Functional Analysis of Human Ornithine Decarboxylase Alleles

Yongjun Guo, Robin B. Harris, Dan Rosson, David Boorman, and Thomas G. O’Brien

Abstract

It has been known for >10 years that there are two alleles of the human ornithine decarboxylase (ODC) gene, defined by a polymorphic PstI RFLP in intron 1. We have sequenced a large portion of each of the two alleles, including some of the 5′ promoter region, exon 1, intron 1, and exon 2, and determined that a single nucleotide polymorphism at base +317 (relative to transcription start site) is responsible for the presence or absence of the PstI restriction site. We have developed two genotyping assays, a PCR-RFLP assay and a high-throughput TaqMan-based method, and determined the ODC genotype distribution in >900 North American DNA samples. On the basis of its location between two closely spaced Myc/Max binding sites (E-boxes), we speculated that the single nucleotide polymorphism at base +317 could have functional significance. Results of transcription assays with allele-specific reporter constructs support this hypothesis. The promoter/regulatory region derived from the minor ODC allele (A allele) was more effective in driving luciferase expression in these assays than the identical region from the major allele (G allele). Our results suggest that individuals homozygous for the A allele may be capable of greater ODC expression after environmental exposures, especially those that up-regulate c-MYC expression.

Introduction

The enzyme ODC is a critical regulatory enzyme in the polyamine biosynthesis pathway. In both humans and animal models, ODC has been implicated as an important gene during the early stages of tumor progression (1–3). Among numerous inducers of ODC expression, the strong mouse skin tumor promoter 12-O-tetradecanoylphorbol-13-acetate is one of the most potent (4). In normal skin, ODC expression is tightly regulated, even after multiple 12-O-tetradecanoylphorbol-13-acetate applications, whereas in skin tumors, ODC is aberrantly regulated (5). Prevention of ODC overexpression by various means inhibits tumor development in both mouse skin and other animal models (6–9). On the basis of these results, targeted overexpression of ODC to the skin of mice was found to greatly increase susceptibility to tumorigenesis (10). These results suggest that ODC would be an attractive target for chemoprevention of cancer, and indeed the specific inhibitor of ODC, α-difluoromethylornithine, is a promising human chemopreventive agent (11).

In terms of its regulation, the ODC gene is a transcriptional target of c-MYC (12). Strong transactivation of ODC transcription is accomplished by virtue of two closely spaced Myc-binding elements (E-boxes) in the proximal region of intron 1 of the ODC gene. Thus, in the many physiological and pathological contexts in which c-MYC expression is up-regulated, ODC expression is also up-regulated. Indeed, in several studies, usually involving malignant cells or tissues, there has been good correlation between c-MYC and ODC expression (13–15).

In humans, there are two known ODC alleles, defined by a PstI RFLP (16). Of the three PstI sites in the human ODC gene, the polymorphic site has been shown to be in intron 1, between the two closely spaced E-boxes (17). The positions of these two E-boxes in intron 1 are highly conserved across species, whereas the sequences flanking the core CACGTG are not. Because the sequence context flanking the E-boxes has been shown to influence binding of Myc (18, 19), we explored the possibility that sequence differences between the two human ODC alleles might influence transcriptional activation by Myc. To test this idea, we determined the DNA sequence around the polymorphic site in the two alleles and asked whether the promoter/regulatory regions derived from the two alleles exhibit any functional differences.

Materials and Methods

DNA and Leukocyte Samples. A collection of 450 DNA samples from North American individuals representing diverse ethnicities was obtained from the Human Polymorphism Discovery Resource (20) at the Coriell Institute for Medical Research (Camden, NJ). A set of 518 leukocyte cell pellets from individuals participating in the Southeastern Arizona Health Study was obtained from the University of Arizona Cancer Center (Tucson, AZ). DNA was isolated from cell pellets via use of QIAamp Blood DNA Mini kits (Qiagen).

Our use of the samples from the Polymorphism Discovery Resource and leukocytes from patients in the Southeastern Arizona Health Study was approved by the Institutional Review Boards of the Lankenau Institute for Medical Research and the University of Arizona.

Genotyping Assays. Genomic DNA (0.1 μg) was subjected to a nested PCR-RFLP procedure to detect the presence or absence of the polymorphic PstI site. For the first PCR amplification, we used primers 5′-ATCGTGG-GCTGTGTGAAGCTG3′ and 5′-GTATCTGTCTGTAGACACACAC-3′. For the second PCR reaction, a 1/10th volume of the first PCR amplification was used as template DNA with primers 5′-GGTGCTATAAGTGGGAGGACGGC-3′ and 5′-CGAAGGTGTTGGAAGAGGACGC-3′. Each reaction contained 1× PCR buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl; 1.5 mM MgCl2, and 0.001% gelatin), 200 μM nucleotides, 10 μM each primer, 5% glycerol, 2% DMSO, and 0.5 units of Taq DNA polymerase in a volume of 25 μl. After an initial denaturation step at 95°C for 2 min, samples were cycled 30 times as follows: 95°C for 30 s, 55°C for 30 s, and 72°C for 1.5 min. The final extension was for 5 min at 72°C. The expected sizes of the amplification products were 757 bp for the first PCR reaction and 547 bp for the second PCR reaction. After amplification, 10–20 μl of each reaction were digested with 10 units of PstI in 30 μl for 2 h at 37°C. Loading dye was then added to each sample, and the samples were electrophoresed through a 1% agarose gel. DNA from individuals homozygous for the allele containing the PstI site yielded two fragments of 351 and 196 bp, whereas DNAs from homozygotes of the other allele yielded only the uncut 546-bp fragment. The presence of all three fragments was indicative of heterozygotes.

An allelic discrimination assay was developed based on the sequence difference at the polymorphic PstI site (see "Results and Discussion"). Oligonucleotide primers were designed with the assistance of Primer Express Software (PE Biosystems, Foster City, CA) to amplify a 172-bp fragment containing the polymorphic base at +317. Allele-specific TaqMan probes were synthesized with different 5′ fluorescent labels (6-carboxyfluorescein or VIC) and 3′ nonfluorescent labels. Oligo-primer sets were designed with the assistance of Primer Express Software (PE Biosystems, Foster City, CA) to amplify a 172-bp fragment containing the polymorphic base at +317. Allele-specific TaqMan probes were synthesized with different 5′ fluorescent labels (6-carboxyfluorescein or VIC).
and the same 3' quencher dye (6-carboxytetramethylrhodamine). Each reaction included 0.1 μg of genomic DNA, 30 pmol of each primer, 12.5 pmol of each TaqMan probe and 1× TaqMan Universal Master Mix (PE Biosystems) in a volume of 50 μl. PCR cycling conditions were 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 10 min, and 40 cycles at 95°C for 30 s and 62°C for 1 min. The results were analyzed on a PE Biosystems ABI Prism Model 7700 Sequence Detection System using allelic discrimination software supplied by the manufacturer. All DNA samples were analyzed in duplicate or triplicate. One hundred sixty-nine samples were analyzed by both the PCR-RFLP assay and the TaqMan-based assay, and results from both methods were 100% concordant. Probe and primer sequences are available from the authors upon request.

**Generation of Luciferase Reporter Constructs.** To obtain the luciferase-reporter constructs, a segment of the ODC gene from base −462 to base +3070 was amplified by PCR with primers 5'-NNNNNGAAGCTTGGTCCTTTCGGTCTC-3' and 5'-NNNNNGATCCGGAACACATCGAGTTGAAAGATGG-3'. This 3532-bp fragment was amplified from individuals 1930 was amplified by PCR with primers 5'-PmlI. The top strand was 5'-strands between the two Myc-binding sites, which are also restriction sites for ODC/A was changed to ter Plasmid Maxi kit (Qiagen). The constructs were sequenced prior to use in JM 109 cells with subsequent large-scale plasmid preparation using the Qiagen Plasmid Maxi kit (Qiagen). The constructs were sequenced prior to use in transfection assays. The sequence CTGCAG in pGL3-ODCIA was changed to CTGGCG by synthesizing oligonucleotides for the top and bottom DNA strands between the two Myc-binding sites, which are also restriction sites for pMIL. The top strand was 5'-GGGCCC CGGGTGCA GAGA CACAC-3', and the bottom strand was 5'-CC CGTCGCGCGCAC CGTCGCGCGCAC CGTCGCGCGCAC AC-3'. The Pml recognition sequences (also Myc-binding sites) are in brackets and the mutated nucleotides are underlined. Each oligonucleotide thus contained hybridized sequences between the PmlIL sites with 5'-overhangs. pGL3-ODCIA was digested with PmlI and the large fragment was recovered from a preparative agarose gel. The isolated fragment was incubated with T4 DNA polymerase for 1 min to digest sequences from the 3' ends. This was hybridized to the synthetic double-stranded sequence and ligated, and the mixture was transformed into bacteria. The 317 A→317G mutation was confirmed by loss of restriction with PstI and DNA sequencing.

**Transfection Assays.** NIH 3T3 cells were maintained in DMEM containing 10% fetal bovine serum. Rat-1 MycER (clone YY8ME4) fibroblasts expressing the c-MycER fusion protein (21), which is activated by 4-HT (kind gift of Dr. Amato Giaccia, Stanford University, Stanford, CA) were grown in DMEM containing 10% fetal bovine serum and 2.5 μg/ml puromycin.

For transfection, cells were plated at a density of 1 × 10^4 cells per 35-mm dish and grown overnight to 40–60% confluence. Cells were then transfected using LIPOFECTIN Reagent (Life Technologies, Inc.) in serum-free medium for 6 h with 1 μg of the construct containing either the A or G allele promoter/regulatory region and 100 ng of the pRL-TK control luciferase plasmid (Promega) as an internal transfection efficiency control. After 36–48 h, the cells were harvested, and luciferase activity was determined with a luminometer (Model TD-20/20b; Promega) using the Dual-Luciferase Reporter Assay System (Promega). To normalize luciferase activity, the firefly relative luminescence units/μg of protein were divided by the renilla relative luminescence units/μg of protein measured in the same extract. To determine the effect of inducible Myc, 0.1 μM 4-HT was added to the YSME4 transfectants 3 h before harvest, and luciferase activities were compared in extracts derived from cells cultured in the presence or absence of 4-HT. The promoterless pGL3 vector was used as a control in each experiment. Each transfection experiment was repeated at least once with duplicate samples and multiple DNA prepa-

**Results and Discussion**

**Sequence Analysis of the Two Human ODC Alleles.** DNA was isolated from the normal-appearing colonic mucosa of several colorectal carcinoma patients and subjected to a nested PCR-RFLP procedure (see “Materials and Methods”) to identify individuals homozygous for each of the two ODC alleles. DNA from these individuals was then sequenced from −400 bases upstream of exon 1 into the proximal region of the large intron 1 separating exons 1 and 2. Only two base differences were consistently found: a G/T polymorphism at base +264 in intron 1 and an A/G polymorphism at base +317 in the polymorphic PstI site between the two CACGTG Myc-binding domains (E-boxes) in intron 1 (Fig. 1). We designated the two human ODC alleles as A or G depending on the base at position +317. In the sequence of the G allele shown in Fig. 1, the base at position +264 is A. When 13 DNA samples isolated from normal colonic mucosa of
colorectal cancer patients that were genotyped as homozygous G (according to base at position +317) were resequenced, 11 had a T at position +264 and 2 had a G. The latter may represent minor alleles derived from the G allele or somatic mutations arising de novo. In the only published sequence of the G allele, Hickok et al. (22) reported a G at this position. The source of DNA used by Hickok et al. was a tumor-derived cell line containing amplified copies of the ODC gene. Resequencing of five additional DNA samples genotyped as homozygous A gave a sequence for this allele identical to the one shown in Fig. 1. There were no other sequence differences observed between the two alleles in this important regulatory region of the ODC gene spanning the proximal S’ promoter region, exon 1, and the proximal region of intron 1.

Allele Frequencies in Human Populations. To determine the frequencies of the A and G alleles in a representative sample of the United States population, 450 DNA samples from the Human Polymorphism Discovery Resource (20) were genotyped using two independent methods (see “Materials and Methods”). The results of these assays indicated that 52.4% of the individuals were homozygous for G, 10% were homozygous for A, and 37.6% were heterozygotes (Table 1). The individual donors of DNA for the Polymorphism Discovery Resource were ethnically diverse by design. To determine whether the polymorphic base at +317 has functional significance, we cloned a 3532-bp fragment from each of the ODC alleles (−462 to +3070) into the pGL3 plasmid containing a modified firefly luciferase gene. NIH 3T3 cells were then transiently transfected with the reporter constructs containing either A- or G-allele-derived sequences. The results of five independent experiments (Table 2) indicated that both constructs caused large luciferase inductions relative to empty vector controls (0.12 ± 0.01). However, the important conclusion from these experiments was that the promoter/regulatory sequence from the A allele produced a consistent 3-fold higher luciferase induction than the G-derived sequence (Table 2). When the base at position +317 in the A-allele-derived construct was mutated to G, the luciferase induction was greatly reduced (12.7 units in the 317A→G mutated construct versus 24.0 units in the A construct; compare to values in Table 2). Although we cannot rule out some contribution of the polymorphic base at +264 to these results, it appears that most of the difference in promoter strength of these two allelic sequences is attributable to the base at position +317.

The preceding data do not directly address the role of Myc in mediating the luciferase induction observed. To investigate this question, the same reporter plasmids were transfected into YY8ME4 cells, a rat-1-derived cell line that expresses a MycER fusion protein (21). In the presence of 4-HT, the cytosolic fusion protein is activated and translocates to the nucleus to transactivate Myc target genes. The results of two independent transfection experiments are given in Table 3. Consistent with the results in NIH 3T3 cells, in the absence of 4-HT, the A allele construct gave a much greater luciferase induction than the G allele construct (3.7- and 8.8-fold in the two experiments). In the presence of 4-HT, luciferase induction was enhanced, with a greater enhancement in absolute terms in cells transfected with the A allele construct versus the G allele construct (Table 3). The MycER fusion protein appears to preferentially transactivate the luciferase construct containing the A-allele-derived promoter/regulatory sequence. On the basis of the results of our functional assays and the known ability of MYC to transcriptionally activate ODC (12, 23), we suggest that for a given level of MYC expression, cells of the AA genotype will express more ODC mRNA than cells of the GG genotype. Furthermore, constitutive overexpression of MYC, which occurs in many tumors by a variety of mechanisms (24), would have a greater effect on ODC overexpression in AA cells versus GG cells. Studies to test these hypotheses are in progress.

Is the ODC A Allele a Cancer Susceptibility Gene? The first large-scale ODC genotyping of human populations reported here indicates that ~7–10% of individuals are homozygous for the A allele, 52–54% are homozygous for the GG allele, and 38–40% are heterozygotes. If the results of our functional assays are predictive of differences in ODC expression in vivo between individuals with

<table>
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<th>Source of DNA</th>
<th>No. of Samples</th>
<th>Number with ODC genotype (%)</th>
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<tr>
<td>Polymorphism Discovery Resource</td>
<td>450</td>
<td>45 (10.0) 169 (37.6) 236 (52.4)</td>
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<tr>
<td>Arizona Cancer Center</td>
<td>518</td>
<td>35 (6.8) 202 (39.0) 281 (54.2)</td>
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Luciferase activity

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<th>Experiment</th>
<th>A allele</th>
<th>G allele</th>
<th>A/G ratio</th>
</tr>
</thead>
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<tr>
<td>1</td>
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<td>2</td>
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<tr>
<td>4</td>
<td>22.6</td>
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</tr>
<tr>
<td>5</td>
<td>22.9</td>
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<tr>
<td>Mean ± SD</td>
<td>30.5 ± 8.36</td>
<td>9.94 ± 2.68</td>
<td>3.08 ± 0.3</td>
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* Ratio of relative firefly luminescence units/μg of protein to relative renilla luminescence units/μg of protein measured in the same cell extract.

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<td>35 (6.8) 202 (39.0) 281 (54.2)</td>
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</tbody>
</table>

Table 2 Functional analysis of the two human ODC alleles

A fragment (−462 to +3070 bp) of the human ODC gene was isolated by PCR from individuals homozygous for either A or G allele and cloned into the pGL3 plasmid (Promega) in front of a modified firefly luciferase gene. NIH 3T3 cells were transiently transfected using Lipofectin reagent with the above constructs (1 μg) along with 0.1 μg of a renilla luciferase plasmid (pRL; Promega). Luciferase activities were measured in cell extracts 36 h post-transfection. Luciferase activity in cells transfected with empty pGL3 controls was 0.12 ± 0.01 (n = 3).

Table 3 Prevalence of ODC genotypes in human populations

<table>
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<th>Number with ODC genotype (%)</th>
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<td>Arizona Cancer Center</td>
<td>518</td>
<td>35 (6.8) 202 (39.0) 281 (54.2)</td>
</tr>
</tbody>
</table>

Table 3 Role of Myc in ODC promoter-driven luciferase activity

A fragment (−462 to +3070) of the human ODC gene was isolated by PCR from individuals homozygous for either the A or G allele and cloned into the pGL3 plasmid (Promega) in front of a modified firefly luciferase gene. YY8ME4 cells (21) were transiently transfected using Lipofectin reagent with the above constructs (1 μg) along with 0.1 μg of a renilla luciferase plasmid (pRL; Promega). In some dishes, 0.1 μg 4-HT was added 3 h prior to harvesting cells for luciferase assay. Luciferase activities were measured in cell extracts 36 h post-transfection. Values in parentheses indicate the percentage of increase in luciferase activity produced by 4-HT treatment.

Luciferase activity

<table>
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<th>4HT (%)</th>
<th>Δ4HT (%)</th>
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<td>2</td>
<td>38.7</td>
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<td>21.1 (55)</td>
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* Ratio of firefly relative luminescence units/μg of protein to renilla luminescence units/μg of protein measured in the same extract.
different ODC genotypes, could this phenotype influence susceptibility to cancer? Our results from transgenic mouse models demonstrate that overexpression of ODC, even to a relatively modest extent, increases susceptibility to carcinogen-induced skin cancer (10, 25). Mouse in which ODC is overexpressed are, in effect, “genomically promoted,” requiring only an initiation event for tumor development to occur. With appropriate study populations and the high-throughput genotyping assay described here, the relationship of the ODC A allele and cancer susceptibility can be addressed experimentally in large-scale association studies. The ODC A allele may be one of several genetic variants that act in concert with environmental exposures to carcinogens to determine an individual’s risk for cancer.

Acknowledgments

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References

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