATTTTCTCTAGACACTTGGCACCAGCGTAAGTAA
 101  AGGGGACAGAAACAACATAGTGTCTTCCCAGTGTCTCTCCATGCCAAC
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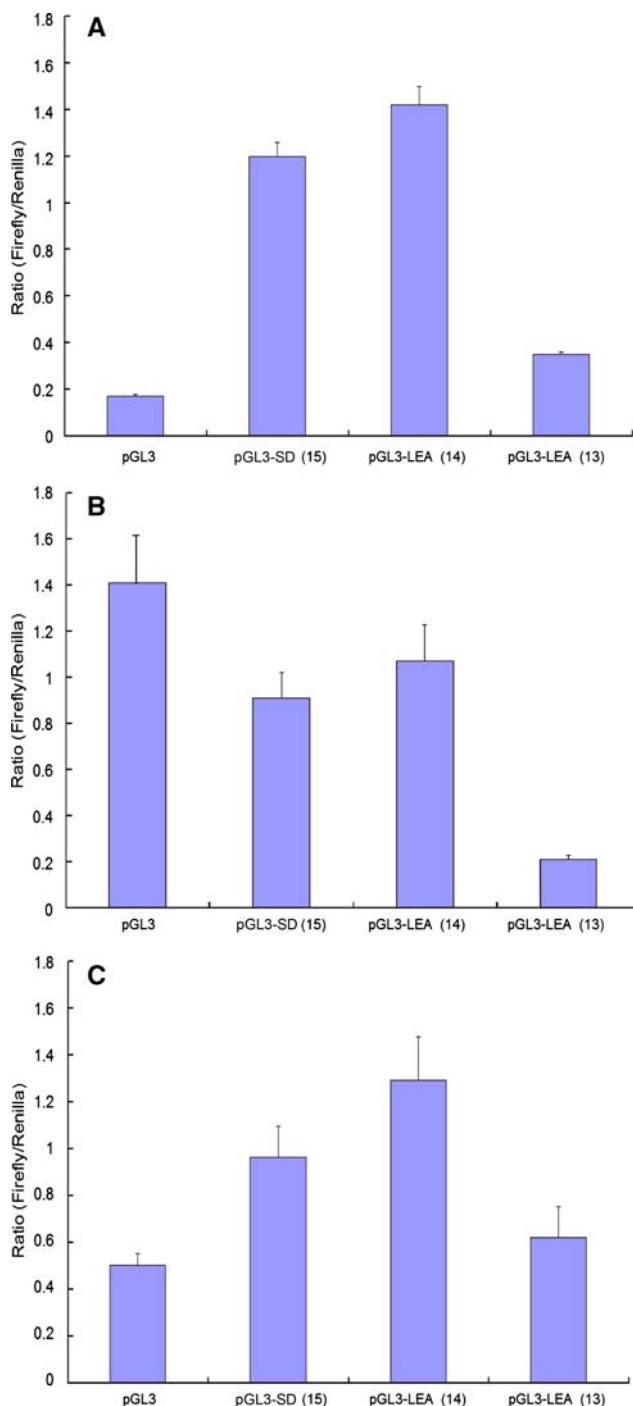
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results were basically the same, though there was a slight difference (Fig. 4b). The vector containing 14 TAA repeats as well as that containing 15 repeats conferred the luciferase activity on the NRK-52E cells, whereas the vector containing 13 TAA repeats did not. Strangely, the basic pGL3 vector without the promoter sequence conferred high luciferase activity on the NRK-52E cells. The reason was not clear. These reporter vectors were further tested on CCL-PK1 cells which were derived from the pig kidney. However, the results were quite similar to those observed in the COS-1 cells, though the baseline was higher in the CCL-PK1 cells (Fig. 4c). Therefore, it was certain that the

fragment containing 14 TAA repeats had promoter activity whereas that containing 13 repeats did not.

Electrophoretic mobility-shift assay

Using electrophoretic mobility-shift assays, we examined whether there were any proteins in the nuclei that bound to the TAA repeat region. An oligonucleotide in which 5 TAA repeats was connected to 7 nucleotides of the 5'-flanking and 11 nucleotides of the 3'-flanking sequences (5'-GGTGT ATTAATAATAATAATAATAGAAAAGGCT-3') and its complementary oligonucleotide were synthesized. They



were biotin-labeled at their 3'-ends and annealed. They were incubated with a nuclear extract premade from COS-7 cells and were separated by electrophoresis. Their mobility was retarded compared with the unbound oligonucleotides (Fig. 5, the left lane and the middle lane). The retardation was prevented with an excess amount of non-labeled oligonucleotides (Fig. 5, the right lane). The same results were obtained when a nuclear extract, which was prepared from COS-1 cells in our laboratory, was employed (data not

◀ **Fig. 4** Promoter activity of the 5'-upstream region of the DAO gene. The 1.3-kb fragment containing the TAA repeats and the transcription start point of the DAO gene from a SD rat (nucleotides -1,087 to +229) and LEA rats (nucleotides -1,084 to +229 and -1,081 to +229) were inserted into the upstream of the firefly luciferase gene of a pGL3 vector. The construct was transfected into COS-1 cells (a) NRK-52E cells (b) and CCL-PK1 cells (c) with the control pRL-TK vector carrying the *Renilla* luciferase gene. Two days after the transfection, the cells were harvested and examined for luciferase activity. For normalization, a ratio (firefly luciferase activity/*Renilla* luciferase activity) was calculated. pGL3 is a basic vector without a promoter sequence. pGL3-SD (15) indicates the construct that had the upstream sequence of the DAO gene derived from the SD rat. It had 15 TAA repeats. pGL3-LEA (14) indicates the construct that had the upstream sequence of the DAO gene derived from the LEA rat. It had 14 TAA repeats. pGL3-LEA (13) indicates the construct that had the upstream sequence of the DAO gene derived from the LEA rat. It had 13 TAA repeats. The reporter assays were independently carried out five times. The promoter activity of pGL3-LEA (13) was significantly lower than that of pGL3-SD (15) and pGL3-LEA (14)

shown). Therefore, it was clear that there were some proteins in the nuclei that bound to the TAA repeat region.

Discussion

The LEA/SENDAI rats did not have DAO activity and the DAO proteins. This was because DAO mRNA was not present in these rats. These results were in accordance with the finding that the expression of the DAO gene was extremely low in the LEA rats compared with LEC rats in the DNA microarray experiments (Klein et al. 2003). Consequently, LEA/SENDAI rats were suspected to have a defect in the transcription of the DAO gene. Sequencing of the 5'-upstream region of the DAO gene revealed that the LEA/SENDAI rats had 14 repeats of TAA triplet whereas the normal SD rats had 15 repeats located at -721 to -677 from the transcription start point. Since no other difference was detected between the LEA/SENDAI and SD rats in the 5'-upstream sequences up to 1.8-kb from the transcription initiation site, we considered that the deletion of one repeat unit would be responsible for the defect in the transcription of the DAO gene in the LEA/SENDAI rats.

The 5'-upstream sequence containing 14 TAA repeats was expected to lack promoter activity. However, the reporter assay indicated that the 5'-upstream fragment containing 14 repeats did have promoter activity as high as that containing 15 repeats (Fig. 4a). These unexpected results were thought to be a reflection of incompatibility due to the species difference because the fragment was derived from the rat genome while the transfected COS-1 cells were from the monkey. However, it was not the case. The fragment containing the 14 repeats had the promoter activity in the NRK-52E cells as well (Fig. 4b), a cell line derived from the rat kidney. The same results were obtained in CCL-PK1

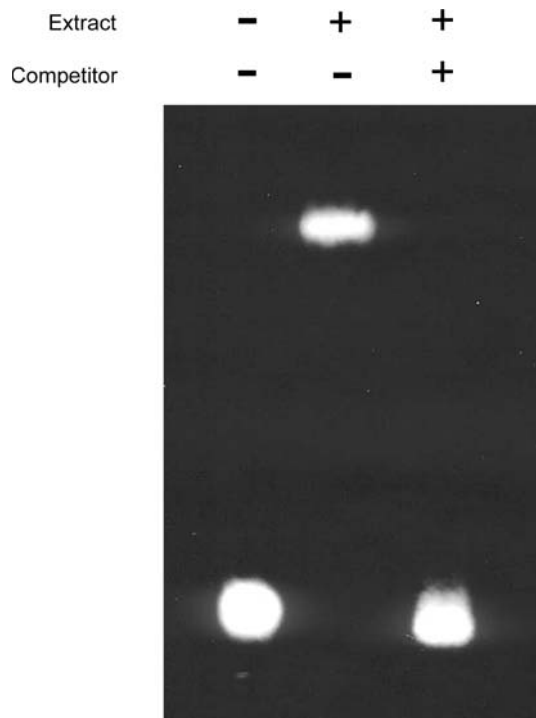


Fig. 5 Electrophoresis mobility-shift assay. Biotin-labeled double-stranded oligonucleotides (5'-GGTGTATTAATAATAATAATAA TAGAAAAGGCT-3') were incubated with a nuclear protein extract from COS-7 cells. The mixture was separated by electrophoresis and transferred to a nylon membrane. The membrane was treated with streptavidin-horseradish peroxidase conjugates. The positions of the oligonucleotides were detected by the production of chemiluminescence. *Left lane:* the nuclear protein extract was not added to the binding reaction. *Middle lane:* the nuclear protein extract was added to the binding reaction. *Right lane:* an excess amount of the non-labeled oligonucleotides in addition to the nuclear protein extract was added to the binding reaction as a competitor

cells (a cell line derived from the pig kidney). Therefore, it is not necessarily clear that the 5'-upstream region containing 14 TAA repeats is the cause of the defect in the transcription of the DAO gene in the LEA/SENDAI rats. Following explanations may be considered: (1) The reason may be ascribed to the difference between the in vivo and in vitro experiments. The transcriptional control in the rat kidney may be different from the cells in culture. (2) In addition to the 5'-upstream sequence, other genome sequence may be necessary for the correct transcription. (3) COS-1, NRK-52E and CCL-PK1 cells may not be the pertinent cells for the expression of DAO because this enzyme is expressed only in the cells localized in the proximal tubules of the kidney (Usuda et al. 1986; Perotti et al. 1987). In any case, it is clear that the TAA repeats are associated with the regulation of the transcription of the DAO gene because the lack of one unit from 14 TAA repeats drastically reduced the promoter activity in any of the cells tested (COS-1, NRK-52E and CCL-PK1) (Figs. 4a-c).

Alterations in triplet repeats are known to cause diseases, such as Huntington chorea (The Huntington's disease collaborative research group 1993), spinocerebellar atrophy (Orr et al. 1993) and the fragile X syndrome (Verkerk et al. 1991). These triplet repeats are present in the exons or 5'-untranslated region of the genes. In the present case, the TAA triplet repeats were present in the promoter region of the DAO gene. In this context, the present results may be similar to those reported by Itokawa et al. (2003). They found that the length of GT repeats in the promoter region affected the transcriptional activity of the *N*-methyl-D-aspartate receptor 2A subunit gene. They found that the longer repeats have the weaker promoter activity.

The 5'-upstream sequence of the rat DAO gene was determined by genome walking. When we searched the database for similar nucleotide sequences, an identical sequence was present in two bacterial artificial chromosome clones (AC114218 and AC097543) in GenBank. In these clones, the TAA triplet is repeated 15 times as seen in the SD rats. We also sequenced the 5'-upstream region of the DAO gene of Fisher 344 and Wistar rats and confirmed that they had 15 TAA repeats (data not shown).

In the 5'-upstream region of the DAO gene, the typical TATA, CCAAT or GC boxes were not present. This was the same in the human DAO gene (Fukui and Miyake 1992). This may be the reason for that DAO is a non-inducible enzyme. It is known that DAO activity was not significantly changed by various stimuli (see Konno and Yasumura 1992). It is not clear whether the 5'-upstream region of the DAO gene has the specific sequences responsible for the expression of this gene in the cells, such as the proximal-tubular cells in the kidney, the hepatocytes in the liver and the glial cells in the hindbrain.

Schizophrenia is one of the most frequent and devastating mental diseases of humans. Hypofunction of NMDA receptors is recognized as one of the most plausible causes of this disease. Since DAO metabolizes D-serine which stimulates glutamatergic neurotransmission via the binding to the NMDA receptor, it causes hypofunction of NMDA receptors. Indeed, a polymorphism in the DAO gene is shown to be one of the risk factors for schizophrenia (Chumakov et al. 2002; Schumacher et al. 2004; Liu et al. 2004). However, this hypothesis has to be rigorously verified. The LEA/SENDAI rats that do not have DAO would be useful for this study although the gene for the inferred DAO activator pLG72 is only present in mammals.

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