Regulation of mouse ornithine decarboxylase activity by cell growth, serum and tetradecanoyl phorbol acetate is governed primarily by sequences within the coding region of the gene

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Received July 28, 1989; Revised and Accepted October 4, 1989

ABSTRACT

To determine the genetic elements required for modulation of ornithine decarboxylase (ODC) activity in response to cell growth or treatment with serum or with tetradecanoyl phorbol acetate, ODC-deficient cells were transfected with a series of recombinant DNAs encoding mouse ODC. All of the transfected cells expressing an intact mouse ODC protein displayed regulation of ODC activity, including those expressing a construct deprived of all ODC-specific sequence information except the protein-coding region. ODC mRNA changed much less than enzymatic activity. A mutation of the protein-coding region that converted ODC from an unstable to a stable intracellular protein attenuated the regulatory response. We conclude that post-transcriptional events associated with ODC degradation dominate the response to these stimuli.

INTRODUCTION

Ornithine decarboxylase (EC 4.1.1.17) catalyzes the conversion of ornithine to putrescine, the initial step in polyamine biosynthesis [1]. Genetic or pharmacologically induced deficiency in ODC leads to cell death, suggesting an absolute requirement for polyamines. Their function in cells, however, is not known. The level of ODC activity is finely tuned and directly correlated with cell proliferation [2]. Thus promotion of cell growth by mitogens, growth factors and oncogenic transformation induces ODC activity, whereas slowing of cell growth by contact inhibition or terminal differentiation decreases it. These correlations hint at a relationship between the mechanisms that regulate ODC expression and those that affect cell growth.

Speculation that such a relationship exists is fueled by observations that the initial and landmark events associated with the onset of cell proliferation share salient characteristics with the changes in ODC expression. ODC mRNA levels, like those of the proto-oncogenes *c-fos* and *c-myc*, undergo a rapid but transient increase when quiescent cells are treated with serum [3,4] or 12-O-tetradecanoyl-phorbol-13-acetate (TPA) [3,5,6,7]. Furthermore, ODC mRNA is also subject to superinduction by simultaneous treatment of cells with mitogens and inhibitors of protein synthesis [3,4,8].

The question of to what degree and under what conditions changes in ODC mRNA account for changes in ODC intracellular activity is unresolved. To better understand the nature of regulation of ODC activity, we and others have cloned mouse ODC genomic DNA and cDNA from cells in which that gene was selectively amplified [9–13]. We showed the cloned ODC gene to be functional by stably transfecting [9,14] ODC-deficient (ODC⁻) Chinese hamster ovary (CHO) cells [15]. The ODC⁻ cells are auxotrophic for putrescine; transfection with the cloned gene restores them to prototrophy. The transfectants

also produce correctly initiated and sized mouse ODC mRNA transcripts and enzymatically active ODC polypeptide [9].

Here we show further that the cloned gene includes elements sufficient for its regulation by growth and by serum or TPA stimulation of quiescent cells. We show that this information resides primarily within the ODC protein-coding region and that mitogenic regulation of ODC expression is carried out predominantly at the post-transcriptional level.

MATERIALS AND METHODS

Cells

NIH3T3 mouse cells (from Mike Verderame, Univ. Calif. San Francisco) were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS). C55.7 ODC⁻ CHO cells [15] were grown in DMEM supplemented with 5% FCS, non-essential amino acids and 0.5 mM putrescine. CHO wild type (clone 10001, from Michael Gottesman, National Cancer Institute) and stably transfected cells were grown in the same medium but without putrescine. Quiescent cells were obtained by growing cells to confluence and then incubating them for 30 hours in fresh DMEM supplemented with 0.5% FCS. Stimulation of quiescent or confluent cells was with 30% FCS, 1.6 μ M TPA or 100 μ g/ml cycloheximide.

Cell lysates for enzyme assays were prepared by twice washing cell monolayers with phosphate-buffered saline (PBS), harvesting cells by scraping and low-speed centrifugation and quick-freezing the pellets. These were resuspended in 25 mM Tris-HCl, pH 7.5, freeze-thawed twice and the microfuged supernatants used for enzyme assay.

Total cellular RNA was isolated from PBS-washed monolayers by the urea-LiCl method [16].

Protein content of cellular extracts was determined by the method of Lowry et al [17].

Construction of recombinant ODC plasmids

The structures and descriptions of the recombinant plasmids used in this analysis are given in Figure 2. A detailed description of their construction will be provided upon request by the authors.

Transfections

Cells were transfected by calcium-phosphate co-precipitation [18]. For transient expression assays, cells were plated in 100mm dishes. The following day, the growth medium was replaced with 10ml fresh medium and four hours later, 1ml of co-precipitate containing 5 μ g of each plasmid DNA was added to each dish. After incubation for 4 hours, the medium was removed, the cells shocked for 2 minutes with 20% glycerol, washed, and incubated with fresh medium. The cells were harvested 48 hours after transfection. Stable transfections were carried out in 150cm² tissue culture flasks. Two $\times$ 10⁶ cells per flask were co-transfected with 15 μ g of ODC plasmid DNA and 15 μ g pSV2neo DNA [19]. Two days later the cultures were subjected to selection in medium containing 450 μ g/ml G418 (GIBCO), but not putrescine. Selective medium was replaced every 3–4 days until the flasks were repopulated. Transfected cells were maintained in putrescine-deficient medium thereafter. A 'pool' of stable transfectants consisted of 100 or more colonies that appeared under selective conditions of incubation.

Enzymatic Assay

The ODC assay quantitates the release of [¹⁴C]CO₂ during ODC-catalyzed conversion of ornithine to putrescine and CO₂ [20]. Reaction volumes of 250 μ l include 100 μ l of cell lysate and, at final concentrations: 100 mM glycyl-glycine, pH 7.2, 0.2 mM pyridoxal

Figure 1: nuclease S1 protection assay. The assay for cellular ODC mRNA content was carried out as described in Materials and Methods. (A) Diagram of the mouse-specific products generated by the assay and detected on sequencing gels. (B) Detection of ODC mRNA in total cellular RNA extracted from cells expressing endogenous and transfected ODC genes. Lane a. 842 nt probe; b. unprotected probe subjected to S1 assay conditions; assays shown in lanes c–m contained total cellular RNA as noted: c, f. 1.0 μ g D4.1; d, g. 0.1 μ g D4.1; e, h 0.01 μ g D4.1; assays f, g & h contained, in addition, 10 μ g of C55.7 RNA; i. 10 μ g NIH3T3; j. 10 μ g 10001; k. 10 μ g C55.7; l. 10 μ g OD12.7; m. 10 μ g TK-ODC3'. The 484 and 358nt mouse-specific products are indicated. The band marked by an asterisk is of a size consistent with that for a hamster ODC fragment as predicted by sequence comparison [46,47].

phosphate, 4 mM dithiothreitol, 0.4 mM l-ornithine and 0.5 μ Ci (50 mCi/mmol) [14 C] l-ornithine (Amersham). Reactions were incubated at 37°C for 2 hours and terminated by heating at 85°C for 5 minutes. [14 C]CO₂ was collected and counted as described [20].

CAT [21] and β -galactosidase [22] assays were carried out as described. β -galactosidase activity is expressed as OD₄₂₀ $\times$ 100/ minutes $\times$ mg protein [22].

Nuclease S1 protection assay

[35 S]CTP-labelled cRNA was transcribed from the T7 promoter of ScaI-linearized

pT3od(rt)rna (Fig. 2j) using T7 RNA polymerase as recommended by the supplier (Bethesda Research Labs).

The nuclease S1 protection assay used to determine ODC mRNA content in total cellular RNA was carried out essentially as described [23]. In general, 10 μ g of cellular RNA, 10⁶ cpm (~10⁹ cpm/ μ g) of probe cRNA and 50 μ g of yeast carrier tRNA were co-precipitated, dissolved in 20 μ l hybridization buffer (80% formamide, 0.4 M NaCl, 1mM EDTA, 40 mM PIPES, pH 6.5) and hybridization allowed to proceed by stepwise heating at 85° for 15 minutes, then at 65°, 60°, 55° and 50° for 1 hour each. The reactants were diluted by addition of 160 μ l H₂O, 20 μ l of 10 $\times$ S1 buffer (300 mM sodium acetate, pH 4.6, 1M sodium chloride, 20mM zinc sulfate, 50% glycerol) and 1,000 U S1 nuclease (Sigma), then incubated at 37°C for 2 hours. The digests were extracted once each with phenol and chloroform, ethanol precipitated with 50 μ g carrier tRNA and re-dissolved in 10 μ l Maxam-Gilbert gel loading dye [24]. Hybridized strands were separated by heating at 90° for 3 minutes, resolved on 6% polyacrylamide sequencing gels and visualized by autoradiography.

The cRNA probe used was transcribed from a bacteriophage T7 promoter and is complementary to 842 bases at the 3' end of mouse ODC mRNA (Fig. 1A). The probe was constructed so as to contain a 150 nt insert of heterologous nucleic acid (derived from the Rous sarcoma virus reverse transcriptase (rt) gene) inserted within the 842 nt homology

Figure 2: Structure of the ODC Gene and of Derived Recombinant Constructs. A. pOD12.7 is a genomic ODC clone whose isolation from ODC-overproducing S49 mouse lymphoma cells has been described [9]. The 10 kb fragment includes 6.3 kb of transcribed DNA flanked 5' and 3' by 2.7 kb and 1.0 kb, respectively, of untranscribed, contiguous DNA. B. pOD-CAT was constructed by replacing the HSV-TK promoter of CAT expression vector pLSTK1ΔS/N [28] with ODC flanking region DNA extending 4.7 kb 5' from the Sal I site of exon 2. C. pTK-gODC was constructed by ligating sequentially into the polylinker of pUC18 a 160 bp Sst I HSV-TK promoter fragment and the 5.2 kb Sal I-Eco RI transcript fragment from pOD12.7. D. pOW16.5 is a genomic ODC construct in which the 5' flanking region of the cloned amplified gene (pOD12.7) is replaced with an analogous but larger fragment cloned from wild type S49 cell DNA. It was constructed by inserting the 6.4 kb Eco RI transcript fragment from pOD12.7 into the Eco RI site of the cloning vector, pOW8, which carries w.t. flanking region DNA extending 8 kb 5' to the Eco RI site of exon 1. The properly oriented insert reconstructs exon 1 of the transcript region. E. pOW12N is analogous to pOW16.5 but carries additionally, a 150 bp Nco I Rous sarcoma virus reverse transcriptase fragment inserted at the Nco I site of exon 12. The 5' junction of the insert creates a UGA opal codon so that translation of ODC mRNA stops prematurely at a.a. 425. Full length ODC is 461 a.a. long [46]. F. pOD-ODC3' is an ODC minigene. It carries in the polylinker of pUC19 sequentially from the 5' end of ODC, 280 bp of genomic DNA including the TATA homology and transcription start sites, 1.4 kb of ODC cDNA including the entire coding region, and 1.3 kb of genomic ODC DNA including the 3' untranslated (UT) region, two natural polyadenylation sites and 1kb of untranscribed DNA. G. pTK-ODC3' is analogous to pOD-ODC3' but substitutes the 160 bp HSV-TK promoter for the 280 bp ODC promoter. H. Construction of pODM3 has been described [14]. This construct uses the SV40 early region promoter to drive ODC cDNA expression. Signals for polyadenylation and transcription termination are provided by the hepatitis virus surface antigen gene 3' end. I. pODM3Δ3' was derived from pODM3 by in vitro mutagenesis. It has deleted all 3' UT ODC cDNA and contains of ODC only its coding region. J. pT3od(rt)rna is a dual promoter vector used to transcribe radio-labelled cRNA probes for the S1 protection assay. It was constructed by ligating into the polylinker of pT3/T7-18 a 1.6 kb ODC cDNA fragment that includes the entire coding region and 240 bp of 3' UT DNA. To allow distinction between transfected and endogenous ODC transcripts in NIH3T3 cells, a 150 bp Nco I fragment of the RSV reverse transcriptase gene was cloned into the Nco I site of ODC cDNA. K. p(OD)TK-CAT and p(DO)TK-CAT carry the ODC coding region DNA in both possible orientations 5' to the TK promoter-CAT gene construction of plasmid pLSTK1ΔS/N. p(OD)-CAT is a promoterless vector which ODC cDNA precedes the CAT gene. Thin lines: ODC non-transcribed DNA. Open-boxed regions: ODC un-translated mRNA. Filled-boxed: ODC coding. Sawtoothed Line: TK promoter. Wavy Line: SV40 early region promoter. Cross-lined box: CAT coding region. Cross-hatched box: HBV 3' end. Restriction sites indicated are: L-Sal I; R-EcoR I; T-Stu I; N-Nco I; P-Pst I; C-Sca I. RT = reverse transcriptase.

region for ODC. Correct hybridization of mouse ODC mRNA to the radioactive probe and nuclease S1 digestion yields labelled fragments of 484 and 358 nts (Fig. 1A). These fragments are detectable only in cells expressing mouse ODC; figure 1B shows that mouse NIH3T3 and CHO transfectant OD12.7 cells but not wild type CHO (10001) nor ODC⁻ CHO (C55.7) cells expressed such fragments.

To test the accuracy of the assay, ODC mRNA content was measured using 10-fold decrements of total RNA from D4.1 cells, a derivative of S49 mouse lymphoma cells. These cells have a greatly amplified ODC gene copy number and express about 300 times as much ODC mRNA as growing wild type S49 cells [13], as measured by Northern and dot blot analysis. Gel resolution and autoradiographic visualization of S1 digestion products showed that 10 μ g of RNA from exponentially growing OD12.7 and NIH3T3 cells produced 3–5 times greater signal intensity than produced by 0.01 μ g of RNA from D4.1 cells (Fig. 1B). The ratio for relative ODC mRNA levels in D4.1 versus NIH3T3 or OD12.7 cells found using the present assay is therefore comparable to our previous determinations using Northern blot analysis. The S1 assay does not lose sensitivity at lower relative ODC mRNA abundance; 10-fold decrements of D4.1 RNA mixed or not mixed with 10 μ g of RNA from ODC⁻ CHO cells produced similar signal intensities (Fig. 1B). The signal intensities produced by D4.1 RNAs indicate further that this assay for cellular ODC mRNA abundance was carried out in probe excess. We conclude that the S1 assay as used here accurately measures mouse ODC mRNA content in cellular RNA preparations. Intensity of autoradiographic bands was quantitated by densitometric scanning and weighing of the paper wedges representing the areas under the peaks.

RESULTS

The cloned mouse ODC gene is regulated by growth, serum and TPA.

The structure of the mouse ODC gene [9] is shown in Fig. 2 as pOD12.7. Included in this figure as well are the structures of the derivative recombinant DNAs constructed to analyze the regulation of ODC gene expression. The DNAs were transfected into ODC⁻ CHO cells [15]. These cells contain no detectable ODC activity and are auxotrophic for putrescine; they likely produce a mutant enzymatically inactive form of ODC.

When cultures of wild type CHO or of mouse NIH3T3 fibroblast cells grow to stationary phase, ODC expression decreases to low, even undetectable levels [2]. Stimulation of quiescent cultures by serum or TPA causes ODC expression to increase. We determined whether sequence information necessary for a response to TPA and serum was present in the cloned ODC gene. A population of ODC⁻ CHO cells was stably transfected with pOD12.7 genomic ODC DNA (Fig. 2a); these cells are designated OD12.7. We then compared the responses to serum and TPA elicited in the transfected cells to those of NIH3T3 cells expressing the endogenous mouse ODC gene. A time-course for the effects of TPA and serum on ODC enzyme activity and mRNA levels in quiescent cultures of OD12.7 and NIH3T3 cells is shown in Figure 3. When quiescent cells were treated with serum both the magnitude and time of induction of ODC activity were similar in NIH3T3 cells expressing an endogenous mouse ODC gene (Fig. 3A) and in OD12.7 cells expressing a transfected mouse ODC gene (Fig. 3B). In both cell populations ODC activity peaked in 4–6 hours in response to serum, then declined rapidly. Likewise, in response to TPA,

ODC activity in NIH3T3 (Fig. 3C) and OD12.7 cells (Fig. 3D) peaked after 4 hours, and then declined; in TPA-treated NIH3T3 cells, but not in OD12.7 cells, ODC activity rose a second time. These data argue that the molecular mechanisms responsible for serum- and TPA-mediated regulation in mouse and hamster fibroblasts are largely conserved. They indicate as well that the cloned mouse ODC gene carries the information necessary to be operated on by these mechanisms.

Figure 3 also shows the effect of serum and TPA treatment on ODC mRNA levels in NIH3T3 and OD12.7 cells. As described (Materials and Methods; Fig. 1), the protection assay used is sensitive and specific for mouse ODC mRNA. The mRNA levels increased to a maximum value at about the same time as the peak in activity, 4–6 hours after stimulation. However, the increases in ODC mRNA levels were small relative to the changes in enzymatic activity. At their peaks, the ODC activity in response to serum was induced 8-fold in OD12.7 cells and 13-fold in NIH3T3 cells relative to untreated cells, whereas mRNA levels changed by 1.5-fold and 3-fold, respectively. Similarly, in response to TPA, ODC activity increased by 13-fold in OD12.7 cells and 90-fold in NIH3T3 cells, but mRNA levels increased by 2- and 5–6 fold, respectively. The secondary peak in activity seen uniquely in TPA-treated NIH3T3 cells was not accompanied by a corresponding change in ODC mRNA level. It is clear from these comparisons that ODC activity changes to a greater extent than does the level of its mRNA when expression of either the endogenous or the transfected mouse gene is stimulated by treatment with serum or TPA.

Role of genomic 5' flanking sequence in regulation.

Serum and TPA-mediated changes in ODC mRNA levels could be carried out by transcriptional, transcript-processing or mRNA stabilization mechanisms. Precedents exist for serum [25] and TPA [26,27] response elements located near and influential on transcriptional promoters. We determined whether the 5' flanking region of the ODC gene could confer inducibility on a heterologous gene. A 4.7 kb fragment of ODC genomic DNA was inserted so as to activate the bacterial chloramphenicol acetyltransferase (CAT) structural gene (Fig. 2b, pOD-CAT). The fragment of the ODC gene extends 2.7 kb 5' to the TATA homology and transcription start sites and terminates at its 3' end at a Sal I site within the second exon encoding part of the untranslated leader sequence. We have shown previously that this fragment of the ODC gene is comparable in promoter activity to the Rous sarcoma virus long terminal repeat promoter and more active than the SV40 early region promoter in the transient expression assay using mouse cell recipients [9].

The chimeric gene, pOD-CAT, was tested for regulation in transient expression experiments [21] utilizing NIH3T3 and OD12.7 cells as recipients. As shown in Figure 2, the ODC gene, pOD12.7, used to transfect ODC⁻ cells to produce OD12.7 cells has

Figure 3: Time-course for effects of serum and TPA on ODC expression in OD12.7 and NIH3T3 cells. Cells were seeded at 3×10^6 per 100mm dish and allowed to grow to confluence (5 days). NIH3T3 cells were then treated with 1.6 μ M TPA final concentration and duplicate pairs of plates harvested periodically for assays of ODC enzyme and mRNA content. After cells reached confluence and 30 hours prior to TPA-stimulation of OD12.7 cells and to serum-stimulation of NIH3T3 and OD12.7 cells, their growth medium was replaced with fresh medium containing 0.5% fetal calf serum. Then TPA or serum were added to 1.6 μ M and 30% final concentrations respectively, and cells harvested as above. A,B: Serum-treated NIH3T3 and OD12.7 cells, respectively. C,D: TPA-treated NIH3T3 and OD12.7 cells, respectively. The insets of each graph show the effects of treatment times on ODC mRNA as reflected by the 358 nt protected cRNA fragment.

a 5' flanking sequence that is identical to that of pOD-CAT. By supertransfecting OD12.7 cells and measuring both CAT and ODC activities, we can therefore assess within the same cells the relative contribution of the ODC 5' flanking sequence to the overall regulation of ODC gene expression. NIH3T3 and OD12.7 cells were co-transfected with pOD-CAT and pRSV- β gal DNAs, the latter serving as a control for transfection efficiency [22]. The cells were then grown to quiescence, stimulated with TPA or serum for four hours, harvested and their extracts assayed for CAT, ODC and β -galactosidase activities.

When confluent cells were treated with serum or TPA, endogenous ODC activity in each cell type increased significantly (Table 1). CAT activity, however, was virtually unresponsive. Growth of cells to stationary phase greatly decreased ODC activity relative to that in exponentially growing cells, with no concomitant decrease in CAT activity. Additional evidence presented below will show that the effects of serum and TPA on ODC mRNA levels is partially determined by sequences present in ODC cDNA. We conclude that the cellular mechanisms that change ODC mRNA levels in response to TPA or serum

TABLE 1. Regulation of OD-CAT by Serum and TPA^a

Cells	Condition	CAT ^b Activity	β gal Activity	CAT/ ^c β gal	ODC ^d Activity
NIH3T3					
	growing	8	119	1.8	89
	confluent	14	165	2.2	4
	serum 4 hrs	16	166	2.4	156
	8	18	211	2.1	2
	12	15	144	2.6	17
	TPA 4	14	153	2.3	85
	8	12	144	2.1	9
	12	14	176	2.0	23
	serum + TPA 4 hrs	15	163	2.3	139
OD12.7					
	confluent	4.3	9	13	1
	+ 0.5% serum	6.1	13	12	8
	+ serum 4hrs	4.0	11	10	34
	6	5.0	12	10	46
	8	2.7	9	8	20
	+ TPA 4	5.6	11	13	26
	6	6.6	12	14	13
	8	9.0	15	15	8

a. 3×10^6 NIH3T3 or OD12.7 cells were seeded per 100 mm dish. The cells were transfected 24 hours later with 5 μ g each of pOD-CAT and pRSV- β gal DNA per plate. 'Growing' NIH3T3 cells were harvested 24 hours after transfection. Four days after transfection, confluent NIH3T3 cells were treated for the times indicated with 30% serum, 1.6 μ M TPA or both. Four days after transfection, the medium of confluent OD12.7 cells was changed to that containing 0.5% serum and 30 hours later the cells were treated with 30% serum or 1.6 μ M TPA. ODC, CAT and β gal assays were performed as described in Material and Methods. β -gal assays measured the activities in 50 μ l of cytosolic extract, CAT assays measured the activity of 40 μ g cytosolic protein.

b. % conversion of chloramphenicol to acetylated chloramphenicol forms

c. CAT activity normalized to protein content then divided by β gal activity.

d. pmol/ min/ mg protein

stimulation of cells have no components detectable in the cloned 5' flanking region of the ODC gene tested here.

Role of sequences present in ODC cDNA in regulation

We next tested ODC sequences distal to the promoter for regulation. Specific regions of the ODC gene were systematically removed or replaced with heterologous DNA. The responses to serum and TPA of such manipulated genes were tested in stably transfected cells. Fig. 2 depicts five constructs used in the analysis: First, pTK-gODC is a chimeric fusion gene that includes a 160 bp promoter region fragment from the herpes simplex virus thymidine kinase (HSV-TK) gene [28] placed 5' to a 5.2 kb fragment of ODC genomic DNA that extends 3' from the Sal I site within exon 2. This 3' transcribed region of the ODC gene exactly complements the un-inducible promoter region fragment of the ODC gene used in the CAT transient expression experiments described above. Second, pOD-ODC3' is an ODC intronless 'minigene' which has 280 bp of the ODC promoter region, including the TATA homology sequence and transcription start site, ODC cDNA including

Figure 4: Regulation of ODC by cell growth in cDNA transfectants. Growing and confluent cells were harvested and their extracts assayed for ODC enzyme and mRNA content as described in the text. (Above) ODC activity in growing (+) and stationary (-) cells; (Below) ODC mRNA in corresponding extracts from growing (+) and stationary (-) cells. The 484nt and 358nt mouse ODC bands are indicated. Note the absence of the protected 358nt fragment in lanes corresponding to cells transfected with pODM3Δ3, from which sequences protecting that portion of the probe were deleted.

all signals necessary for translational initiation and termination, poly A addition and about one kilobase of untranscribed 3' sequence. Third, pTK-ODC3' is the same minigene as pOD-ODC3', but substitutes the 160 bp HSV-TK for the 280 bp ODC promoter region. Fourth, pODM3 uses the SV40 early region promoter to drive an ODC cDNA that terminates just 5' to its natural polyadenylation site; polyadenylation and transcription termination signals are provided by the 3' end of the hepatitis B virus surface antigen gene. Fifth, pODM3Δ3' was obtained by deleting from pODM3 all 3' untranslated ODC cDNA. Therefore, pODM3Δ3' contains, of the ODC gene, only the protein-coding region. Stably transfected populations of CHO ODC⁻ cells were established with each of these constructs.

We assessed whether regulation of ODC expression by growth is retained in these constructs. ODC activity and mRNA levels were measured in growing and confluent transfectants. In this case, 3×10⁶ cells were plated per 100 mm dish and, without further treatment, harvested either 1 or 5 days later. The data of figure 4 shows that all constructs are growth-regulated. It can be seen also that ODC activity undergoes very large changes in magnitude (> 1000-fold for construct pTK-gODC for example) accompanied by only modest changes in ODC mRNA level. Lastly, this experiment shows that regulation of ODC activity in these constructs is very 'tight' in that activity in non-growing cells decreases to zero, or close to it.

Is regulation of ODC by mitogens encoded within the gene sequence?

Transfectants grown to quiescence were stimulated for four hours with serum or TPA, harvested and their extracts assayed for ODC activity and mRNA content (Table 2). ODC activity was low in all transformants not subjected to mitogen treatment. ODC activity in all transfectants increased following serum or TPA stimulation. It follows, therefore, that the ODC protein coding region itself encodes information sufficient to manifest these forms of regulation. Analysis of mRNA content showed, for these constructs, as for the intact ODC gene, that changes in ODC mRNA levels may occur, but account for only a fraction of the change in ODC enzymatic activity (Table 2).

Superinduction of ODC mRNA by cycloheximide in transfectants

The fact that we observed induction of ODC mRNA in cells expressing these constructs, even to such a small degree, was somewhat unexpected. We asked whether other manipulations known to induce ODC mRNA would also do so in these transfectants. ODC

Table 2: Regulation of ODC by Serum and TPA in cDNA Transfectants^a

Transfectants	Stationary	ODC Expression				
		Enzyme Activity ^b		Relative mRNA levels ^c		
		FCS	TPA	Stationary	FCS	TPA
TK-gODC	7	81	59	1.00	1.63	1.16
OD-ODC3'	2	66	23	1.00	1.12	1.08
TK-ODC3'	3	38	13	1.00	1.04	1.32
ODM3	9	69	16	1.00	1.60	N.D.
ODM3Δ3'	5	69	23	1.00	1.00	N.D

a C_{55.7} ODC⁻ CHO cells were stably transfected with the indicated plasmids. Cells were brought to a stationary condition by growth to confluence, followed by maintenance in medium containing 0.5% FCS for 30 hours. Stationary cells were treated for 4 hours with serum or with TPA, as described in Materials and Methods.

b pmol/ min/ mg protein

c determined by densitometric scanning of autoradiograms

mRNA levels increase when protein synthesis is inhibited [3,4]. Simultaneous treatment of cells with mitogen and cycloheximide, a translational inhibitor, appears to super-induce ODC mRNA [3,4]. We used the chimeric fusion genes to ask what regions of ODC were required to super-induce ODC mRNA in response to serum plus cycloheximide treatment. Quiescent transfectants were treated for 4 hours with serum or serum plus cycloheximide, total RNAs were isolated and relative ODC mRNA levels were determined. ODC mRNA was super-induced in all transfectants examined (fig. 5). The nature of the flanking sequences used to govern transcription initiation and termination proved irrelevant. The minimum information required for super-induction appears to be encribed within ODC cDNA. Similar results were obtained when cells were treated with TPA and cycloheximide (data not shown).

Does ODC cDNA contain an inducible enhancer?

ODC mRNA levels are regulatable in CHO cells stably transformed with constructs containing mouse ODC cDNA. This result suggested that ODC cDNA might contain a serum or growth responsive enhancer element. To test this, we asked if ODC cDNA could affect the activity of a heterologous, constitutive promoter using CAT as a reporter gene. A 1.6 kb DNA fragment encompassing ODC cDNA was inserted 5' to a HSV-TK promoter-CAT fusion gene [28], as depicted in Fig. 2k (p(OD)TK-CAT). Wild-type CHO (ODC⁺) cells were co-transfected with this and pRSV- β gal DNAs. Two days later, the cells were treated with serum. After another four hours, the cells were harvested and their extracts assayed for ODC, CAT and β -galactosidase activities. The response of the activity encoded by the endogenous ODC gene to growth and serum treatment is marked (Table 3). However, no condition that was found to change ODC activity produced a change in CAT expression

Figure 5: Effect of cycloheximide and serum treatment on ODC mRNA in cDNA transfectants. The growth medium of confluent cells was replaced with medium containing 0.5% serum for 30 hours. Serum and cycloheximide (CHX) were then added at 30% and 100 μ g/ml final concentrations, respectively, and incubated an additional 4 hours. The cells were then harvested, their RNAs extracted and assayed for ODC mRNA abundance. The bands depicted represent the 484 nt products of the S1 assay. N.D.: not done.

for any of the constructs examined. We conclude that this assay detected no growth- or serum-responsive enhancer element in ODC cDNA.

ODC Lability and its Effect on Regulation

The rapid and transient nature of ODC induction by mitogens associates it with growth regulatory phenomena. Such behavior does not, however, imply the presence of a mechanism that is specifically regulatory. Schimke [29] noted that a polypeptide with a short half-life could rapidly and transiently rise in abundance simply as the result of an increase in the overall rate of protein synthesis. The turnover of ODC is among the most rapid known for an enzyme, with a half-life of less than one hour [30]. Mitogenic stimulation increases the rate of protein synthesis. We tested the idea that the characteristic induction of ODC by mitogens is a byproduct of its short half-life.

We have shown that an ODC polypeptide with a carboxyl terminal deletion is enzymatically active and stable when expressed in animal cells [14]. Two genomic constructs were employed that encoded wild type (labile, pOW16.5) and mutant (stable, pOW12N) ODCs (Fig. 2d,e). Stable transfectants bearing these plasmids were grown to quiescence and then stimulated with serum or TPA and the time course for induction of ODC activity followed. As shown in figure 6, serum-stimulation of cells expressing stable ODC resulted in a rapid but persistent induction of ODC activity. TPA-stimulation of the same cells resulted in ODC induction that had kinetics similar to those of cells expressing unstable ODC, but the decline from peak levels was slowed. Peak levels of enzymatic activity were similar in both cell types, but initial levels were higher in the transfectants expressing stable

TABLE 3. Test of ODC cDNA for presence of an enhancer^a.

Construct	Condition	CAT ^b Activity	β gal ^b Activity	CAT ^b / β gal	ODC ^b Activity
TK-CAT	growing	1.0	.12	8.3	103
	confluent	1.4	.22	6.4	7
	" + serum	1.2	.44	2.7	80
(OD)-CAT	growing	.4	.30	1.3	113
	confluent	.3	.48	.6	13
	" + serum	.3	.62	.5	92
(OD)TK-CAT	growing	1.4	.42	3.3	116
	confluent	1.7	.43	4.0	14
	" + serum	1.6	.59	2.7	73
(DO)TK-CAT	growing	1.0	.17	5.9	102
	confluent	1.1	.38	2.9	7
	" + serum	1.2	.58	2.1	87

a. 100mm plates were each seeded with 3×10^5 or 3×10^6 10001 cells. The cells were transfected 24 hours later with 5 μ g/plate of the relevant CAT plasmid plus 5 μ g/plate pRSV- β gal DNA. After 44 hours, the medium of the confluent cells was replaced with 10ml fresh medium plus 20% serum. Four hours later all cells were harvested and their extracts prepared for enzyme assay.

b. As in Table 1.

ODC. The magnitude of induction in response to both serum and TPA was therefore less in those cells. The loss of some but not all induction of ODC that results from conversion of the enzyme from an unstable to a stable form indicates that mitogen-dependent induction is largely but not wholly dependent on enzymatic lability.

DISCUSSION

A series of recombinant DNAs encoding mouse ODC were transfected into CHO cells. All of the transfectants displayed regulation of ODC activity and shared faithfully two salient characteristics of that regulation exhibited by mouse NIH3T3 cells expressing the endogenous gene: First, ODC mRNA and ODC enzymatic activity were induced with similar kinetics and second, the latter changed much more than the former. These two attributes persisted even when the recombinant constructs used were deprived of all ODC-specific sequence information except the protein-coding region. We found no evidence

Figure 6: Induction of ODC activity by serum and TPA in cells expressing stable ODC polypeptide. The growth medium of confluent cells was changed to 0.5% serum 30 hours prior to stimulation. Serum or TPA were then added to 30% and 1.6 μ M final concentrations, respectively. The panels show the effects of serum (A) and TPA (B) treatments on ODC activity in cells transfected with pOW16.5 (filled symbols) and pOW12N (open symbols).

that the sequences flanking the coding region participate in regulation of ODC activity when cells are treated with serum or TPA or undergo cell- density-dependent changes in growth.

We and other workers have shown that post-transcriptional and post-translational mechanisms as well as transcriptional changes determine ODC steady-state enzymatic activity [3,4,7,31,32]. (There is good evidence that indicates that enzymatic activity is exclusively determined by the number of ODC protein molecules [6,33]). In any given system in which ODC activity can be made to change, those changes may be dependent to a greater or lesser extent on alterations in mRNA levels.

There are several published descriptions of induction of ODC mRNA and enzymatic activity containing sufficient quantitative data to assess whether changes in the two are similar in timing and extent. The question was answered affirmatively by Feinstein et al. [34], who studied the response of PC12 cells to nerve growth factor and by Hsieh and Verma [7], who examined the process of induction by TPA in T24 human bladder carcinoma cells. In other cases the magnitude of induction of activity exceeded that of mRNA. These instances include treatment of BALB/c 3T3 fibroblasts by TPA or by purified peptide growth factors [3], treatment of HE68BP transformed hamster fibroblasts with TPA [6] and Reuber H35 rat hepatoma cells with TPA [35]. A number of investigators have shown using run-on transcription that inductions of ODC mRNA are associated with enhanced transcription [3,4,8,32]. In contrast, investigation of mRNA induction by androgen in mouse kidney revealed no change in transcription rate as assessed by run-on studies [36,37]. In that same system, *in vivo* labelling with [³H]-orotate indicated a 5.9-fold induction of synthesis [38]. The increased levels of ODC mRNA appeared to be due, at least in part, to its stabilization.

NIH3T3 cells or transfected CHO cells grown to quiescence and then treated with TPA or with serum, the systems we describe here, exhibit changes in mRNA that account for only a minor portion of the induction of activity. Translational regulation or regulation carried out at the level of the protein itself could plausibly account for the inductive process. Because ODC protein stability is subject to regulation [31] and because ODC contains a structural domain that mediates stability [14], the latter hypothesis deserved particular attention. We recently described a 37 amino acid carboxyl-terminal truncation of ODC that leaves enzymatic activity intact but converts ODC from an unstable to a stable protein within cells (14). We have shown here that the same mutation greatly attenuates the response of ODC to serum and TPA stimulation. This result strongly suggests that post-translational mechanisms that alter the intracellular stability of ODC are responsible for much of the change in activity elicited by serum and TPA.

In the system examined here, mRNA levels are also modulated, although to a minor degree compared to changes in activity. This modulation persists even in transfectants expressing recombinant constructs shorn of elements of the ODC gene that plausibly determine transcription rate. Conversely, fragments of ODC genes that were alluring candidates as promoter or enhancer elements failed to display regulated activity with respect to heterologous genes. This suggests that the protein-coding region of the ODC gene also determines a regulated process that may affect the efficiency of conversion of transcripts to mRNA or the stability of mRNA.

Simultaneous treatment of quiescent cells with cycloheximide and serum or TPA super-induces ODC mRNA [3,4]. This effect was inferred to result from an increase in ODC transcription rate [4]. We have shown here, however, that ODC mRNA levels in similarly treated cDNA minigene transfectants can also be superinduced. Since the response does

not depend on the presence of ODC-specific promoter elements, translational inhibition can also act to increase ODC mRNA levels by post-transcriptional as well as transcriptional mechanisms.

Induction of ODC activity and mRNA by TPA appears uniformly reduced in transfectants (2–13 fold induction of activity; fig. 3, table 2) as compared to NIH3T3 cells (20–90 fold induction of activity; fig 3, table 1). The loss of inducibility may reflect: 1. a species difference that results in less efficient interaction of hamster regulatory molecules with mouse nucleic acids or polypeptides; 2. a competition for limiting numbers of regulatory molecules between the products expressed from transfected and endogenous genes in transfected cells; 3. the existence of additional regulatory sequences that lie outside the regions we have cloned; 4. a tissue-specific difference in regulation between fibroblastic (NIH3T3) and ovarian (CHO) cells.

ODC shares a series of interesting regulatory properties with thymidine kinase (TK). Like ODC, serum-induced changes in chicken [39] and mouse [40] TK mRNAs are smaller than changes in activity. Regulation of cytosolic TK in mouse L-MTK⁻ [40] and rat-4 [41] cells has been shown, by methods similar to those reported here, to have components localized within the protein coding region. The mouse TK gene contains additional determinants outside the coding region that regulates an additional 4 to 5-fold change in mRNA levels. This remaining component may not be transcriptional, because chicken TK mRNA levels were found to change with no alteration in transcription run-on rates [42]. Human TK changes during cell-cycle progression could be accounted for almost entirely by changes in polypeptide synthetic rate and stability [43].

Little is known of the specific mechanism of each of the multiple components that can determine ODC activity. The relative contribution of each component in modulating enzymatic activity has been determined in only a few specific cases. It is important to note that ODC expression is diversely regulated in different systems. In particular, transcriptional regulation of ODC was found in several mammalian cell types to be associated with a response of ODC to diverse stimuli. It is necessary to apply genetic and reverse genetic tests to determine the significance of these findings. This paper, to our knowledge, represents the first application of such methods to mammalian ODC.

We initiated a study of the response of ODC to mitogens anticipating that it would share important elements of regulation with the proto-oncogenes *c-fos* and *c-myc*. Instead, the dissimilarities proved more apparent. TPA and serum act through well-described transcriptional mechanisms to drive *c-fos* and *c-myc* expression [25,26,27,44,45]; we found little evidence that similar mechanisms regulate ODC. We have shown instead that TPA and serum can exert a strong regulatory influence on the expression of ODC through post-transcriptional mechanisms and that mitogenic induction of ODC activity depends at least in part on lability of the enzyme. This suggests that ODC activity is modulated in these cases by mechanisms that alter the rate or extent of enzymatic degradation, a hypothesis that requires further experimental investigation.

ACKNOWLEDGEMENTS

This work was supported by grants to P.C. from the NIH (CA29048 and CA47721) and NSF (DCB 8707375).

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