Importance of the 3' untranslated region of ornithine decarboxylase mRNA in the translational regulation of the enzyme

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Translational regulation of ornithine decarboxylase (ODC), which catalyses the first step in the biosynthesis of polyamines, appears to be an important mechanism in the strong feedback control as well as in the hypotonic induction of the enzyme. However, the exact mechanisms are not yet understood. The ODC mRNA has long 5' and 3' untranslated regions (UTRs) which may be involved in the translational control of the enzyme. In the present study we have used a series of stable transfectants of Chinese Hamster ovary cells expressing ODC mRNAs with various truncations in the 5' and 3' UTRs to investigate the importance of these regions. It is demonstrated that neither the 5' UTR nor the 3' UTR appears to be involved in the polyamine-mediated feedback control of ODC synthesis.

The hypotonic induction of ODC, on the other hand, was shown to be highly dependent on the presence of the 3' UTR, but not on the 5' UTR, of ODC mRNA. Cells expressing ODC mRNAs lacking the 3' UTR showed no, or only a very slight, induction of ODC whether the 5' UTR was present or not, whereas the cell lines expressing ODC mRNAs containing the 3' UTR (with or without the 5' UTR) markedly induced ODC after a hypotonic shock. The present finding of a role for the ODC mRNA 3' UTR in the hypotonic induction of ODC is the first demonstration of a specific effect of the 3' UTR in the regulation of ODC.

Key words: Chinese Hamster ovary cells, feedback regulation, osmotic stress, polyamines.

INTRODUCTION

The polyamines putrescine, spermidine and spermine are known to play essential roles in the proliferation and differentiation of cells of various species [1,2]. The importance of the polyamines in cell function is reflected in a strong regulation of their intracellular levels [1,3]. Mammalian ornithine decarboxylase (ODC; EC 4.1.1.17), which catalyses the first and rate-limiting step in the polyamine biosynthetic pathway, is subject to a tight regulation. The regulation of ODC takes place at several levels, including transcriptional, translational and post-translational [1,3,4]. The enzyme has a very high turnover rate with a half-life usually ranging from a few minutes to an hour [5]. ODC is, like other mammalian proteins with a short half-life, degraded by the 26 S proteasome [6]. However, in contrast with other proteins degraded by the 26 S proteasome, the degradation of ODC is not triggered by ubiquitination [6,7]. Instead, the degradation of ODC is induced by the binding of a specific protein, named antizyme, to the enzyme [8]. The production of antizyme, and thus the degradation of ODC, is stimulated by polyamines in a process involving ribosomal frameshifting [9,10]. This was the first example of a mammalian ribosomal frameshifting to be described. The induction of ODC by a variety of growth factors is, at least partly, explained by an increased transcription of the gene [4], although a stabilization of the ODC mRNA has also been described to contribute in some systems [11]. The cellular increase in ODC activity is often also partly explained by a stabilization of the enzyme against degradation. However, in some experimental systems the increase in ODC activity cannot

be fully explained by an increase in ODC mRNA and/or a stabilization of the enzyme protein, indicating also regulation at the translational level [12,13].

The polyamines exert a strong feedback regulation of ODC, which appears to partly occur at the translational level [1,3]. Treatment with inhibitors of polyamine synthesis results in a compensatory increase in the synthesis of ODC, as measured by pulse-labelling. Conversely, ODC synthesis is repressed when there is a rise in the cellular polyamine content. These polyamine-mediated changes in ODC synthesis are not correlated with any change in the amount of ODC mRNA, indicating a translational mechanism.

Cellular ODC activity has also been shown to be strongly affected by the osmolarity of the growth medium [14–17]. A marked increase in ODC activity is usually observed after exposure to a hypotonic medium. The mechanisms by which a hypotonic shock induces ODC activity are not fully understood. In some experimental systems, the phenomenon is explained by a change in the ODC mRNA content [16], whereas in others it appears to involve mainly, if not exclusively, translational and post-translational events [15,17].

Like many other mRNAs that code for proteins that are important for cell viability and proliferation, ODC mRNA has a long (close to 300 nt) GC-rich 5' untranslated region (UTR), which is predicted to form extensive secondary structures [18,19]. The 5' UTR of ODC mRNA has been shown to greatly hamper the translation of the message [20,21]. Most of the ODC mRNA in the cell is associated with monosomes or ribosomal subunits indicating a poor translation [22,23]. The strong repression *in*

Abbreviations used: ODC, ornithine decarboxylase; UTR, untranslated region; CHO, Chinese Hamster ovary; DFMO, α -difluoromethylornithine; ARE, AU-rich element.

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vivo of ODC mRNA by the secondary structure in the 5' UTR was further supported by the finding of a markedly elevated ODC activity in cells overexpressing the initiation factor eIF-4E, which is believed to be involved in the melting of secondary structures of mRNA 5' UTRs [24].

The 3' UTR of ODC mRNA is also relatively long (300 nt), but has less potential to form stable secondary structures than the 5' UTR [25,26]. However, it has been demonstrated in various expression systems that the 3' UTR may interact with the 5' UTR of ODC mRNA in such a way that the repressive effect of the 5' UTR on translation is relieved [20,27].

In the present work we have studied the importance of the ODC mRNA 5' UTR and 3' UTR in the translational control of the enzyme exerted by polyamines or occurring after exposure to hypotonic shock.

MATERIALS AND METHODS

Materials

The expression vector pSVL, which contains the simian virus 40 late promoter as well as origin of replication, was obtained from Pharmacia. ODC-deficient Chinese Hamster ovary (CHO) C55.7 cells and the cDNA encoding hamster ODC mRNA 5' UTR were kindly provided by Dr Immo E. Scheffler (University of California, San Diego, CA, U.S.A.) [28,29]. Human ODC cDNA was a generous gift from Dr Olli A. Jänne (University of Helsinki, Finland) [30]. Restriction enzymes, DNA ligase and *Taq* DNA polymerase were obtained from Boehringer Mannheim. Pfu polymerase was purchased from Stratagene. L-[1-¹⁴C]Ornithine (57 Ci/mol) and L-[³⁵S]methionine (1000 Ci/mmol) were obtained from New England Nuclear and Amersham, respectively. α -Difluoromethylornithine (DFMO) was a gift from Marion Merrel Dow (Cincinnati, OH, U.S.A.).

Cell culture

ODC-deficient CHO C55.7 cells [28] and parental wild-type CHO cells were grown in Dulbecco's minimal essential medium/Ham's F12 (1:1) containing 10% fetal-calf serum, non-essential amino acids and antibiotics (50 units/ml penicillin and 50 μ g/ml streptomycin). The CHO C55.7 cells were routinely grown in the presence of 0.5 mM putrescine [28]. The cells were cultured at 37 °C in a humidified incubator with 5% CO₂. All cells were seeded at a density of 10–20 $\times 10^3$ /cm², and subcultured every 3 or 4 days. DFMO-resistant cells were selected by growth in medium containing increasing concentrations (up to 5 mM) of DFMO. These cells were then routinely grown in the presence of 2–5 mM DFMO. For experiments, the growth medium was supplemented with 1 mM aminoguanidine to inhibit the production of toxic products by serum amine oxidases. In order to achieve a hypotonic shock, the cells were exposed to an NaCl-free RPMI 1640 medium for 6 h. The hypotonic medium had an osmolality of about 100 mOsm/l, when compared with the normo-osmotic value of 325 mOsm/l.

DNA constructs

All plasmid constructs are illustrated schematically in Figure 1. The various truncations in the ODC cDNA were made either by PCR or by restriction enzyme digestion. The cDNAs were then subcloned into the mammalian expression vector pSVL. The following constructs were made: pODC, giving rise to a full-length hybrid ODC mRNA containing the hamster 5' UTR ligated to the human ODC coding region and 3' UTR (using a

common *StyI* site); pODC- Δ (29)5', corresponding to an ODC mRNA containing the 29 most 3' nucleotides of the 5' UTR, the complete coding sequence and the 3' UTR; pODC- Δ 5', which was devoid of the 5' UTR but contained the coding sequence and the 3' UTR; pODC- Δ 3', which contained the complete 5' UTR and the coding sequence but lacked the 3' UTR; pODC- Δ 5'-3', which was devoid of both the 5' UTR and 3' UTR but contained the complete coding sequence; pODC- Δ 5'-tr, giving rise to an ODC mRNA containing the 3' UTR but lacking the 5' UTR and the first 30 nucleotides of the coding sequence; pODC- Δ 3'-tr, corresponding to an ODC mRNA in which the last 111 nucleotides of the coding sequence as well as the complete 3' UTR were deleted.

Transfection

Transfection was performed by electroporation using a BioRad gene pulser. Before transfection, CHO C55.7 cells grown for 3 days were harvested, washed and resuspended in 0.8 ml of fresh growth medium at a density of 10 $\times 10^6$ cells/ml. The cells were mixed with 15 μ g of linearized DNA, incubated on ice for 5 min, and then pulsed with 0.3 kV at 250 μ F. Following a 10 min recovery on ice, the cells were seeded in fresh growth medium. Cells stably expressing an active ODC enzyme were selected by growing the cells without putrescine, and cloned cell lines were thereafter obtained by dilution cloning.

Determination of ODC activity

The ODC activity was determined by measuring the release of ¹⁴CO₂ from carboxy-labelled ornithine. Briefly, cells were sonicated in ice-cold 0.1 M Tris/HCl (pH 7.5), containing 2.5 mM dithiothreitol and 0.1 mM EDTA. After centrifugation at 30000 g for 20 min at 4 °C, ODC activity was determined in aliquots of the supernatants by measuring the release of ¹⁴CO₂ from L-[1-¹⁴C]ornithine in the presence of saturating levels of pyridoxal 5'-phosphate (0.1 mM) and ornithine (0.5 mM).

Determination of ODC synthesis

Synthesis of ODC was determined by measuring the incorporation of [³⁵S]methionine into the enzyme. The cells were thoroughly washed and pre-incubated for 10 min at 37 °C in methionine-free medium. Thereafter L-[³⁵S]methionine (10–50 μ Ci/ml) was added to the medium. In the experiments measuring ODC synthesis in cells treated with hypotonic medium, the medium was changed into a methionine-free medium diluted with water to an osmolality of about 100 mOsm/l before L-[³⁵S]methionine (30–50 μ Ci/ml) was added. The incubation with L-[³⁵S]methionine was carried out for 20 min. The incorporation of radioactivity was stopped by the addition of one volume of ice-cold medium containing an excess of methionine. The cells were harvested, on ice, and collected by centrifugation at 1000 g for 10 min at 4 °C. Extracts prepared by sonication in 0.1 M Tris/HCl (pH 7.5) containing 2.5 mM dithiothreitol, 0.1 mM EDTA and 1 mM methionine were centrifugated at 30000 g for 20 min at 4 °C. The supernatants were collected and aliquots containing equal amounts of acid-insoluble radioactivity were incubated with an excess of anti-ODC antibody [31] for 30 min at room temperature. The antibody-ODC complexes were precipitated by incubation with bacterial Protein A adsorbent for an additional 30 min. After thorough washing in 10 mM Tris/HCl (pH 7.5) containing 0.1 mM EDTA, 0.5 mM dithiothreitol, 0.02% (w/v) BSA, 0.1% (v/v) Triton X-100, 0.1% (w/v) SDS and 0.1% (v/v) Tween 80, the precipitate was separated by

Figure 1 Schematic representation of the various DNA constructs used for transfection

SDS/PAGE (10 % gel). Proteins were revealed by fluorography after incubating the gels in Amplify (Amersham). Relative measurements of ODC synthesis were obtained by densitometric scanning.

Northern-blot analysis

Total cellular RNA was isolated using a SV total RNA isolation system (Promega) and size-fractionated on 1 % agarose gel electrophoresis in the presence of formaldehyde. The RNA was blotted on to a Hybond N membrane and hybridized with a ^{32}P -labelled hamster ODC cRNA. After washing the membrane at high stringency, the radioactive bands were revealed by autoradiography. Relative measurements of ODC mRNA were obtained by densitometric scanning.

Miscellaneous methods

Cellular polyamine content was determined essentially as described in [32]. Protein concentrations were measured by the method of Bradford [33].

RESULTS

In order to elucidate potential roles of mammalian ODC mRNA UTRs in the translational control of the enzyme, ODC cDNA constructs with various truncations in the 5' UTR, coding region and 3' UTR were made and subcloned into the mammalian expression vector pSVL (for details see the Materials and methods section). ODC-deficient CHO cells were then stably transfected with the various ODC cDNA constructs. Cell lines expressing active ODC were isolated by selection for growth in the absence of putrescine. It should be noted that the two constructs, pODC-Δ-5'-tr and pODC-Δ-3'-tr, coding for partly truncated ODCs, each gave rise to enzymically active enzymes. The various stable transfectants exhibited slight differences in their ODC activity, presumably reflecting changes in the site of genomic incorporation. However, the cells transfected with pODC-Δ-3'-tr

contained much higher ODC activity than all of the other cell lines (including wild-type CHO cells) (Figure 2). The reason for this was most likely that pODC-Δ-3'-tr gave rise to a C-terminally truncated ODC, which was metabolically stable (results not shown).

Polyamine-mediated downregulation of ODC activity

Wild-type CHO cells and stable transfectants expressing ODC mRNAs with the various truncations were grown in the absence or presence of 10 μM spermidine for 24 h, whereupon the ODC activity was measured (Figure 2). In all of the stable transfectants, except in cells expressing pODC-Δ-3'-tr, ODC activity was downregulated to almost undetectable levels after treatment with spermidine. In the cells expressing an ODC mRNA devoid of its 3' UTR and coding for a C-terminally truncated ODC (pODC-Δ-3'-tr) ODC activity was only reduced by 36 % when spermidine was added to the medium (Figure 2). Otherwise, no major differences in ODC response to spermidine were observed between the stable transfectants and the wild-type CHO cells.

Table 1 shows the changes occurring in cellular polyamine content when the cells were grown in the presence of 10 μM spermidine for 24 h. As expected, there was an increase in spermidine content, which presumably caused the downregulation of ODC. In addition, spermidine treatment appeared to cause a minor decrease in putrescine and spermine content (Table 1). The polyamine pattern and the changes induced by spermidine did not differ markedly between the various cells used in the study.

Polyamine-mediated downregulation of ODC synthesis

The downregulation of ODC by polyamines has been shown to be partly due to increased degradation of the enzyme and partly due to decreased ODC mRNA translation [1,3,5]. In order to determine the importance of the ODC mRNA 5' UTR and 3' UTR in the feedback control of ODC translation, a measure of the synthesis of the enzyme was required. However, synthesis

Figure 2 Effects of spermidine on ODC activity in CHO cells and stable transfectants of the C55.7 CHO cell line expressing ODC mRNAs with various truncations

Cells were grown in the absence or presence of 10 μ M spermidine (SPD) for 24 h before being analysed for ODC activity (means $\pm$ S.E.M., $n = 6$).

Table 1 Effects of spermidine treatment on cellular polyamine content

CHO cells and stable transfectants of the C55.7 CHO cell line expressing ODC mRNAs with various truncations were grown in the absence or presence of 10 μ M spermidine (Spd) for 24 h before being analysed for polyamine content (nmol/mg of protein; means $\pm$ S.E.M., $n = 4$).

Cells	Treatment	Polyamine content (nmol/mg)		
		Putrescine	Spermidine	Spermine
CHO	—	1.58 $\pm$ 0.12	11.5 $\pm$ 0.3	5.5 $\pm$ 0.3
CHO	Spd	0.10 $\pm$ 0.06	14.1 $\pm$ 0.6	5.3 $\pm$ 0.1
pODC	—	1.17 $\pm$ 0.17	10.5 $\pm$ 1.4	7.0 $\pm$ 0.4
pODC	Spd	1.23 $\pm$ 0.19	16.3 $\pm$ 1.9	5.0 $\pm$ 0.3
pODC- Δ (29)5'	—	0.99 $\pm$ 0.06	9.0 $\pm$ 0.7	6.0 $\pm$ 0.3
pODC- Δ (29)5'	Spd	0.82 $\pm$ 0.13	12.5 $\pm$ 1.0	4.2 $\pm$ 0.4
pODC- Δ 5'	—	2.92 $\pm$ 0.18	12.3 $\pm$ 0.2	5.1 $\pm$ 0.3
pODC- Δ 5'	Spd	0.63 $\pm$ 0.12	15.3 $\pm$ 1.3	4.6 $\pm$ 0.3
pODC- Δ 5'-tr	—	0.56 $\pm$ 0.02	7.5 $\pm$ 0.1	6.3 $\pm$ 0.1
pODC- Δ 5'-tr	Spd	0.87 $\pm$ 0.06	13.0 $\pm$ 1.2	4.4 $\pm$ 0.3
pODC- Δ 3'-tr	—	1.40 $\pm$ 0.12	9.0 $\pm$ 1.0	5.9 $\pm$ 0.7
pODC- Δ 3'-tr	Spd	0.70 $\pm$ 0.04	12.6 $\pm$ 1.4	4.5 $\pm$ 0.3
pODC- Δ 3'	—	1.51 $\pm$ 0.09	9.8 $\pm$ 0.7	5.6 $\pm$ 0.3
pODC- Δ 3'	Spd	0.51 $\pm$ 0.04	13.7 $\pm$ 0.7	4.7 $\pm$ 0.2
pODC- Δ 5'-3'	—	1.45 $\pm$ 0.09	9.4 $\pm$ 0.9	6.8 $\pm$ 0.7
pODC- Δ 5'-3'	Spd	0.21 $\pm$ 0.12	15.3 $\pm$ 1.3	6.3 $\pm$ 0.5

of ODC in the cell is normally very low and is therefore difficult to measure. Furthermore, even though the cell line used for transfection is deficient in ODC activity, it produces an enzymically inactive ODC, which is regulated in a normal manner by polyamines [28,34]. Since this inactive ODC has approximately

the same molecular mass as the products of the transgenes (except for the ODCs from pODC- Δ 5'-tr and pODC- Δ 3'-tr) the method used for measuring ODC synthesis did not distinguish between the endogenous and recombinant ODCs. In order to circumvent this problem, the cell lines were made resistant to the antiproliferative effect of the ODC inhibitor DFMO, by growing the cells in the presence of increasing concentrations of the inhibitor. This procedure is known to cause resistance to DFMO by ODC gene amplification [35–37]. Although the expression of ODC is greatly increased in DFMO-resistant cells, the feedback control of the enzyme appears to work normally [36,37].

The polyamine-mediated regulation of ODC synthesis was assayed by pulse-labelling of cells grown in the presence or absence of 2–5 mM DFMO. When cells are seeded in the presence of DFMO there is usually an upregulation of ODC synthesis [38]. Furthermore, when DFMO-resistant cells are grown in the absence of the inhibitor there is a marked downregulation of ODC synthesis [37,39]. These changes in ODC synthesis are most likely due to the changes occurring in cellular polyamine content when DFMO is added or omitted from the medium. As shown in Table 2, deletion of DFMO from the growth medium of the DFMO-resistant cells resulted in a dramatic increase in cellular putrescine specifically, but also in cellular spermidine content. There was no major difference in growth rates between the various DFMO-resistant cells grown in the absence or presence of DFMO, except for the cells expressing a stable C-terminally truncated ODC (pODC- Δ 3'-tr-DFMO^r). These cells died when DFMO was omitted from the growth medium (results not shown). However, since it is possible to distinguish between the C-terminally truncated ODC and the endogenous ODC expressed in the same cell, because of the size difference, it was possible to

Table 2 Effects of DFMO on cellular polyamine content

CHO cells and stable transfectants of the C55.7 CHO cell line expressing ODC mRNAs with various truncations were grown with or without 2–5 mM DFMO for 4 days and then reseeded in the presence or absence of the inhibitor. At 1 day after reseeding the cells were analysed for polyamine content (nmol/mg of protein; means $\pm$ S.E.M., $n = 4$; N. D., not detectable). CHO-DFMO^r, DFMO-resistant variant of the wild-type CHO cells; pODC- Δ -5'-tr-DFMO^r, DFMO-resistant CHO cells expressing an ODC mRNA (without 5' UTR) coding for an N-terminally truncated ODC; pODC- Δ -3'-tr, CHO cells expressing an ODC mRNA (without 3' UTR) coding for a C-terminally truncated ODC; pODC- Δ -5'-3'-tr-DFMO^r, DFMO-resistant CHO cells expressing an ODC mRNA without the 5' UTR and 3' UTR.

Cells	Treatment	Polyamine content (nmol/mg)		
		Putrescine	Spermidine	Spermine
CHO-DFMO ^r	DFMO	0.4 $\pm$ 0.2	9.3 $\pm$ 1.1	5.8 $\pm$ 0.5
CHO-DFMO ^r	–	22.0 $\pm$ 1.4	12.8 $\pm$ 0.6	4.2 $\pm$ 0.2
pODC- Δ -5'-tr-DFMO ^r	DFMO	N.D.	0.7 $\pm$ 0.1	7.7 $\pm$ 1.3
pODC- Δ -5'-tr-DFMO ^r	–	23.6 $\pm$ 0.5	7.9 $\pm$ 0.4	3.6 $\pm$ 0.2
pODC- Δ -3'-tr	DFMO	N.D.	2.7 $\pm$ 0.3	6.2 $\pm$ 0.7
pODC- Δ -3'-tr	–	0.9 $\pm$ 0.2	5.4 $\pm$ 0.8	3.5 $\pm$ 0.4
pODC- Δ -5'-3'-tr-DFMO ^r	DFMO	N.D.	3.9 $\pm$ 1.2	8.2 $\pm$ 0.5
pODC- Δ -5'-3'-tr-DFMO ^r	–	13.0 $\pm$ 0.7	10.9 $\pm$ 0.4	3.9 $\pm$ 0.2

Figure 3 Effects of DFMO on ODC synthesis in CHO cells and stable transfectants of the C55.7 CHO cell line expressing ODC mRNAs with various truncations

(A) DFMO-resistant variant (CHO-DFMO^r) of the wild-type CHO cells; (B) DFMO-resistant CHO cells expressing an ODC mRNA (without 5' UTR) coding for an N-terminally truncated ODC (pODC- Δ -5'-tr-DFMO^r); (C) CHO cells expressing an ODC mRNA (without 3' UTR) coding for a C-terminally truncated ODC (pODC- Δ -3'-tr); (D) DFMO-resistant CHO cells expressing an ODC mRNA without the 5' UTR and 3' UTR (pODC- Δ -5'-3'-tr-DFMO^r). The cells were grown with or without 2–5 mM DFMO for 4 days, and then reseeded in the presence (lanes 1) or absence (lanes 2) of the inhibitor. At 1 day after reseeding the cells were pulse-labelled with [³⁵S]methionine. Extracts from the cells were precipitated with an ODC-antiserum and analysed by SDS/PAGE. ¹⁴C-Methylated proteins were used as molecular-mass markers (kDa), as shown on the left of each gel.

carry out a pulse-labelling experiment using the non-amplified variant of the cell line. DFMO treatment of these cells resulted in a marked reduction in their putrescine and spermidine content (Table 2). The spermine content, on the other hand, increased after addition of DFMO to the growth medium.

As shown in Figure 3(A), ODC synthesis was significantly downregulated in the DFMO-resistant CHO cells (CHO-DFMO^r) when the cells were grown in the absence of DFMO. This effect on ODC synthesis was similar to that observed earlier in a DFMO-resistant variant of stable CHO transfectants expressing full-length ODC mRNA [39]. ODC synthesis was also markedly reduced in the DFMO-resistant cells expressing the N-terminally truncated ODC (pODC- Δ -5'-tr-DFMO), indicating that the 5' UTR, as well as the first 30 nt of the coding sequence of the ODC mRNA, was unimportant for feedback control of ODC synthesis (Figure 3B). In the cells expressing a C-terminally truncated ODC (pODC- Δ -3'-tr), two different sized ODC forms were clearly visible (Figure 3C). Most likely, the larger one was the full-length endogenous ODC and the smaller one was the C-terminally truncated ODC. When DFMO was omitted from the growth medium a marked downregulation of the synthesis of endogenous ODC was observed. However, the synthesis of the truncated ODC appeared to be unaffected by this treatment (Figure 3C). The mRNA coding for the C-terminally truncated ODC was also devoid of the 3' UTR. However, as shown in Figure 3(D), this part of the mRNA did not seem to be involved in the feedback control of ODC synthesis, since the DFMO-resistant cells expressing an ODC mRNA devoid of both the 5' UTR and the 3' UTR (pODC- Δ -5'-3'-tr-DFMO) responded with a decrease in ODC synthesis, which was comparable with that of the CHO-DFMO^r cells expressing full-length ODC mRNA, when DFMO was omitted from the medium.

The effects on ODC synthesis were mainly due to changes in the translational efficiency, as indicated by the fact that the ODC mRNA level did not change when the cells were grown in the absence of DFMO (results not shown), although there was a marked downregulation in ODC synthesis.

Hypotonic induction of ODC activity

Exposure of cells to a hypo-osmotic environment has been shown to greatly induce ODC activity in the cells [14–17]. This induction seems to be, at least in part, caused by an increased efficiency of ODC mRNA translation [15,17]. In order to elucidate potential roles for the different parts of ODC mRNA in the hypotonic induction of ODC, the various stable transfectants were exposed to hypotonic shock for 6 h and analysed for ODC activity. As shown in Figure 4, marked induction of ODC activity occurred in wild-type CHO cells subjected to this treatment. A similar, or even larger, increase in ODC activity was observed in cells expressing ODC mRNAs partly [pODC- Δ -(29)5'] or completely (pODC- Δ -5') devoid of the 5' UTR. In cells expressing an N-terminally truncated ODC (pODC- Δ -5'-tr), the hypotonic induction of ODC appeared smaller than in the wild-type CHO cells (Figure 4). However, in other clones expressing the same ODC mRNA no such difference was observed, indicating that the finding was non-specific. Cells expressing a C-terminally truncated ODC (pODC- Δ -3'-tr), on the other hand, did not induce ODC following hypotonic shock. Instead a 35% decrease in ODC activity was observed in these cells when they were exposed to the hypotonic medium (Figure 4). Interestingly, in the other cell lines expressing ODC mRNAs devoid of the 3' UTR (pODC- Δ -3' and pODC- Δ -5'-3') the hypotonic induction

Figure 4 Effects of hypotonic stress on ODC activity in CHO cells and stable transfectants of the C55.7 CHO cell line expressing ODC mRNAs with various truncations

At 1 day after seeding in fresh medium, the cells were exposed to an isotonic or hypotonic medium for 6 h before being analysed for ODC activity (means $\pm$ S.E.M., $n = 6$).

of ODC was much less than that found in the wild-type CHO cells (Figure 4). In cells expressing an ODC mRNA without the 3' UTR (pODC-Δ-3'), only a 50% increase in ODC activity was seen after the hypotonic shock. In cells expressing an ODC mRNA completely devoid of both the 5' UTR and the 3' UTR (pODC-Δ-5'-3') no hypotonic induction of ODC activity was observed (Figure 4). The observation that the cell lines expressing ODC mRNAs lacking the 3' UTR showed no, or only a very slight, induction of ODC activity after hypotonic shock was confirmed using several independent cell clones.

Hypotonic induction of ODC synthesis

The induction of ODC synthesis after hypotonic shock was determined by pulse-labelling of the DFMO-resistant variants of the cell lines. As shown in Figure 5, ODC synthesis increased markedly in the DFMO-resistant CHO cells (CHO-DFMO^r) when these were exposed to hypotonic medium for 6 h. A similar hypotonic induction of ODC synthesis was observed in the cells expressing an N-terminally truncated ODC (pODC-Δ-5'-tr-DFMO^r) (Figure 5). However, in the DFMO-resistant cells expressing ODC mRNAs devoid of the 3' UTR (pODC-Δ-3'-tr-DFMO^r and pODC-Δ-5'-3'-DFMO^r) no major change in ODC synthesis was observed when the cells were exposed to a hypotonic medium (Figure 5), confirming the importance of the ODC mRNA 3' UTR in the osmotic regulation of ODC expression. Northern-blot analysis revealed that the cellular ODC mRNA level was essentially unchanged during the hypotonic shock (results not shown), confirming that the hypotonic induction of ODC synthesis in the CHO cells was mainly a result of increased ODC mRNA translation.

DISCUSSION

Results of earlier studies indicate that the strong polyamine-mediated feedback regulation of ODC occurs mainly by two separate mechanisms [1,3,5]. It has long been known that the polyamines stimulate the degradation of the enzyme. The underlying mechanisms of the rapid turnover of ODC and its stimulation by polyamines are presently being unravelled [3,8]. ODC has been shown to be degraded by the 26 S proteasome in a process stimulated by the binding of a specific protein, named antizyme, to the enzyme [6,8]. Antizyme has a very strong affinity for ODC and also inhibits the enzyme activity. The synthesis of antizyme is induced by polyamines by a unique mechanism involving ribosomal frameshifting [9,10]. Molecular analysis of mammalian ODC has revealed sequences which are important for the rapid turnover of the protein. The C-terminal part of ODC has been shown to contain an important degradation domain and truncations/deletions in this part of the enzyme give rise to stabilization of the protein against degradation [40]. This was also shown in the present study and most likely explains the finding that the cells expressing the C-terminally truncated ODC, which was stable, had much higher levels of ODC activity than the other cell lines examined. In addition to stimulating the degradation of ODC, the polyamines appear to exert an inhibitory effect on ODC synthesis [1,3]. These polyamine-mediated changes in ODC synthesis are not correlated with a change in the ODC mRNA level, indicating a translational mechanism. However, as demonstrated in the present study, the feedback control of ODC synthesis appears to be independent of the 5' UTR as well as the 3' UTR of ODC mRNA. Furthermore, in the stable transfectants expressing a C-terminally truncated ODC no apparent effect was observed on the synthesis of this protein when the cells were grown in the presence or absence of DFMO, even

Figure 5 Effects of hypotonic stress on ODC synthesis in DFMO-resistant CHO cells and stable transfectants of the C55.7 CHO cell line expressing ODC mRNAs with various truncations

(A) DFMO-resistant variant (CHO-DFMO) of wild-type CHO cells; (B) DFMO-resistant CHO cells expressing an ODC mRNA (without 5' UTR) coding for an N-terminally truncated ODC (pODC- Δ 5'-tr-DFMO); (C) DFMO-resistant CHO cells expressing an ODC mRNA (without 3' UTR) coding for a C-terminally truncated ODC (pODC- Δ 3'-tr-DFMO); (D) DFMO-resistant CHO cells expressing an ODC mRNA without the 5' UTR and 3' UTR (pODC- Δ 5'-3'-DFMO). At 1 day after seeding in fresh medium, the cells were exposed to an isotonic (lanes 1) or hypotonic (lanes 2) medium for 6 h before being analysed for ODC synthesis, as described in the Materials and methods section. 14 C-Methylated proteins were used as molecular-mass markers (kDa), as shown on the left of each panel.

though there was a marked change in the synthesis of the endogenous full-length ODC expressed in the same cells. Therefore it appears that the C-terminal region of ODC (or its coding sequence) is, in some way, important for the apparent feedback control of ODC synthesis. It has been suggested that the polyamine-mediated effect on ODC synthesis is actually a co-translational stimulation of degradation, induced by the binding of antizyme to newly synthesized ODC [22]. Thus newly synthesized ODC would partly be degraded during the labelling time, giving rise to an apparent decrease in the synthesis of ODC. The present finding that the synthesis of the C-terminally truncated ODC, which is a stable protein, is not affected by DFMO treatment may support the existence of such a mechanism. Nevertheless, a minor decrease in ODC activity was observed in the cells expressing the C-terminally truncated ODC when the cells were grown in the presence of spermidine, indicating that some stimulation of ODC degradation may still occur.

In addition, the osmolality of the growth medium has been shown to strongly affect cellular ODC activity [14–17]. Exposure to a hypotonic medium gives rise to a rapid increase in ODC activity, whereas exposure to a hypertonic medium results in a marked down-regulation of ODC activity. Interestingly, the increase in putrescine content caused by the rise in ODC activity after a hypotonic shock appears to render the cells more resistant to the decrease in osmolality [41]. The mechanisms involved in the hypotonic induction of ODC are not yet fully understood.

In some systems, the phenomenon is explained by a change in the ODC mRNA content [16], whereas in others it appears to involve mainly, if not exclusively, translational and post-translational events [15,17]. In the present study, we have shown that hypotonic induction of ODC is highly dependent on the presence of the 3' UTR in the ODC mRNA. The ODC mRNA 5' UTR, on the other hand, does not seem to be essential for induction of ODC by hypotonicity. Since the expressed proteins from the mRNAs lacking the 3' UTR are full-length ODCs, we can rule out any post-translational effects.

In recent years, the importance of the mRNA 3' UTRs in the translational control of a variety of regulatory proteins has attracted increased attention [42,43]. The 3' UTR of ODC mRNA has been suggested to be involved in the translational control of the enzyme by interfering with the inhibitory effect of the ODC mRNA 5' UTR [20,27]. However, this conclusion is based on indirect studies using various chimaeric reporter mRNAs and until now no physiological function for the 3' UTR has been demonstrated. The present finding of a role for the ODC mRNA 3' UTR in the hypotonic induction of ODC is the first demonstration of a specific effect of the 3' UTR in the regulation of ODC. The observation that the hypotonic induction of ODC was independent of the ODC mRNA 5' UTR suggests that the mechanism by which ODC synthesis is increased after hypotonic shock does not involve an interaction between the 3' UTR and the 5' UTR. Interestingly, the 3' UTR of mammalian ODC mRNA contains regions corresponding to what is known as AU-rich elements (AREs). These elements have been shown to be located in the 3' UTR of a number of growth-related mRNAs, including mRNAs coding for various proto-oncogenes and cytokines [44]. The AREs are *cis*-acting motifs known to be involved in the control of mRNA stability and translational efficiency. The AREs in the 3' UTR of mammalian ODCs are well conserved and it is thus conceivable that they fulfil some function in relation to the post-transcriptional control of ODC expression.

The induction of ODC by hypotonicity has been shown to be inhibited by the presence of polyamines, indicating a common mechanism [15,17]. It has been suggested that the increase in cell volume or change in intracellular ion concentrations may affect the polyamine levels or their interactions with proteins/nucleic acids in such a way that their inhibitory effect on ODC mRNA translation is reduced. However, as shown in the present study, the osmotic regulation of ODC can be separated from the polyamine-mediated control of ODC synthesis, suggesting two different mechanisms. Nevertheless, further studies are needed to clarify these mechanisms.

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