Co-operation of the 5′ and 3′ untranslated regions of ornithine decarboxylase mRNA and inhibitory role of its 3′ untranslated region in regulating the translational efficiency of hybrid RNA species via cellular factor(s)

Erna C. LORENZINI*†[1] and Immo E. SCHEFFLER†

*Institute of General Pathology and C.N.R. Centre for Cell Pathology, via L. Mangiagalli 31, 20133 Milan, Italy, and †Department of Biology-0322, University of California, San Diego, La Jolla, CA 92093-0322, U.S.A.

The 5′ untranslated region (UTR) has an inhibitory role in the translatability of ornithine decarboxylase (ODC) mRNA and of hybrid mRNA species, whereas the ODC 3′ UTR causes a partial release of this inhibition. We designed experiments to explore whether the co-operation between ODC 5′ UTR and 3′ UTR in the translational regulation is due to a direct interaction of those sequences or whether it is mediated by their interaction with cellular factor(s). We stably transfected Chinese hamster ovary (CHO)-K1 cells and transiently transfected COS-1 cells with expression vectors carrying different chimaeric DNAs having the luciferase (LUC) coding sequence as reporter gene, the ODC 5′ UTR or the ODC 3′ UTR, or both, in the appropriate positions. We compared the results obtained by assaying the LUC activities of both transfected cell lines with each chimaeric DNA with those observed by translating the hybrid RNAs in a translation system *in vitro*. When the ODC 3′ UTR was present, we observed a partial release of the translation inhibition owing to the ODC 5′ UTR only *in vivo*. The releasing effect was restored *in vitro* by the addition of cytoplasmic extracts from wild-type CHO-K1 or COS-1 cells, prepared 2 and 8 h after their release from serum starvation. We also observed a partial inhibition of the translatability of the hybrid RNA owing to the presence of the ODC 3′ UTR itself; the translational efficiency could be rescued by cell extract from 8 h serum-stimulated cells. The co-operation between the ODC-UTRs might be mediated by factors expressed by cells during particular phases of the cell cycle. Excess copies of the ODC-UTRs, expressed *in trans*, could compete in binding limited amounts of such regulatory factors and remove them from interaction with the endogenous ODC mRNA. This phenomenon should be reflected by modifications of the kinetics of ODC and/or LUC activities during serum stimulation. The overexpression of the ODC 3′ UTR determined an increase in both endogenous ODC activity and LUC activity. Moreover, in the transfectants expressing the hybrid RNA species bearing the ODC 3′ UTR the basal ODC activity is higher than that observed in control cells. We suggest that excess copies of the ODC 3′ UTR mis-regulate the endogenous ODC translatability, probably by tying up regulatory molecules expressed by cells in limited amounts and sequestering them from the ODC mRNA species they should interact with.

INTRODUCTION

Ornithine decarboxylase (ODC) (EC 4.1.1.17) is the key point and the rate-limiting enzyme in the polyamine biosynthetic pathway. The activity of mammalian ODC is regulated by a complex array of control mechanisms [1,2]. Changes in ODC mRNA levels in a variety of systems and under various conditions frequently cannot account for the strong fluctuations in ODC activity [3,4]. It has recently been demonstrated how disturbances of regulatory mechanisms governing ODC expression at different steps could be associated with neoplastic transformation [5–8]. Thus post-transcriptional and post-translational mechanisms have been postulated, and it is clear that the control of the stability of the ODC protein is one important and widely recognized phenomenon. Less clear and only poorly understood is the possibility of regulating the translatability of ODC mRNA. This transcript has a relatively long 3′ untranslated region (UTR), and a 5′ UTR with the potential for extensive and stable hairpin formation. Complex regulatory mechanisms at the translational level could be involved in governing the growth and differentiation of different organisms, including mammalian cells and tissues. The existence of sequence elements in the 5′ and 3′ UTRs that regulate mRNA translatability (and also stability and even subcellular localization), frequently through interactions with *trans*-acting proteins [e.g. poly(A)$^+$-binding protein, translation initiation factors, eIFs], has been amply documented and reviewed [9]. The importance of the 3′ UTR of some RNA species in controlling the translation and stability of mRNA [10–12] and its location within the cell has recently been emphasized [13–16].

Specifically, the 5′ UTR of ODC mRNA has been demonstrated to have a strongly inhibitory effect on the translation of this mRNA, or of chimaeric transcripts translated *in vivo* or *in vitro*. Some investigations have attempted to overcome this inhibitory effect by the activation or overexpression of translation initiation factors (eIF4E, eIF4B) that might help in unwinding the 5′ UTR secondary structure by means of their helicase activity [7,17]. So far, the 3′ UTR of ODC has received less attention. However, it has been reported by Grens and Scheffler [18] that the addition of the 3′ UTR to a construct of luciferase (LUC) coding sequence and ODC 5′ UTR can relieve the inhibition of the 5′ UTR alone to a significant extent. Thus there are indications that the 5′ UTR and the 3′ UTR might interact directly or indirectly *in vivo*.

The present studies were undertaken as a follow-up to gain a

Abbreviations used: CHO, Chinese hamster ovary; FCS, fetal calf serum; LUC, luciferase; ODC, ornithine decarboxylase; RSV, Rous sarcoma virus; UTR, untranslated region.

[1] Present address and address for correspondence: Cattedra di Immunologia, Universita degli Studi di Milano, Via Venezian, 1, 20133 Milan, Italy.

better understanding of the nature of this interaction. The question was also raised whether the ODC 3′ UTR has a role in controlling translation by itself, either directly or through its binding with cellular factors. First of all, the relevant transcripts to be tested were expressed from vector constructs that had been stably integrated into the genome of the host cells. This made it possible to study the expression of these chimaeric genes and their transcripts in cells synchronized by serum starvation, and compare their expression with that of the endogenous ODC. Cytoplasmic extracts from synchronized cells were added to a reticulocyte translation system *in vitro* to test for the presence of factors that might promote the interaction between the ends of these chimaeric transcripts. Finally, we hypothesized that the presence of an excess of 5′ UTR or 3′ UTR sequences in the transfected cell might influence the expression of the endogenous ODC, owing to competition with limiting cellular factors during the cell cycle.

MATERIALS AND METHODS

Cells, cell culture and serum starvation

Wild-type Chinese hamster ovary (CHO-K1) or COS-1 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum (FCS), non-essential amino acids, gentamycin and amphotericin B (Gibco BRL) in a humidified incubator under air/CO_2 (9:1) for CHO-K1 or air/CO_2 (47:3) for COS-1 cells. For serum starvation, cells were rendered quiescent by first rinsing them twice with PBS and then incubating them for 48 h with the routinely used medium with 0.1% FCS. Serum-starved cells were stimulated by the addition of 10% FCS (Gibco BRL).

Chimaeric DNA species

We used chimaeric constructs cloned in eukaryotic expression vectors. All had the Rous sarcoma virus (RSV) promoter, the polyadenylation signal from simian virus 40 and the complete coding sequence from firefly LUC gene. In the control (pRSV/L) no other sequences were present; in a second construct (pODC/L1) the hamster ODC 5′ UTR was placed upstream of the LUC coding sequence; a third construct (pODC/L4) had both the ODC 5′ and 3′ UTRs in the appropriate positions; the fourth construct (pODC/L9) had the ODC 3′ UTR downstream of the LUC coding sequence (see Figure 1). For more details of the construction of the chimaeric DNAs see Grens and Scheffler [18].

Transient and stable transfections

COS-1 cells were seeded at 20% confluence the day before transfections. They were transfected at 50% confluence with 10 μg of DNA per 10 cm plate of each different chimaeric DNA by using the chloroquine/DEAE-dextran/DMSO protocol. The transfectants were then returned to Dulbecco's modified Eagle's medium with 10% FCS for 48 h to allow expression before they were harvested for the assays. Stable CHO-K1 transfectants were generated by co-transfection of chimaeric DNA constructs along with pSV2Neo, followed by a selection of cells in G418 (600 μg/ml) (Gibco BRL). Cells were transfected by electroporation (Electro Cell Manipulator 600; BTX, San Diego, CA, U.S.A.) following the method described by Dunn et al. [19]. Multiple colonies, transfected with each of the constructs, were pooled to ensure a population of cells with a heterogeneity of integration sites. Asynchronously growing cells or serum-starved cells at 0, 2, 4 or 8 h after serum stimulation were harvested for LUC, ODC and protein assays, for the preparation of cytoplasmic S100 cell extracts and for DNA and RNA extractions.

Southern, Northern and slot-blot analysis

Genomic DNA from asynchronously growing stably transfected CHO-K1 and wild-type CHO-K1 cells was obtained as previously reported [20], digested with *Eco*RI and fractionated in agarose gels by electrophoresis. Gels were blotted on membranes and hybridized with a radiolabelled 650 bp *Eco*RI fragment of the LUC coding sequence. Total RNA was obtained from eight plates, transfected with each chimaeric DNA at each experimental time point, from wild-type CHO-K1 and COS-1 cells, from asynchronously growing transfected CHO-K1 cells and from synchronized CHO-K1 cells after serum stimulation by using the guanidinium isothiocyanate method [21]. Total RNA (20 μg) from each set of transfectants was used for Northern blots, slot-blots or to perform translation reactions *in vitro*. Filters were probed for Northern blot analysis with a *Hin*dIII/*Hin*dIII fragment of pODC16 corresponding to 953 bp of the mouse ODC coding sequence [22]; slot-blots were hybridized with the same probe or with a radiolabelled 650 bp *Eco*RI fragment of the LUC coding sequence obtained from pRSV/L [23]. Probes were labelled by nick translation (Amersham) by using [α-^{32}P]dCTP. After hybridization, filters were washed and exposed for autoradiography as previously described [18]. Each autoradiogram was scanned for densitometric analysis with a laser densitometer (Model 300A computing densitometer; Molecular Dynamics, Sunnyvale, CA, U.S.A.).

ODC, LUC and protein assays

Cells for analysis of ODC and LUC activities and of protein content measurements were pelleted at 1000 ***g*** for 10 min at 4 °C and immediately stored at −80 °C before analysis. ODC activity was measured by the release of $^{14}CO_2$ from carboxy-labelled L-ornithine as previously described [24]. LUC activity was determined from transfectants or after translation reactions *in vitro* by using a LUC assay system (Promega). LUC activities from pRSV/L-transfected cells or after translation reactions of pRSV/L transcripts *in vitro* were taken as 100%. Protein contents were determined in each supernatant by using the Bio-Rad Coomassie Brilliant Blue assay in accordance with the manufacturer's instructions.

Translation and complementation *in vitro*

Total RNA species obtained from asynchronously growing transfected cells were quantified by hybridizing slot-blots with the LUC probe as above reported. Translation reactions were performed in a nuclease-free reticulocyte lysate system (Promega) in accordance with the manufacturer's instructions at 30 °C for 2 h after the addition of RNase inhibitor (RNAsin; Promega). S100 cell extracts (10, 20 or 40 μl), obtained as previously described [25] from quiescent wild-type CHO-K1 or COS-1 cells and obtained from the same cells at different times after their release from serum starvation, were added to the reticulocyte lysate at the beginning of each translation reaction. At the end of the reactions samples were used to perform LUC and protein assays. The amount of each hybrid RNA was quantified by slot-blot at the beginning and at the end of each reaction.

All the results shown are representative of at least triplicate experiments, except for experiments in which induction of ODC and LUC activities after serum stimulation were analysed, which were performed at least six times. Results are presented as

means ± S.E.M. The statistical analysis used is indicated in the legend of each figure.

RESULTS

The different chimaeric constructs used in the present analysis are shown in Figure 1. They all have an RSV promoter and simian-virus-40 polyadenylation site, and an LUC coding sequence, with the ODC 5′ UTR or 3′ UTR, or both, inserted as indicated. Stable CHO-K1 cell lines with these transgenes were established as described in the Materials and methods section. A Southern blot analysis (Figure 2A) shows the presence and relative amounts of the transgene in each line, and a slot-blot analysis of total RNA (Figure 2B) established the expression of the endogenous ODC gene and the LUC transgene. There was no apparent effect on the endogenous ODC mRNA levels. COS-1 cells were transiently transfected with the same set of constructs. Slot-blot analyses (results not shown) were used to establish comparable levels of expression of these chimaeric genes.

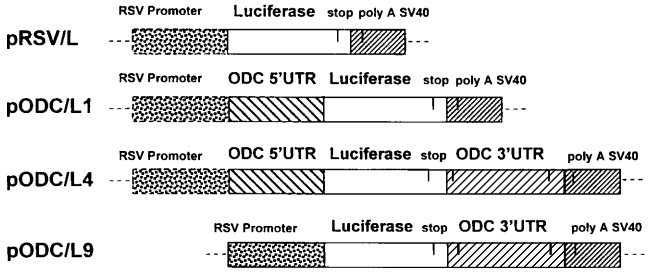

Figure 1 Chimaeric reporter genes

The transcription start site, the translation termination codon and the polyadenylation sites are indicated. pRSV/L plasmid contains the LUC coding sequence [23]. The chimaeric constructs contain the ODC-UTR sequences derived from hamster ODC cDNA. pODC/L1 and pODC/L4 contain the ODC 5′ UTR, which contains a GC-rich region of approx. 140 nt and a short open reading frame starting approx. 150 nt upstream of the true translation initiation codon. pODC/L4 and pODC/L9 contain the ODC 3′ UTR comprising approx. 900 bp. For more details of the chimaeric gene construction see Grens and Scheffler [18]. Each plasmid has been used to transfect CHO-K1 cells stably and to transfect COS-1 cells transiently.

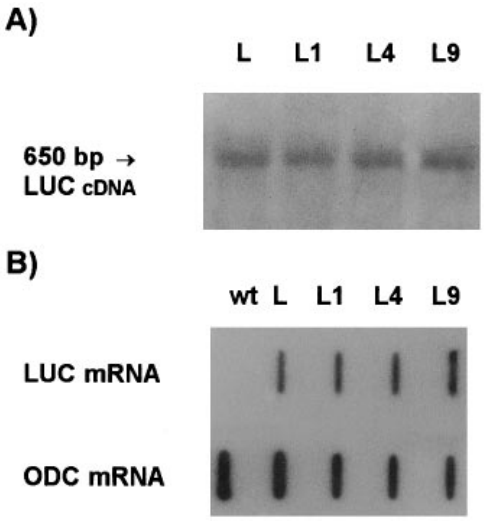

Figure 2 Southern blot and slot-blot analyses of stably transfected CHO-K1 cells

(**A**) Equal amounts of *Eco*RI-digested genomic DNA, obtained from wild-type CHO-K1 cells and cells transfected with pRSV/L (L), pODC/L1 (L1), pODC/L4 (L4) or pODC/L9 (L9) were loaded. The hybridization was performed by using the 650 bp *Eco*RI fragment of the LUC coding sequence. (**B**) Total RNA (20 μg) obtained from asynchronously growing wild-type (wt) CHO-K1 and transfected cells was spotted on membranes and hybridized with the LUC probe or with the *Hin*dIII fragment of the ODC cDNA (see the Materials and methods section).

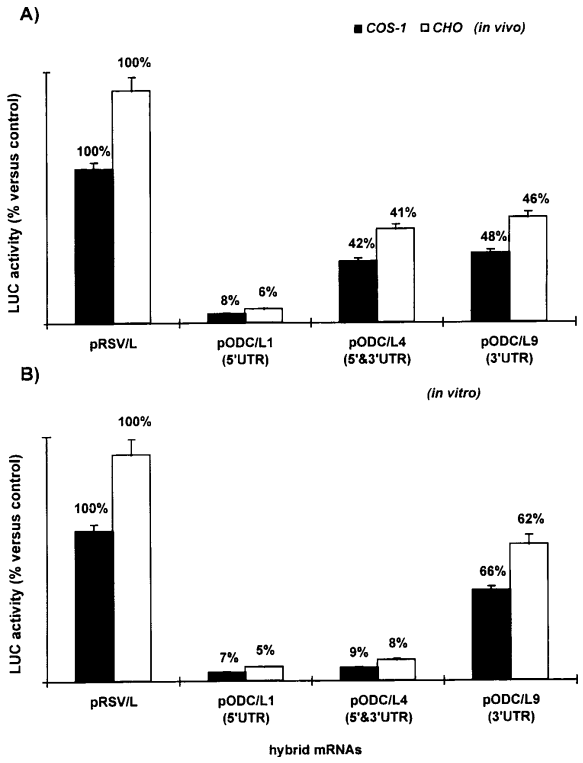

Figure 3 Effects of the hamster ODC UTRs on the translatability of the hybrid transcripts *in vitro* or in intact transfected cells

(**A**) LUC activity *in vivo* in CHO-K1 (open bars) and COS-1 (filled bars) transfected cells with the indicated constructs (abscissa). (**B**) LUC activity derived from the translation in a reticulocyte lysate system of the hybrid transcripts expressed from CHO-K1 and COS-1 transfected cells. Each bar is the mean ± S.E.M. for duplicate determinations from eight similar experiments. The LUC activity has been divided by the total protein contents of the supernatants from which the LUC assay was performed. The percentages shown are referred to the LUC activity obtained from pRSV/LUC-CHO-K1 or pRSV/LUC-COS-1. Control wild-type CHO-K1 and COS-1 cells did not express any LUC activity.

Influence of the ODC 5′ and 3′ UTR on the translation efficiency of hybrid RNA species *in vivo* and *in vitro*

By assaying the LUC activity expressed *in vivo* in each set of transfectants, both CHO-K1 and COS-1 cells during unsynchronized growth (Figure 3A), we evaluated the influence of the ODC-UTRs on the translatability of hybrid transcripts carrying those sequences. We set the LUC activity of control cells (pRSV/L-CHO-K1 or pRSV/L-COS-1) at 100%. In this first series of experiments we observed both a strong translational inhibition imposed by the ODC 5′ UTR and a partial release of this inhibition when the ODC 3′ UTR was also present (Figure 3A). These results in COS-1 cells parallel those obtained by Grens and Scheffler [18] in transiently transfected CHO-K1 cells, and they also extend and confirm the phenomenon for stably transfected CHO-K1 cells. A decrease to less than one-tenth was observed with the 5′ UTR alone; when the 3′ UTR was added to the transcript, the inhibition was only 60%. If the pODC/L1

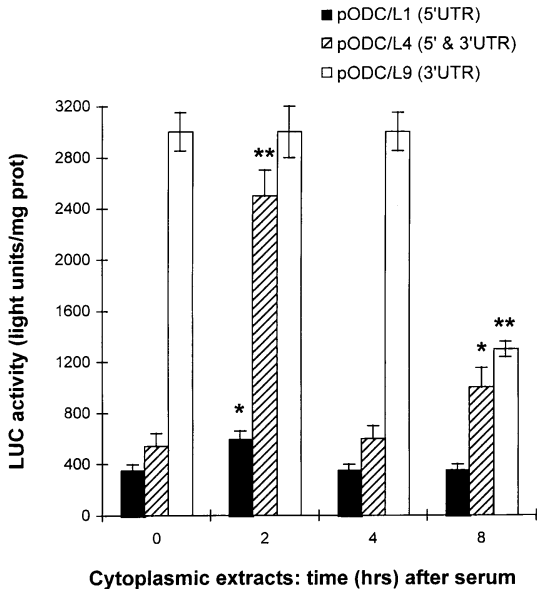

Figure 4 Effects of the addition of cytoplasmic extracts from serum-stimulated wild-type cells on the translation of hybrid transcripts *in vitro*

Cell extracts were obtained from wild-type CHO-K1 cells after their release from serum starvation. Zero time is the time at which the 48 h serum-starved cells were washed with PBS and harvested. S100 supernatant (20 μl), obtained at the indicated times, was added to the translation mixture *in vitro* at the beginning of the translation reaction. Total RNA (20 μg), obtained from each transfected cell line, was translated. The LUC activity assay was performed on the supernatant obtained from the translation mixture *in vitro* at the end of the translation reaction. Filled bars, hatched bars and open bars respectively represent the means of LUC activities observed from the translation of the hybrid RNA species derived from pODC/L1, pODC/L4 and pODC/L9. $^{*}P < 0.05$ compared with the appropriate control (zero time); $^{**}P < 0.01$ compared with the appropriate control (zero time) (Student's *t* test).

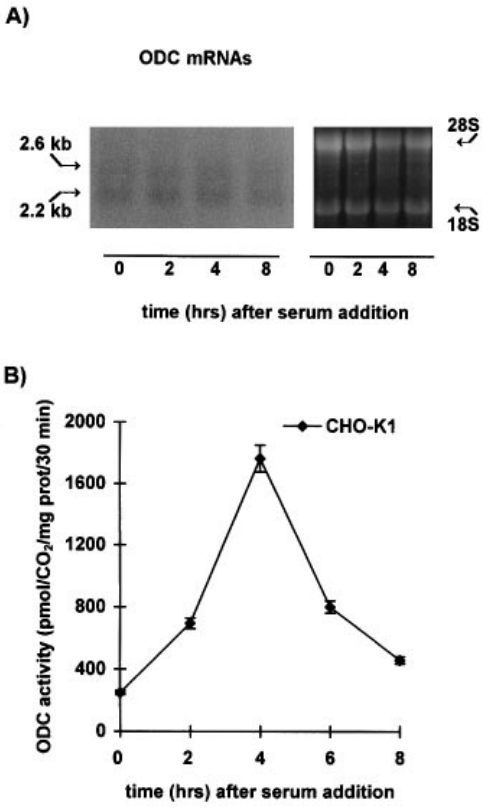

Figure 5 ODC mRNA expression and ODC activity levels after serum stimulation of quiescent wild-type CHO-K1 cells

(**A**) Total RNA (20 μg), obtained from serum-starved (0 h) or serum-stimulated cells 2, 4 or 8 h after the addition of FCS, was loaded. The positions of 18 S and 28 S RNA species are indicated at the right. The hybridization conditions and the ODC probe used are described in the Materials and methods section. The ODC mRNA species are indicated at the left. (**B**) Wild-type CHO-K1 cells were synchronized by serum starvation. Serum was added at zero time. At the indicated times, cells were collected and the ODC activity and protein content were measured. Results are means $\pm$ S.E.M.

and pODC/L4 results are compared alone, there was a 5–6-fold stimulation in the presence of the 3′ UTR. The ODC 3′ UTR by itself (pODC/L9) determined a partial decrease in the translatability of the transcript (Figure 3A).

Total RNA species from the same cultures were translated *in vitro* in a reticulocyte system (see the Materials and methods section), with the results shown in Figure 3B. With the same normalization, the inhibitory effect of the 5′ UTR was equally great. However, the partial release of this inhibition by the presence of the 3′ UTR was not observed in this *in vitro* system. The LUC activity reached approx. 60 % after the translation *in vitro* of the hybrid RNA carrying the ODC 3′ UTR alone. From these results it is tempting to speculate that cellular factors, through which the 5′ and 3′ UTRs could interact to release the inhibition of translation, are not present, or are present only at insignificant levels, in the reticulocyte lysate, and that the same or other cytoplasmic factor(s) could interact with the ODC 3′ UTR and cause the inhibition due to the 3′ UTR itself.

Complementation *in vitro*

To investigate whether such factor(s) could possibly be synthesized by cells during the cell cycle we added cytoplasmic extracts obtained from wild-type CHO-K1 or COS-1 cells at different times after release from serum starvation to each translation reaction *in vitro* of the hybrid RNA species. The concentrated extracts were prepared as described in the Materials and methods section, and three different amounts (10, 20 and 40 μl) were tested. In general, 20 μl was maximally effective. We added cell extracts obtained from quiescent cells (0 h) or from serum-stimulated cells 2, 4 and 8 h after serum stimulation to each translation reaction *in vitro*. Our observations can be summarized as follows. (1) There was a slight increase in the translation of the hybrid mRNA containing the ODC 5′ UTR when the cellular extract from 2 h-stimulated cells was added (Figure 4, filled bars). (2) The most marked and interesting result was obtained with the transcript pODC/L4 having both 5′ and 3′ UTRs flanking the LUC coding sequence. An extract made from cells 2 h after serum stimulation had a significant stimulatory effect compared with extracts made at earlier or later times (Figure 4, hatched bars). (3) A decrease in the translatability of the hybrid RNA containing the ODC 3′ UTR, comparable to that observed *in vivo*, was determined by the addition of the extract from 8 h serum-stimulated cells (Figure 4, open bars). The addition of cell extracts at any time after serum stimulation did not affect the translatability of the control transcript (pRSV/L) carrying the LUC coding sequence alone (results not shown). All the results obtained by testing CHO-K1 transfected cells were the same as those obtained from COS-1 transfected cells.

Figure 5(A) shows the ODC mRNA levels in wild-type cells corresponding to the time points at which extracts were made from these cells. They seem to be quite constant, whereas ODC

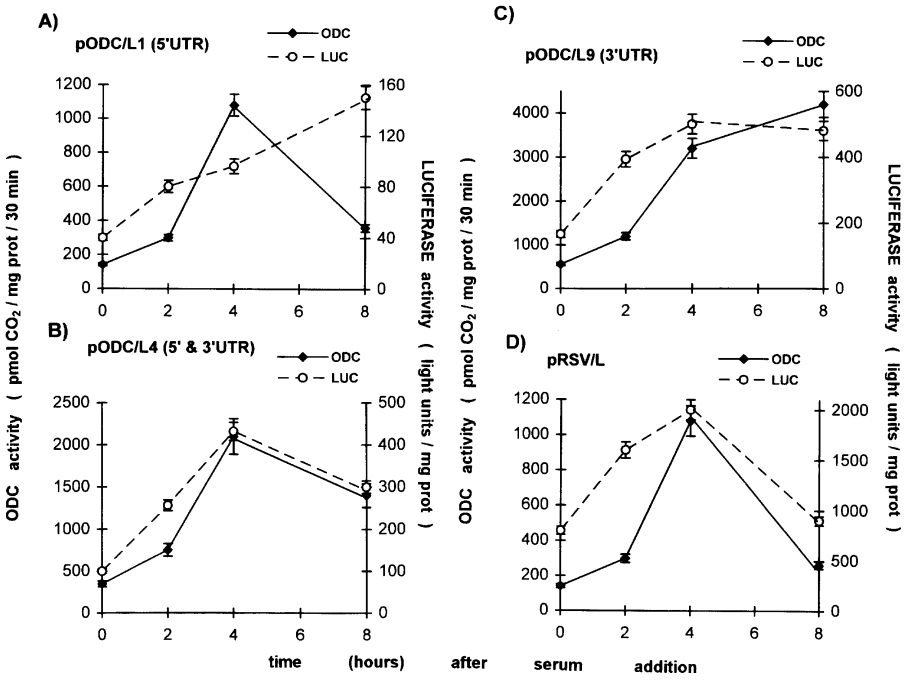

Figure 6 Effects of overexpression of the ODC 5′ UTR and/or 3′ UTR on ODC and LUC activities in serum-stimulated transfected cells

Cells were synchronized by serum starvation. Serum was added at zero time. At the indicated times, cells were collected and the ODC activity (◆) (left ordinate), LUC activity (○) (right ordinate) and protein content were measured. CHO-K1 cells were transfected with (**A**) pODC/ L1, which resulted in the overexpression of the ODC 5′UTR; (**B**) pODC/L4, expressing both the ODC 5′ and 3′ UTR; (**C**) pODC/L9, expressing the ODC 3′ UTR; (**D**) pRSV/L, which expressed the LUC coding sequence only. Results are means ± S.E.M.; error bars smaller than the symbols are not shown.

activity increased sharply between 0 and 4 h, and decreased to almost the basal level 4 h later.

Kinetics of ODC and LUC activity in stably transfected, synchronized CHO-K1 cells

If, as suggested by the results shown above, there are cellular factors induced in cells by serum stimulation, one might ask about their relative abundance, and hence about a potential competition between the endogenous ODC mRNA and the chimaeric transcripts from the transgenes. Specifically, either the 5′ or the 3′ UTR of the transcripts from the transgenes might sequester regulatory factors synthesized in limited amounts during the cell cycle, and hence the translation of the endogenous ODC mRNA might be affected. In these CHO-K1 cells ODC activity normally increases 5–10-fold 4 h after the addition of serum (Figure 5B), whereas mRNA levels remain constant (Figures 5A and 7). Therefore, ODC activity as well as LUC activity were measured in serum-starved and -stimulated cells. These results are shown in Figure 6.

In the presence of excess 5′ UTR sequences from the pODC/L1 construct, ODC activity varied in a completely normal pattern over the first 8 h (Figure 6A). At the same time there was a steady accumulation of LUC activity (Figure 6A). The corresponding chimaeric mRNA levels are shown in Figure 7, and they do not change appreciably during this period for any of the constructs. We conclude that the 5′ UTR *in trans* does not affect the translation of ODC mRNA. When the pODC/L4 transcript was present (5′ and 3′ UTRs), the behaviour of the ODC activity was again almost normal, whereas LUC activity seemed to reach a maximum at 4 h, followed by a decrease between 4 and 8 h (Figure 6B). A similar pattern for both enzymes was also observed

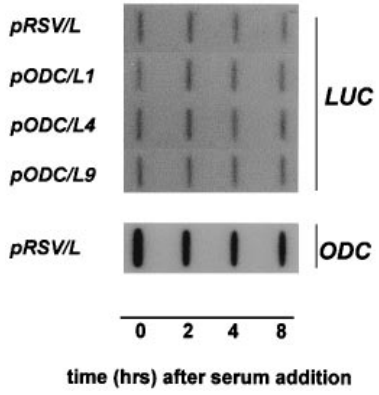

Figure 7 Effect of serum stimulation on the expression of the hybrid and ODC transcripts

Slot-blot analyses. Total RNA (20 μg) from each set of CHO-K1 transfectant rendered quiescent by serum withdrawal (zero time) and at different times after serum addition was used. RNA samples were spotted on membranes by using a slot-blot apparatus and hybridized with the radiolabelled LUC probe (shown at the right) or with the *Hin*dIII fragment of the ODC cDNA (see the Materials and methods section).

when the pRSV/L transcript without additional flanking sequences was expressed (Figure 6D). It should be noted that LUC activity was considerably higher in Figure 6(D). A possible explanation for the observed decrease at later times might be that when LUC is in excess its import into peroxisomes might be

limited; LUC remaining in the cytosol has a significantly shorter half-life, and at later times an increased rate of turnover might be responsible for the observed decline.

Finally, somewhat unusual results were observed for ODC activity when the pODC/L9 transcript was expressed. The basal level of ODC activity (0 h, Figure 6C) was higher than in the control or the other transfected lines, there was further accumulation over 8 h, and no decline in activity was observed between 4 and 8 h. It is tempting to speculate that excess 3′ UTR sequences were directly responsible for this abnormal behaviour. A careful titration or systematic variation in the 3′ UTR sequence levels will be required to confirm such an effect. It should be noted that all of these results were obtained with a pooled set of clones and are therefore not the result of a single exceptional clone of CHO-K1 cells selected in the course of the transfection and selection for the transgene.

DISCUSSION

The marked rise and fall in ODC levels during the early phases of the cell cycle in serum-stimulated cells has attracted attention for a long time. Post-transcriptional mechanisms involving either protein turnover or translational control have been suggested, and both might contribute to the overall kinetics [1–4]. A particular challenge has been to understand the significance, if any, of the exceptionally long 5′ UTR of ODC mRNA, and more significantly the presence of the GC-rich and hence very stable hairpin loop [1,2,17,18,26]. Numerous investigators have demonstrated that the 5′ UTR alone has a strong inhibitory effect on the translation of ODC mRNA and various chimaeric mRNA species [18]. It is clear that the rate of translation initiation with ODC mRNA is poor [26–28]. It is much less clear whether this inhibitory function has physiological significance and whether it can be subject to modulation as a means of regulating ODC expression.

In the first set of experiments we compared the translatability of hybrid transcripts, bearing the ODC-UTRs, *in vivo* and *in vitro*. Our data *in vivo* confirmed the inhibition of the translational efficiency owing to the presence of the ODC 5′ UTR and the relieving effect imposed by the ODC 3′ UTR described previously [18]. Furthermore we noted a decrease in LUC activity when only the ODC 3′ UTR was carried by the transcript. We observed a different pattern of translatability of the same hybrid RNA species, obtained from the transfectants, by translating them in a translation system *in vitro* (reticulocyte lysate).

It can be hypothesized that cellular factors are responsible for the co-operative interaction between the ODC 5′ and 3′ UTRs. Such factors might not be present in the rabbit reticulocyte lysate, or might be present only in insufficient amounts or in an inactive state. Furthermore their presence in CHO-K1 or COS-1 cells might be cell-cycle-dependent. To assay such factors, attempts were made to restore such a function to the reticulocyte system by complementing it with extracts from CHO-K1 or COS-1 cells. To relate such function(s) to the observed translational regulation of endogenous ODC mRNA, extracts were prepared at different times after serum stimulation of quiescent cells. In our experimental conditions only the cytoplasmic extract from 2 h serum-stimulated cells contained factor(s) that enabled a more efficient translation of the hybrid mRNA that carries the ODC 5′ UTR alone, and the effect was relatively minor (Figure 4). In contrast, a striking effect of different extracts was observed with the pODC/4 transcript (5′ and 3′ UTR). There was a significant stimulation with the 2 h extract that disappeared at later times. Translation of a transcript with only the 3′ UTR was relatively insensitive to extracts from 0 to 4 h, but at longer times an inhibitory effect became apparent.

What is the relationship of these factors to the UTRs and to known or previously described factors involved in translation? The role of the various eukaryotic initiation factors (such as eIF2 and eIF4) is now established, but what is less well understood is the precise modulation of the activity of these factors by phosphorylation, and how such modifications can influence mRNA translation either globally or even in a transcript-dependent manner [29]. Thus *cis*-acting sequences such as the 5′ UTR also have specific roles. For example, extensive secondary structure (e.g. hairpin loops) in the RNA might have to be melted by a helicase activity associated with eIF4A and eIF4B. Either the overexpression of such factors or the expression of factors mutated to lack phosphorylation sites has demonstrated the importance of such regulatory mechanisms [29]. Neoplastic transformation might result either from a modification of such factors or from a change (alternative splicing or transcription initiation) in the 5′ UTR [2,7,29,30].

A computer-assisted comparison has failed to reveal extensive complementary regions between the 5′ and 3′ UTRs of ODC mRNA, and it is most likely that proteins are involved in the indirect interaction between the ends of ODC mRNA, as demonstrated here and in previous studies. It is becoming increasingly clear that the ends of at least some mRNA species interact in complex ways that might determine either the stability or the translatability of these transcripts [9,10]. Circular polyribosomes have been observed by electron microscopy [31]. An interaction of the poly(A)$^+$-binding protein (PABP) and other factors with factors associated with the 5′ end is strongly suggested by the role of polyadenylation either in translation or in mRNA turnover, with different emphasis depending on the particular transcript [10,12]. As with other RNA species [9,12,32], an ODC 5′ UTR-binding protein has been described but its role is still obscure [33]. Factors binding to the coding sequences or the 3′ UTR (for example, AUUUA-binding factors controlling mRNA stability) [34] are believed to control deadenylation and hence the binding of the PABP complex. Such factors might be inducible during the cell cycle, or their activity might depend on stages in the cell cycle. Our results from complementation studies *in vitro* with the pODC/L4 transcript containing both the 5′ and 3′ UTR can be interpreted in terms of a cell-cycle-dependent activity promoting the positive interaction of the ends of ODC mRNA. This interaction could either cause an unwinding of the strong hairpin in the 5′ UTR and hence promote ribosome scanning [35], or it could promote a by-pass or even internal initiation of translation [36]. Thus the stimulatory activity of the 2 h extract on the translation of pODC/L4 mRNA in the reticulocyte lysate might reflect an activity that is also responsible for the induction of ODC after serum stimulation *in vivo*.

Another question that arises in this context is whether the relevant factors are abundant or present in very limiting amounts. For example, it has been reported that the overexpression of the creatine kinase B 3′ UTR causes a translational derepression of the creatine kinase messenger by removing an RNA-binding protein that binds to the 3′ UTR of this transcript, thereby suppressing translation [37]. Our initial results, comparing the expression of endogenous ODC in different cells expressing LUC transcripts with different 5′ and 3′ UTRs, suggest some preliminary answers.

From the kinetics of ODC and LUC activities measured in the transfectant that expresses excess ODC 5′ UTR we concluded that this untranslated segment is apparently strictly involved in the translational regulation of the RNA to which it belongs (*cis*-acting). It does not seem to compete for cellular factors expressed

during the early phase of the cell cycle, when ODC mRNA translation is stimulated significantly (Figure 6A). Such factors are either abundant or do not interact with the 5′ UTR.

Finally, the presence of excess copies of the ODC 3′ UTR expressed *in trans* seemed to modify the basal ODC activity level and the kinetics of both ODC and LUC activities, the latter derived from the translation of the hybrid transcript bearing the ODC 3′ UTR. A competition for cytoplasmic regulatory factors seems to occur between the 3′ UTR of endogenous ODC mRNA and the chimaeric mRNA, which results in a decreased translational repression of ODC mRNA (Figure 6C), either in quiescent cells (0 h), or at later times (8 s). A provocative hypothesis arises from recent observations that indicate that specific 3′ UTRs can act *in trans* and affect the expression of different mRNA species with unexpected consequences for the cells [38,39]. Moreover an increase in ODC and LUC activities is still observed after serum stimulation. Recent developments demonstrate that the 3′ UTRs of an increasing number of mRNA species contain sequences involved in translational control [40]. For example, it has been demonstrated that translation of 15-lipoxygenase mRNA is repressed by a protein expressed by peripheral reticulocytes that specifically binds a segment of the 3′ UTR [41]. A relationship to the changing polyamine levels during the same time interval remains to be established. Nevertheless other authors observed that the polyamines accumulate in significant amounts in later phases of the cell cycle [42]. A currently favoured model suggests that polyamine accumulation leads to an elevation of antizyme, followed by increased ODC turnover, perhaps combined with translational repression [4,43]. In our experiments the translational repression might not be sufficient to cause a net decrease in ODC activity as observed in control cells.

The complex regulatory mechanisms associated with the translation of ODC mRNA have been indicated by numerous experiments *in vivo*. We believe that the experiments reported here for the translation of chimaeric mRNA species in a reticulocyte system, and the observed complementing effects of cytoplasmic extracts from serum-stimulated cells, represent a novel and promising model system for the exploration of the same mechanisms *in vitro*. Such systems *in vitro* will ultimately be essential for the complete purification and characterization of the relevant factors.

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