Expression of Human Chromosome 2 Ornithine Decarboxylase Gene in Ornithine Decarboxylase-deficient Chinese Hamster Ovary Cells

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ABSTRACT

Ornithine decarboxylase (ODC) belongs to a multigene family and some of these may very well be nonfunctional (pseudogenes). We isolated an ODC gene from a human chromosome 2-specific library and transfected the gene into ODC-deficient Chinese hamster ovary cells to directly demonstrate that this ODC gene is functional and ODC is essential for cell proliferation. After screening 2.5 x 10⁷ plaques using a human ODC complementary DNA probe, a typical clone with a 5.4-kilobase insert was isolated and then cloned into the HindIII site of the pGEM-1 vector. One (pHODC 2B1) of these clones containing a 5.4-kilobase ODC gene insert was identified. Restriction enzyme analysis and partial sequencing data revealed that pHODC 2B1 contained the full length protein-coding sequences but lacked first exon and 3'-polyadenylation sequences. Primer extension analysis indicated that human ODC mRNA has homologous sequences with the ODC gene from human chromosome 2. To determine that the chromosome 2 ODC gene is functional, ODC-deficient Chinese hamster ovary cells were transfected with the ODC expression vector (pHSV2B1-neo) and several G418-resistant transfectants were isolated which expressed ~70- to 400-fold more ODC activity than parental or wild-type Chinese hamster ovary cells. Furthermore, these stable transfectants exhibited a higher growth rate than wild-type cells. These results indicate that the ODC gene from human chromosome 2 encodes functional ODC protein, and ODC (and its product putrescine) is required for cell growth.

INTRODUCTION

Eukaryotic cells contain significant amounts of the polyamines spermine, spermidine, and their precursor putrescine. Polyamine levels are highly regulated in cells and are thought to be critical in controlling normal cell proliferation and differentiation, as well as malignant transformation (1-3). ODC (EC 4.1.1.17), which decarboxylates ornithine to form putrescine, is the key enzyme in mammalian polyamine biosynthesis. ODC is induced in response to many growth stimuli including hormones, growth factors, and tumor promoters and is also characterized by a rapid turnover rate (half-life, ~15 min) (2).

Data from Southern blot analysis indicate that the mouse (4-6) as well as the human (7) ODC genes belong to a multigene family and some of these may well represent pseudogenes (4-6). Using the somatic cell hybrid technique, the human ODC gene was found to be localized on chromosomes 2 and 7 (8). Alhonen-Hongisto et al. (6) reported that a human myeloma cell line (Sultan) has an amplified ODC gene residing in the short arm of chromosome 2. Although the results presented by Winquist et al. (8) indicate the possibility that the ODC gene on chromosome 2 is active, direct evidence is lacking that the ODC gene on chromosome 2 encodes functional ODC in normal human cells. In this paper, we isolated a human ODC gene from a chromosome 2-specific library, constructed an ODC gene expression vector, and transfected it into ODC-deficient CHO cells. The stable transfectants have 70- to 400-fold more ODC activity than the wild-type CHO cells. Furthermore, these transfectants exhibited a higher growth rate than parental cells. The results, indicating that the human chromosome 2 contains a functional ODC gene and that putrescine, the product of ODC, is essential for cell growth, are summarized in this paper.

MATERIALS AND METHODS

Materials

TPA was purchased from Life System (Newton, MA). [α-32P]-dATP (~1000 Ci/mmol), [α-32P]-dCTP (~3000 Ci/mmol), and [γ-32P]-ATP (~3000 Ci/mmol) were purchased from Amersham Co. (Arlington Heights, IL). D-[1-14C]Ornithine (49.9 mCi/mmol) was purchased from New England Nuclear (Boston, MA). G418 sulfate (Geneticin) was obtained from Gibco Laboratories (Grand Island, NY). Human chromosome 2 library cloned into Charon 21A vector (HindIII digested; average size of insert, ~5 kilobases) and pSV2-neo (9) were obtained from American Type Culture Collection (Rockville, MD). The ODC-deficient CHO cell line (C55.7), cultured in DMEM containing 10% fetal bovine serum and 500 μM putrescine, was a gift from Dr. I. E. Scheffler (University of California, San Diego, La Jolla, CA) (10). The wild-type CHO cell line, cultured in DMEM containing 10% fetal bovine serum, was a gift from Dr. C. A. Reznikoff (University of Wisconsin, Madison, WI). pODC10/2H, a cDNA clone derived from human ODC mRNA, was a gift from Dr. O. A. Jänne (Rockefeller University, NY) (7). pGEM-1 and the DNA-sequencing kit (K/R) were obtained from Promega Biotechnology (Madison, WI). Propidium iodide was purchased from Calbiochem Co. (La Jolla, CA).

Methods

Screening Human Genomic Library for ODC Sequences. The human chromosome 2 library was screened for ODC sequences by plaque hybridization using the EcoRI insert (1.8 kilobases) from the human ODC cDNA probe pODC10/2H. Plaques (10⁶-10⁷) were grown in Escherichia coli strain K802 on L-B agar plates (25 cm × 25 cm). After overnight incubation at 37°C, the plates were placed at 4°C for at least 1 h. Plaques were transferred onto Genescreen Plus membranes, as described (11), and membranes were then placed in 0.5 N NaOH for 2 min and 1 M Tris-HCl (pH 7.5) for another 2 min. The membranes were air-dried and prehybridized overnight at 42°C. The membranes were subsequently hybridized overnight with a 32P-labeled nick-translated probe and then washed, first with 2x standard saline citrate plus 0.2% sodium dodecyl sulfate at room temperature and then with 0.1 x standard saline citrate plus 0.2% sodium dodecyl sulfate at 65°C. The membranes were air-dried for autoradiography.

End-labeling of Oligonucleotides. Oligonucleotides were end-labeled with [γ-32P]-ATP by T4 polynucleotide kinase, as described (12). The specific activity of the oligonucleotide was 2-5 x 10⁶ cpm/μg.

Dideoxyxynucleotide Sequencing of DNA. Nucleotide sequences of the insert were determined using a DNA-sequencing kit (Promega Biotechnology) by a modification of the dideoxyxynucleotide chain-termination method, as described (13). Two μg supercoiled plasmid were denatured

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2 To whom requests for reprints should be addressed.

3 The abbreviations used: ODC, ornithine decarboxylase; TPA, 12-O-tetradecanoylphorbol-13-acetate; cDNA, complementary DNA; DMEM, Dulbecco's modified Eagle medium; PBS, phosphate-buffered saline; CHO, Chinese hamster ovary.
and ethanol precipitated. The DNA pellet was resuspended in 6 μl of water, to which was added 3 μl of the appropriate primer (stock, 10 ng/μl) and 1 μl of 10× Klenow enzyme buffer, and incubated at 37°C for 2 h for annealing. Sequencing reactions were carried out in four separate reactions. Each reaction contained 3 μl of appropriate dyeoxy nucleotide mix, 5 μl of Klenow enzyme, 2 μl of [α-32P]-dATP, and 3 μl of primer/template mix. After 20 min of incubation at 37°C, 1 μl of chase buffer was added to each reaction for another 15 min, and the reaction was then terminated with 5 μl of stop buffer. After denaturation for 3 min at 70°C, 2.5 μl of each reaction mix were subjected to polyacrylamide gel electrophoresis (8% polyacrylamide, 7 μm urea gel with 1× TBE buffer) and autoradiography.

Primer Extension. Hybridization and reverse transcription were carried out as described by McKnight and Kingsbury (14). The hybridization reaction was carried out in a final volume of 60 μl containing 50 μg total cellular RNA, [32P]-labeled primer (4.5× 10^5 dpm), 0.25 mM KCl, 10 mM Tris-HCl (pH 7.5), and 1 mM EDTA. The reaction was carried out at 60°C for 1 h and then slowly cooled to room temperature. Primer extension reaction mixture (3.0 μl of diethylyglycocolerate-water, 14 μl of 100 mM dithiothreitol, 28 μl of 1.25 mM d(A,T,G,C)TP, 2.0 μl of Actinomycin D (5 mg/ml), 14 μl of 100 mM MgCl2, 5.0 μl of 500 mM Tris-HCl (pH 8.3), 2.0 μl of 40 units/μl RNasin, and 2.0 μl of 7 units/μl avian myeloblastosis virus reverse transcriptase) was added to the annealed primer and RNA. Primer extension assay mix was incubated at 37°C for 1 h. RNA transcripts were removed by hydrolysis in 0.2 N NaOH, 68°C, for 15 min. Reactions were neutralized with 1.0 N HCl, extracted with phenol/chloroform, and ethanol precipitated. The DNA pellet was dissolved in 20 μl formamide dye mix [0.1% (w/v) xylene cyanol, 0.1% (w/v) bromophenol blue, 10 mM EDTA, pH 8.0, and 95% (v/v) deionized formamide] and electrophoresed on a 5 μm urea/5% polyacrylamide gel.

Calcium Phosphate Transfection. Calcium phosphate transfection was carried out as described (15). Cells (5× 10^5) were plated onto 100-mm Petri dishes 1 day before transfection. DNA-calcium phosphate coprecipitation was carried out by adding dropwise 500 μl of 2× HeBS buffer (0.28 mM NaCl, 0.05 mM Mg-2-(hydroxyethyl)-1-piperazineethanesulfonic acid, 1.5 mM NaH2PO4, pH 7.05) to 500 μl of 0.25 M CaCl2/DNA mixture. After incubation for 20 min at room temperature, the precipitate was added onto cells evenly. After 6 h of incubation, medium was removed. Glycerol shock was accomplished by adding 2.0 ml of 10% (v/v) glycerol solution in PBS to each plate for 2 min. Finally, cells were washed with PBS twice and re-fed with complete medium for recovery.

Determination of the Cell Cycle Distribution. Cells were trypsinized, fixed in 70% ethanol for 30 min at 4°C, and then washed once with PBS solution. After centrifugation (500 x g for 10 min), the cell pellet was resuspended in DNase-free RNase A (100 μg/ml), incubated at 37°C for 30 min, washed once with PBS, and centrifuged. The cell pellet was resuspended in propidium iodide (50 μg/ml) solution, incubated at 37°C for 30 min, reashed, and centrifuged. The final cell concentration was adjusted to about 10^6/ml and the DNA content was analyzed using FACScan (Becton-Dickinson, Mountain View, CA).

Assay of ODC Activity. ODC activity from the soluble cell extract was determined by measuring the release of [14C] ornithine hydrochloride (16).

Analysis of Polymine Levels. The level of polyamines in CHO cells was determined according to the method described previously (17). Briefly, at 3 days after plating, cells were homogenized in 2% perchloric acid, centrifuged, and the polyamines were purified on Dowex AG-50W columns (100 to 200 mesh, H+ form, 0.9 x 5 cm; Bio-Rad Laboratories, Richmond, CA) as outlined by Inoue and Mizutani (18). Quantitation of individual polyamines was further accomplished by dansylation, separation of dansyl derivatives by thin layer chromatography, and measurement of the fluorescent intensity of eluates from individual polyamine spots, as described previously (17, 19).

The acid-insoluble pellet obtained after polyamine extraction was washed with 4 ml ethanol/ether (1/1, v/v). DNA was hydrolyzed in 0.5 M perchloric acid for 10 min at 90°C, and DNA content in the clear supernatant was determined by the diphenylamine method of Burton (20).

RESULTS

Screening of Human Chromosome 2 Library for ODC Gene. A human chromosome 2 library, having an average insert of 5.0 kilobases cloned into the HindIII site of Charon 21A vector, was screened. Using a human ODC cDNA probe (pODC10/2H EcoRI site), we obtained five positive clones from the 2.5× 10⁷ plaques screened. After three repeated plaque hybridization screenings, a typical clone (designated as A2B1) containing about 5.4-kilobase insert was isolated. After subcloning into the HindIII site of pGEM-1 vector, one of these clones (phODC 2B1) was identified and its restriction enzyme map was determined (Fig. 1A). Dideoxy partial sequencing data (Fig. 1B) revealed that phODC 2B1 contained the translation initiation site between the EcoRI and PvuII sites and the ODC protein coding sequences had 100% homology with that of pODC10/2H (7). Moreover, phODC 2B1 contained 111 base pairs of noncoding sequence, including 94 base pairs from exon 2 and 17 base pairs from exon 3. Between exons 2 and 3 is intron 2 (105 base pairs), which is 17 base pairs upstream of the translation initiation site. Recently, mouse genomic DNA sequence data (21, 22) revealed that the first intron sequence (~1.9 kilobases) is located between exon 1 (197 nucleotides) and exon 2 (89 nucleotides). By comparison with those sequences, it appears that phODC 2B1 contained only 288 base pairs of the first intron sequences followed by the remaining exon and intron sequences but lacked the first exon sequence and the 3'-end polyadenylation signal. Taken together, we concluded that
phODC 2B1 contains the full length protein-coding sequence of the ODC gene.

The transcription initiation site of the ODC gene was determined by primer extension analysis and revealed about a 320-base pair band (Fig. 2). These results (Fig. 2) indicate that the 5'-noncoding sequence of human ODC mRNA from T24 cells is about 380 nucleotides long and the human ODC gene from chromosome 2 has homologous sequences with ODC mRNA from T24 cells.

Expression of Human ODC Gene from Chromosome 2 in the ODC-deficient CHO Cell. To determine whether phODC 2B1 is a functional gene, we subcloned these 5.4 kilobases of the ODC gene into the HindIII site of pSV2-neo vector. One of the clones, designated as phSV2B1-neo, had a 5' to 3' orientation relative to the SV40 promoter (Fig. 3A), while the other, designated as phi5, had opposite orientation (Fig. 3B). The ODC gene expression vector was transfected into ODC-deficient CHO cells using the calcium phosphate transfection method (15). The typical transfection efficiency was about 10-15 transfecants/µg DNA. After culture of the transfecants in medium containing G418 (400 µg/mL) for 1 month, resistant clones were isolated and transferred into 35-mm Petri dishes for 2 weeks, then into 100-mm Petri dishes for another 2 weeks. As shown in Table 1, the basal level of ODC activity of G418-resistant transfecants ranged from 70- to 400-fold more than wild-type CHO cells. In contrast, ODC activity was not detected in any clone transfected with phi15 plasmid. Interestingly, TPA treatment of positive transfecants resulted in about a 2-fold increase of ODC activity (Table 1).

The possibility was examined that ODC-overproducing transfecants may have increased levels of polyamines. In this experiment, wild-type CHO cells and two (2B1-1 and 2B1-6) of the ODC-overproducing transfecants were cultured for 3 days and then basal polyamine level was analyzed. As shown in Table 2, ODC-overproducing transfecants also overproduce polyamines.

To determine if elevated ODC activity in the transfecants is the result of increased levels of ODC mRNA, a human ODC-specific probe (H6) was used. Interestingly, Northern blot analysis indicated the expression of two different sized RNA transcripts (5.1 and 1.75 kilobases) in these three phSV2B1-neo transfecants (Fig. 4A). Dot blot analysis indicated a dra-

![Fig. 2. Determination of transcription initiation site of human ODC gene. Total cellular RNA (50 µg) isolated 6 h after treatment of T24 cells with 50 nm TPA was hybridized with end-labeled synthetic oligomer H6, which is complementary to nucleotide positions -163 to -183 of the ODC gene (Fig. 1). Primer extension assay was carried out as described in "Materials and Methods."](image-url)
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Table 2 Polyamine levels of ODC-overproducing transfectants

<table>
<thead>
<tr>
<th>CHO cells</th>
<th>Putrescine (nmol/mg DNA)</th>
<th>Spermidine (nmol/mg DNA)</th>
<th>Spermine (nmol/mg DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>29.23*</td>
<td>74.87</td>
<td>49.20</td>
</tr>
<tr>
<td>2B1-1</td>
<td>108.33</td>
<td>155.00</td>
<td>91.67</td>
</tr>
<tr>
<td>2B1-6</td>
<td>104.74</td>
<td>152.75</td>
<td>87.28</td>
</tr>
</tbody>
</table>

*Each value is a determination from the extract prepared from three separate culture plates.

Cells were cultured for 3 days in DMEM containing 10% dialyzed fetal bovine serum. Cellular polyamine levels were determined as described (see "Materials and Methods").

Parental CHO and ph 15-1 cells did not grow in the absence of putrescine. Also, flow cytometrical analysis (Table 3) indicated that parental and ph 15-1 cells contained 60-70% of the cell 16-h doubling time) than the wild-type CHO cell (about 24-26-h doubling time). These three transfectants reached confluence after 5 days of culture in DMEM containing 10% dialyzed serum, while the wild-type and the parental cells (in the presence of putrescine) were still in the exponential phase.

Fig. 4. Northern blot and dot blot analyses of CHO control cell lines and cells transfected with the ODC gene. For Northern blot analysis (A), 10 \( \mu \)g of total cellular RNA were subjected to formaldehyde-agarose gel electrophoresis and then probed with end-labeled H6 oligomer. Lane 1, parental cell; lane 2, wild-type; lane 3, ph 15-1; lane 4, 2B1-1; lane 5, 2B1-2; lane 6, 2B1-6. For dot blot analysis (B), the indicated amounts of total cellular RNA were spotted on GeneScreen and probed with end-labeled H6 oligomer. Higher amounts of total cellular RNA were used for the parental, wild-type, and ph 15-1 samples than for other transfectants.

Fig. 5. Southern blot analysis of ODC-transfectants and control cell lines. Ten \( \mu \)g of DNA from each line were digested with HindIII and subjected to Southern blot analysis on 0.8% agarose gels in 1x TBE buffer. 3P-nick-translated pODC10/2H EcoRI insert was used for hybridization. Lane 1, parental cells; lane 2, wild-type cells; lane 3, ph 15-1; lane 4, 2B1-1; lane 5, 2B1-2; lane 6, 2B1-6.

Fig. 6. Growth curve of control and ODC-transfected cell lines. Cells (1 x 10^6) were plated in 60-mm Petri dishes in DMEM containing 10% dialyzed fetal bovine serum. At the indicated times, cells in triplicate were trypsinized and counted by the trypan blue exclusion method. Each point is a mean ± SE of determinations from three separate culture plates. •, parental cell; △, parental cell plus 500 \( \mu \)M putrescine; ○, wild-type; □, ph 15-1; ▲, 2B1-1; ▼, 2B1-2; ○, 2B1-6.
population in S phase and only a very small amount of cells (about 2–3%) in the G2+M phase. In contrast, the ODC-transfected and parental cells in the presence of 500 μM putrescine showed a significant increase (about 3- to 5-fold) in the G2+M cell population (Table 3).

DISCUSSION

We present here direct evidence that the ODC gene from human chromosome 2 encodes functional ODC and that ODC (and its product putrescine) is essential for cell proliferation. After screening a human chromosome 2 library (cloned in Charon 21A vector) using a human ODC cDNA probe (pODC10/2H), a 5.4-kilobase insert of the ODC gene was isolated (Fig. 1). The primer extension (Fig. 2) and partial sequencing (Fig. 1B) data revealed that the human ODC gene should contain another exon (i.e., exon 1), about 270-base pairs long, in the further 5'-end of ODC gene. In addition, this 5.4-kilobase fragment of the ODC gene contained neither the 3'-polyadenylation signal nor the transcriptional termination sequence. Therefore, we constructed an expression plasmid (Fig. 3A) using pSV2-neo as a vector, which contained the SV40 splicing signal. The cellular polyadenylation polymerase is capable of recognizing this splicing signal and also will add the polyadenylate tail on the 3' end of the nascent RNA transcript. Interestingly, the expression plasmid (pSV2B1-neo) expressed two different sizes (5.1 and 1.75 kilobases) of RNA transcripts. The 5.1-kilobase mRNA represented the fused form of ODC and neo mRNAs, while the 1.75-kilobase mRNA contained only ODC mRNA. It is important to note that the majority (80–90%) of RNA transcripts are 5.1 kilobases in length. These results indicate that there may be an inefficient transcriptional termination signal in the neo gene. It remains unknown which RNA transcript is capable of being translated into ODC protein. Since both transcripts contained a full length protein-coding sequence, it is highly likely that both RNA transcripts are functional. As shown in Table 1, the calcium phosphate transfection technique did not result in any detectable revertant from parental CHO cells. Furthermore, the variable ODC levels in different transfectants (Table 1) may be the result of a different copy number of ODC gene in each transfectant (Fig. 5). However, the level of RNA transcripts from each transfectant (Fig. 4B) was not proportional to the ODC activity (Table 1), suggesting that the translational or post-translational regulation of ODC may vary between the different clones. In this regard, recent results reported by Ghoda et al. (23) are noteworthy. In their findings, ODC is stabilized by truncating 37 residues at its carboxy terminus which contain PEST sequences. Although we have not determined the size of ODC protein, it is unlikely that ODC from the ODC-overproducing transfectants is truncated. Our 5.4-kilobase DNA fragment contains the complete protein-coding sequences, as compared with human cDNA sequences reported by Hickok et al. (7). Therefore, ODC protein from the overproducing transfectants should contain the same PEST sequences as the normal ODC protein. Also, ODC activity was increased (~2-fold) after treatment of ODC-overproducing transfectants with TPA. The mechanisms involved in the induction of ODC activity by TPA in ODC transfectants were not analyzed; it may be the result of either increased transcription or post-transcriptional regulation. It is unclear whether this effect resulted from the AP-1 binding site in the SV40 early promoter region (24). It is also likely that there may be a TPA-responsive element in the 288 base pairs of the first intron sequence of the ODC gene in phSV2B1-neo.

Clearly, the results (Tables 1 and 2 and Fig. 6) are convincing that ODC and its product putrescine are essential for cell growth. CHO cells deficient in ODC required putrescine for growth but, when the ODC gene was introduced into ODC-deficient CHO cells, the transfectants overproduced ODC and putrescine and were able to proliferate in the absence of putrescine. However, the ODC transfectants exhibited similar morphology as their parental cells. Also, cell cycle analysis indicated that these transfectants exhibited more G2+M cells, as compared to parental cells, indicating that putrescine may be essential for the transition from the S phase to the G2+M phase of cell cycle. Taken together, the results further substantiate the conclusion that the elevated level of ODC, the first and the rate-limiting enzyme in polyamine biosynthesis, is required for mammalian cell growth (25). Furthermore, the human ODC gene can express and exert its biological function in a different species, such as hamster. Similar results have been reported by others (26, 27).

REFERENCES


Table 3  Cell cycle distribution of ODC-transfected and parental CHO cells

<table>
<thead>
<tr>
<th>CHO cells</th>
<th>G1</th>
<th>S</th>
<th>G2 + M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parental</td>
<td>33.5 ± 4.3*</td>
<td>64.0 ± 4.6</td>
<td>3.3 ± 1.0</td>
</tr>
<tr>
<td>Parental + 500 μM putrescine</td>
<td>40.5 ± 1.7</td>
<td>51.5 ± 1.5</td>
<td>8.0 ± 1.9</td>
</tr>
<tr>
<td>ph15-1</td>
<td>32.8 ± 0.9</td>
<td>65.0 ± 0.6</td>
<td>2.2 ± 1.0</td>
</tr>
<tr>
<td>2B1-1</td>
<td>38.0 ± 1.4</td>
<td>52.0 ± 0.6</td>
<td>10.0 ± 1.8</td>
</tr>
<tr>
<td>2B1-2</td>
<td>38.0 ± 1.7</td>
<td>54.5 ± 0.9</td>
<td>7.5 ± 1.9</td>
</tr>
<tr>
<td>2B1-6</td>
<td>39.5 ± 1.5</td>
<td>53.4 ± 0.4</td>
<td>7.0 ± 1.8</td>
</tr>
</tbody>
</table>

*Cell cycle distribution was calculated by using the Sum of Broadened Rectangles Model, DNA cell-cycle analysis software (Version C), developed by Becton-Dickinson (Mountain View, CA).

*Each value represents mean ± SE.
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