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Transformation by H-ras Can Result in Aberrant Regulation of Ornithine Decarboxylase Gene Expression by Transforming Growth Factor-β_1

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Abstract Inhibition of DNA synthesis and cell proliferation is frequently lost during malignant transformation and occasionally, tumour cell proliferation is actually stimulated by transforming growth factor β_1 (TGF-β_1). The present study demonstrates a novel link between alterations in TGF-β_1 regulation during cellular transformation and malignant conversion and the expression of ornithine decarboxylase (ODC) which is a key rate limiting activity in the biosynthesis of polyamines and which is an enzyme that plays an important role in cell growth and differentiation. H-ras transformed mouse $10T\frac{1}{2}$ cell lines of varying degrees of malignant potential were examined for possible TGF-β_1-mediated alterations in ODC expression. Selective induction of ODC gene expression occurred. This induction was dependent upon the cellular phenotype expressed and the mechanisms responsible for the regulation of the TGF-β_1-mediated alterations in ODC expression varied as a function of malignant potential. The TGF-β_1-mediated alterations in ODC gene expression involves de novo protein synthesis, transcriptional, and post-transcriptional events. Evidence is also presented to suggest a possible role for protein kinase C-mediated events, protein phosphatases, and G-protein-coupled events in the TGF-β_1-mediated regulation of ODC expression in H-ras transformed cells. Evidence is also presented to suggest a possible role for cellular polyamines in the TGF-β_1-mediated alterations in ODC expression in H-ras transformed cells. Additionally, alterations in cellular polyamines were shown to influence TGF-β_1 gene expression in H-ras transformed cells and that these alterations occurred, in part, through post-transcriptional events. The TGF-β_1-mediated regulation of ODC expression in H-ras transformed cells of varying degrees of malignant potential appears to be complex, multifaceted, and interactive. This study illustrates the importance of TGF-β_1-mediated regulation of ODC expression as a result of H-ras mediated cellular transformation and malignant progression, and further suggests that this TGF-β_1-mediated regulation constitutes an integral part of an altered growth regulatory program. J. Cell. Biochem. 81:39–55, 2001.

Key words: ornithine decarboxylase; TGF-β_1; cellular transformation; malignant progression; altered regulation and expression

During malignant progression, numerous and varied biological and biochemical changes occur [Nicolson, 1984; Weber, 1983; Wright et al., 1993]. These alterations appear to be controlled through modifications in a discrete set of fundamental regulatory genes, especially oncogenes [Egan et al., 1987a,b; Greenberg et al., 1989; Wright et al., 1990a] which appear to be critically important in cellular functions [Greenberg et al., 1989; Wright et al., 1990b, 1993]. These functions are often subject to regulation by growth factors [Weinberg, 1989; Wright et al., 1993].

Growth factors are fundamentally involved in cellular processes that are important in the progression of malignant disease [Wright et al., 1993]. Two growth factor families, transforming growth factor β (TGF-β) and fibroblast growth factor (FGF) are both useful models for examining the involvement of growth factors in mechanisms of malignant progression [Wright et al., 1993]. TGF-β appears to be a fundamental regulator of cellular behaviour. TGF-β represents a family of homodimeric proteins required for the growth, development,

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and differentiation of many types of cells [Sporn and Roberts, 1990]. Although most normal cells are growth inhibited by TGF-β_1, its effects are often dependent on many growth conditions [Roberts et al., 1988; Schwarz et al., 1988]. Previously, it has been reported that while TGF-β_1 inhibited DNA synthesis in the $10T\frac{1}{2}$ pulmonary fibroblast cell line, H-ras transformed $10T\frac{1}{2}$ cell lines that demonstrated a highly malignant phenotype exhibited stimulation of DNA synthesis following exposure to TGF-β_1 [Hurta and Wright, 1995; Schwarz et al., 1988]. Furthermore, we have suggested that this altered response to TGF-β_1 following malignant transformation by ras, combined with an elevated rate of secretion of activated TGF-β_1 by these cells [Hurta et al., 1991, 1993; Schwarz et al., 1990] indicates a role for TGF-β_1 in the autocrine stimulation of cell proliferation in malignancy. In this regard, a link between cellular transformation and alterations in the expression of ribonucleotide reductase, a highly controlled rate-limiting step in DNA synthesis, has been previously demonstrated [Hurta and Wright, 1995; Hurta et al., 1991]. Furthermore, these studies were extended to demonstrate a role for TGF-β_1 in the regulation of ornithine decarboxylase (ODC) gene expression in malignant H-ras transformed cells [Hurta et al., 1993]. Ornithine decarboxylase is the first rate-limiting enzyme in the synthesis of polyamines which are necessary for all animal, plant, and microbial cell proliferation and survival [Pegg, 1988; Tabor and Tabor, 1984], and other studies have shown that altered ornithine decarboxylase regulation can play important roles in mechanism of malignancy with NIH-3T3 fibroblasts [Moshier et al., 1993], in cells containing a mutated ras gene [Hibshoosh et al., 1991] and in mechanisms associated with cellular transformation and malignancy [Auvinen, 1997; Auvinen et al., 1992, 1997; Clifford et al., 1995; Hurta, 1999; Hurta et al., 1996; Kubota et al., 1997; Smith et al., 1997; Tabib and Bachrach, 1998]. Previously, a novel link between alterations in TGF-β_1 regulation during malignant progression, and the expression of ornithine decarboxylase, was demonstrated [Hurta et al., 1993]. A panel of radiation and H-ras transformed mouse $10T\frac{1}{2}$ cell lines with malignant potential was investigated for possible TGF-β_1 mediated changes in ornithine decarboxylase gene expression. Selective induction of gene expression was observed since only H-ras transformed cell lines with malignant potential exhibited marked elevations in ornithine decarboxylase message levels. ODC gene expression in non-transformed $10T\frac{1}{2}$ cells and in cell lines capable of only benign tumour formation was unaffected by TGF-β_1 treatment [Hurta et al., 1993]. These studies were done in exponentially growing cells. The present study was designed to continue to investigate this relationship between TGF-β_1 mediated signalling and changes in ODC expression and this present study confirms and extends these earlier observations and provides further support of a key role for TGF-β_1 in the aberrant regulation of ornithine decarboxylase expression associated with H-ras mediated cellular transformation and malignant progression.

MATERIALS AND METHODS

Cell Lines and Growth Conditions

Mouse cell lines were routinely cultured at 37°C on plastic tissue culture dishes (Life Technologies) in alpha minimal essential medium (α MEM) (Life Technologies) supplemented with antibiotics and 7% fetal bovine serum (Hyclone Laboratories) as previously described [Hurta et al., 1996]. Cells were grown to confluence prior to exposure to TGF-β_1 (R&D Systems, Inc.). In experiments using the growth factor TGF-β_1, a serum-free medium (defined medium) was used that contained 4 μg/ml transferrin (Sigma) and 2 μg/ml of insulin (Sigma) [Hurta et al., 1996; Schwarz et al., 1988]. Cells were grown to confluence, then placed on a defined medium for 24 h prior to exposure to TGF-β_1 for predetermined times. Cells were removed from the surface of tissue culture plates using a 0.3% buffered trypsin solution (Difco Laboratories) [Hurta et al., 1996].

Northern Blot Analysis

Total cellular RNA was prepared by a rapid extraction method using the TRIZOL reagent according to the manufacturer's instructions (Life Technologies) and subjected to electrophoresis through 1% formaldehyde-agarose gels followed by transfer to Nytran nylon membranes (Schleicher and Schuell). Blots were pre-hybridized and hybridized at 65°C using Rapid-Hyb (Amersham) according to the manufacturer's instructions. Hybridization

occurred in the presence of either a ^{32}P-labelled Pst-1 generated fragment of ODC cDNA from pODC934 plasmid (provided by A. E. Pegg, Milton S. Hershey Medical Center, Hershey, PA.) or a ^{32}P-labelled Bgl II generated TGF-β_1 fragment from the pPK9A plasmid (provided by A. E. Greenberg, M.C.T.R.F., University of Manitoba, Winnipeg, Manitoba). Probes were labelled using an oligolabelling kit (Amersham Pharmacia Biotech) or a nick translation kit (Gibco-BRL) and ^{32}P-labelled dCTP (Amersham). Blots were washed and autoradiography performed as previously described [Hurta et al., 1996]. Loading was monitored by either ethidium bromide stained ribosomal RNA bands prior to gel transfer to nylon membranes or by monitoring loading with a Bam H1-Kpn I generated cDNA probe specific for rodent glyceraldehyde-3-phosphate dehydrogenase isolated from the plasmid pBssK$^-$ (provided by L.J.Z. Penn, Ontario Cancer Institute, Toronto, Ontario, Canada) or with a 28S ribosomal RNA probe isolated from the plasmid pA_{BB} by digestion with Bam H1 [Gonzalez et al., 1985]. Densitometric analysis of appropriately exposed autoradiograms was performed using a GS700 Imaging Densitometer (Biorad) and the Molecular Analyst software program (Biorad).

Assay for Ornithine Decarboxylase Enzyme Activity

Ornithine decarboxylase enzyme activity was measured using a modification of the method of Russell and Synder [1968] as described by Yoshida et al. [1992] and Hurta et al. [1996]. Cell pellets were solubilized in 50 mM Tris–HCl (pH 7.5) containing 5 mM dithiothreitol, sonicated briefly, and then centrifuged at 10,000 *g* to remove the insoluble fraction. Protein content was determined using a BioRad protein assay kit, with bovine serum albumin as a standard. The enzyme assay consisted of 0.4 mM L-ornithine, 0.125 μCi of DL-[1-^{14}C] ornithine (58 mCi/mM) (Amersham), 0.02 mM pyridoxal phosphate, 0.4 mM EDTA, 50 mM Tris–HCl (pH 7.5), and cellular extract. Incubation was carried out for 60 min at 37°C. The reaction was terminated by the addition of 0.8 ml of 2 M citric acid. Ornithine decarboxylase activity was determined by measuring the release of [^{14}C] CO_2 which was collected in filter paper soaked with 25% phenylethylamine. Radioactivity was determined by liquid scintillation spectroscopy using a LS6500 multipurpose scintillation counter (Beckman, Mississauga, Canada). ODC activity was calculated and expressed as nmoles CO_2/h/mg protein.

RESULTS

Properties of H-ras Transfected Mouse Fibroblasts

Mouse 10T½ cells were transfected with the plasmid pAL8A which contains T-24-H-ras and the neo® gene. Cell lines were established that were either morphologically transformed (C2) or morphologically non-transformed (NR3) [Egan et al., 1987a; Schwarz et al., 1988]. A summary of the biological characteristics of these cells is presented in Table I. It is important to note that the 10T½ cell is not tumourigenic, the NR3 cell is capable of forming benign tumours in syngeneic hosts, while the C2 cell line exhibits malignant characteristics.

TABLE I. Tumourigenic and Metastatic Properties of Mouse 10T½ Cell Lines[a]

			Experimental metastases	
Cell line	Tumourigenic frequency	Frequency	Number of lung nodules (Mean ± SE)	Degree of malignancy
10T½	0 / 12	0 / 12	0	Normal
NR3	6 / 8	1 / 13	0.1 ± 0.1	Benign
C2	11 / 11	8 / 8	118 ± 6	Metastatic

[a]The data in this table was summarized from previously reported observations [Egan et al., 1987; Hurta et al., 1996].

Selective Induction of Ornithine Decarboxylase Gene Expression by TGF-β_1

The effects of TGF-β_1 (10 ng/ml) on ODC gene expression in 10T$\frac{1}{2}$ and C2 cell lines described in Table I are shown in Figure 1. Obvious alterations in ODC mRNA levels were observed following TGF-β_1 treatment, but these elevations occurred only in the malignant C2 cell line and did not occur in the parental, non-transformed 10T$\frac{1}{2}$ cell line (Fig. 1). Marked alterations in ODC gene expression were observed in the highly malignant C2 cells following TGF-β_1 treatment. Densitometric evaluation of appropriately exposed autoradiograms revealed 8.4-, 9.2-, and 9.4-fold increases in ODC mRNA levels in C2 cells following exposure to TGF-β_1 for 2, 4 and 8 h, respectively (Fig. 1).

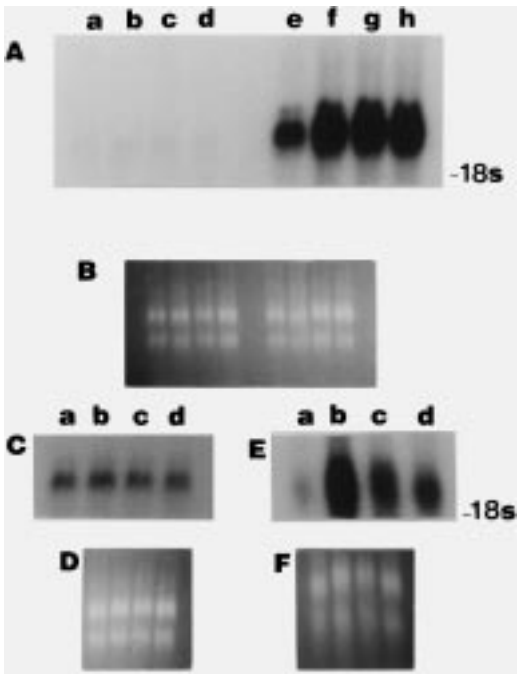

Fig. 1. ODC gene expression following treatment with TGF-β_1 (10 ng/ml). Northern blot analysis of ODC mRNA levels in (**A**) confluent 10T$\frac{1}{2}$ cells in the absence of TGF-β_1 (a), and following exposure to TGF-β_1 for 2 (b), 4 (c), and 8 (d) h, respectively, and in confluent C2 cells in the absence of TGF-β_1 (e), and following exposure to TGF-β_1 for 2 (f), 4 (g), and 8 (h) h, respectively. (**B**) Ethidium bromide stained ribosomal RNA bands as a loading control. (**C**) Northern blot analysis of ODC mRNA levels in exponentially growing NR3 cells in the absence of TGF-β_1 (a), and following exposure to TGF-β_1 for 2 (b), 4 (c), and 8 (d) h, respectively. (**E**) Northern blot analysis of ODC mRNA levels in confluent NR3 cells as described above. (**D**) and (**F**) Ethidium bromide stained ribosomal RNA bands as loading controls. The autoradiograms shown in (A), (C), and (E) were exposed for 48, 24, and 24 h, respectively, at −70°C with intensifying screens.

These observations confirm our previously reported results [Hurta et al., 1993]. Previously, it was demonstrated that ODC mRNA levels were not affected by TGF-β_1 treatment in exponentially growing NR3 cells (These cells have low levels of ras expression and are only capable of forming benign tumours) [Hurta et al., 1993]. The effect of TGF-β_1 (10 ng/ml) on ODC gene expression was examined in exponentially growing and in confluent cultures of NR3 cells. As shown in Figure 1, in agreement with previous observations, no change in the ODC mRNA levels occurred in response to TGF-β_1 treatment in exponentially growing NR3 cells. However, in NR3 cells, which were grown to confluence prior to exposure to TGF-β_1, a marked but transient increase in ODC mRNA levels occurred (Fig. 1). Densitometric evaluation of appropriately exposed autoradiograms revealed 11.2-, 6.6- and 3.8-fold increases in ODC mRNA levels occurred in confluent cultures of NR3 cells following exposure to TGF-β_1 (at 10 ng/ml) for 2, 4, and 8 h, respectively. Based on these observations, the status of the NR3 cells' growth is important in determining the response which occurs when these cells are exposed to TGF-β_1.

Ornithine Decarboxylase Enzyme Activity in Cells Treated with TGF-β_1

To determine if the increases in ODC message observed in these TGF-β_1-treated cells lead to elevated enzyme activity, the level of ODC enzyme activity in exponentially growing and in confluent cultures of 10T$\frac{1}{2}$, NR3, and C2 cells was measured following TGF-β_1 treatment for 2, 4, and 8 h, respectively. No elevation in ODC enzyme activity was found in non-transformed 10T$\frac{1}{2}$ cells (Table II). Marked elevations in ODC enzyme activity were found in high ras, malignant C2 cells (Table II). These increases in ODC enzyme activity occurred in both exponentially growing and in confluent C2 cell cultures, although the induction of ODC enzyme activity was more pronounced in confluent C2 cell cultures. The elevations of ODC enzyme activity were maintained over the time course evaluated. Interestingly, in exponentially growing NR3 cell cultures, no elevation in ODC enzyme activity occurred (Table II), however, increased ODC enzyme activity was found in response to TGF-β_1 treatment of confluent cultures of NR3 cells (Table II). These alterations in enzyme activity were transient

TABLE II. Ornithine Decarboxylase Enzyme Activity in Nontransformed and in H-ras-Transformed Cells in Response to TGF-β_1 (10 ng/ml)

Cell line	Exposure time (h)	ODC enzyme activity[1]	
		Exponentially growing cells	Cells at confluence
$10T\frac{1}{2}$	0	0.14 ± 0.04	0.09 ± 0.05
	2	0.14 ± 0.08	0.08 ± 0.01
	4	0.15 ± 0.02	0.11 ± 0.07
	8	0.17 ± 0.02	0.06 ± 0.02
NR3	0	0.37 ± 0.04	0.28 ± 0.06
	2	0.32 ± 0.08	2.96 ± 0.11
	4	0.41 ± 0.12	1.93 ± 0.11
	8	0.40 ± 0.05	1.02 ± 0.08
C2	0	0.80 ± 0.15	0.63 ± 0.06
	2	3.69 ± 0.51	6.41 ± 0.18
	4	5.77 ± 0.28	20.8 ± 0.47
	8	7.63 ± 0.55	25.2 ± 0.77

[a]The ODC enzyme activity is expressed as nmoles CO_2/h/mg protein.

and paralleled the alterations observed in ODC mRNA expression in response to TGF-β_1 treatment (Table II; Fig. 2). No changes in either ODC mRNA levels or enzyme activity were noted in confluent cultures of either $10T\frac{1}{2}$ or NR3 cells (following exposure to TGF-β_1 for 24 h), however, ODC mRNA and enzyme activity remained elevated in confluent C2 cells, in response to treatment with TGF-β_1 for 24 h (data not shown).

Effect of TGF-β_1 Treatment on Transcription of the Ornithine Decarboxylase Gene

The possibility that the elevations in ODC message observed in NR3 and in C2 cells following exposure to TGF-β_1 were due to changes in gene transcription was examined by pretreating NR3 and C2 cells with the transcription blocker, actinomycin D [Phillips and Crowthers, 1986] prior to exposure to TGF-β_1 (10 ng/ml) for 2 h. As shown in Figure 2, actinomycin D inhibited the elevation in ODC message previously observed following exposure of these cells to TGF-β_1. In confluent NR3 cells, in the absence of actinomycin D, a 6.5-fold increase in ODC mRNA level occurred in response to exposure to TGF-β_1 for 2 h, whereas in the presence of actinomycin D, (5 μg/ml), an increase of only 2.5-fold occurred in these cells in response to TGF-β_1 treatment (Fig. 2). On the other hand, in confluent C2 cells, in the absence of actinomycin D, a 7.4-fold increase in ODC mRNA level was noted, whereas in the presence of actinomycin D, the level of ODC mRNA expression in response to TGF-β, was similar to that found in unstimulated C2 cells (Fig. 2). In the presence of actinomycin D, alone (1 h exposure), the level of ODC mRNA expression was similar to or not appreciably different from (taking into consideration differences in loading as indicated and by GAPDH expression

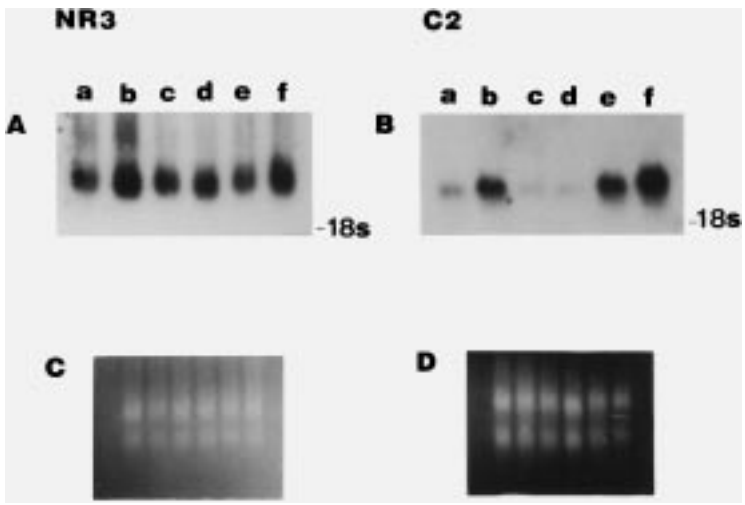

Fig. 2. Northern blot analysis of ODC message expression in NR3 cells and in C2 cells. (**A**) ODC message levels in NR3 cells, control cells (a), in the presence of only TGF-β_1 for 2 h (b), in the presence of only actinomycin D (5 μg/ml) (1 h) (c), in the presence of actinomycin D and TGF-β_1, (d), in the presence of cycloheximide (10 μg/ml) (e), in the presence of cycloheximide and TGF-β_1 (f). (**B**) ODC message levels in C2 cells as described above. (**C**) and (**D**) Ethidium bromide stained ribosomal RNA bands as loading controls. The autoradiogram shown in (A) and in (B) were exposed for 24 and 6 h, respectively at −70°C with intensifying screens.

(data not shown)) that found in unstimulated C2 cells (Fig. 2). Actinomycin D (5 μg/ml) treatment of cells for 1 h was found to inhibit RNA synthesis, in general, by approximately 90% (data not shown). Actinomycin D treatment for 1 h, however, does not generally affect the basal levels of ODC mRNA expression to an appreciable amount in either NR3 or in C2 cells. This observation is entirely consistent with previous studies with these cells which illustrated that actinomycin D (at this concentration and for this duration of time) treatment does not affect the basal level of expression of ODC mRNA in these cells [Hurta and Wright, 1992; Hurta et al., 1993]. Presumably higher concentrations and longer exposure times may affect the basal level of ODC expression in both of these cells. Whether this is so remains to be determined. Inhibitors are commonly used to investigate questions of gene expression control at the level of transcription [Hurta and Wright, 1994, 1995; Hurta et al., 1996; Phillips and Crowthers, 1986;], and although this approach is somewhat indirect, the findings obtained in this study suggest that the TGF-β_1 effects on ODC gene expression are mediated, at least partly, by changes in the transcriptional process.

Effect of Protein Synthesis Inhibition on Ornithine Decarboxylase mRNA Induction by TGF-β_1

To determine whether or not the TGF-β_1 mediated elevations in ODC mRNA determined to occur in confluent cultures of NR3 and C2 cells required protein synthesis, the effect of the protein synthesis inhibitor, cycloheximide [Edwards and Mahadevan, 1992; Hurta et al., 1996] on TGF-β_1 mediated alterations in ODC gene expression was investigated in NR3 cells and in C2 cells. Figure 2 shows that in the absence of cycloheximide, in response to TGF-β_1 treatment, a 6.5-fold increase in ODC mRNA levels occurred in NR3 cells, whereas in the presence of cycloheximide (10 μg/ml), a 3.5-fold increase in ODC mRNA level occurred in NR3 cells in response to TGF-β_1 treatment (Fig. 2). Cycloheximide alone did not affect ODC mRNA levels in NR3 cells. These observations suggest that the TGF-β_1 modulated elevation of ODC mRNA expression in NR3 cells is a de novo protein synthesis dependent process. This is quite different from what occurs in highly malignant C2 cells. In the absence of cycloheximide, in response to TGF-β_1 treatment, a 7.4-fold increase in ODC mRNA expression occurred (Fig. 2). Following exposure of C2 cells to cycloheximide and TGF-β_1 for 2 h, a 17-fold elevation in ODC mRNA levels was observed (Fig. 2). Figure 2 also illustrates that cycloheximide treatment alone markedly elevated ODC message levels in C2 cells, with a 7.3-fold increase in ODC message levels. This observation is consistent with previous observations showing that cycloheximide treatment can elevate ODC mRNA levels in some mammalian cells [Hurta et al., 1996; Olson and Spizz, 1986]. This type of regulation appears to be cell type-specific since a reduction in ODC message levels in cycloheximide treated cells has also been reported [Katz and Kahana, 1987]. These observations suggest that ODC gene expression in the highly malignant C2 cell line is controlled in a positive manner by protein synthesis inhibition following cycloheximide treatment. The exact mechanism(s) associated with this regulation remains to be investigated further, however, part of the mechanism involved in the cycloheximide mediated induction of ODC mRNA expression in C2 cells involves post-transcriptional stabilization of the ODC mRNA transcript (unpublished observations).

Stability of Ornithine Decarboxylase Message in H-ras Transformed Cells Following Exposure to TGF-β_1

Although TGF-β_1 mediated elevations of ODC mRNA levels may occur via changes in the transcriptional process (Fig. 2), growth factors, including TGF-β_1, can also regulate gene expression post-transcriptionally through alterations in message stability [Amara et al., 1995, 1996; Hurta et al., 1993, 1996]. Therefore, the possibility that changes at the post-transcriptional level in response to TGF-β_1 can also occur was investigated in NR3 and C2 cells. Since a common mechanism for regulating message levels post-transcriptionally takes place via alterations in the decay rates of mature message in response to external stimuli [Amara et al., 1994; Chen et al., 1993], the rate of decay of mature ODC message was evaluated in untreated and in TGF-β_1 treated NR3 and C2 cells after exposure to an inhibitor of transcriptional initiation, 5,6,-dichloro-1-β-D ribofuranosylbenzimidazole (DRB) [Hurta and Wright, 1995; Hurta et al., 1996; Mukherjee and Molloy, 1987;]. As shown in Figure 3, ODC

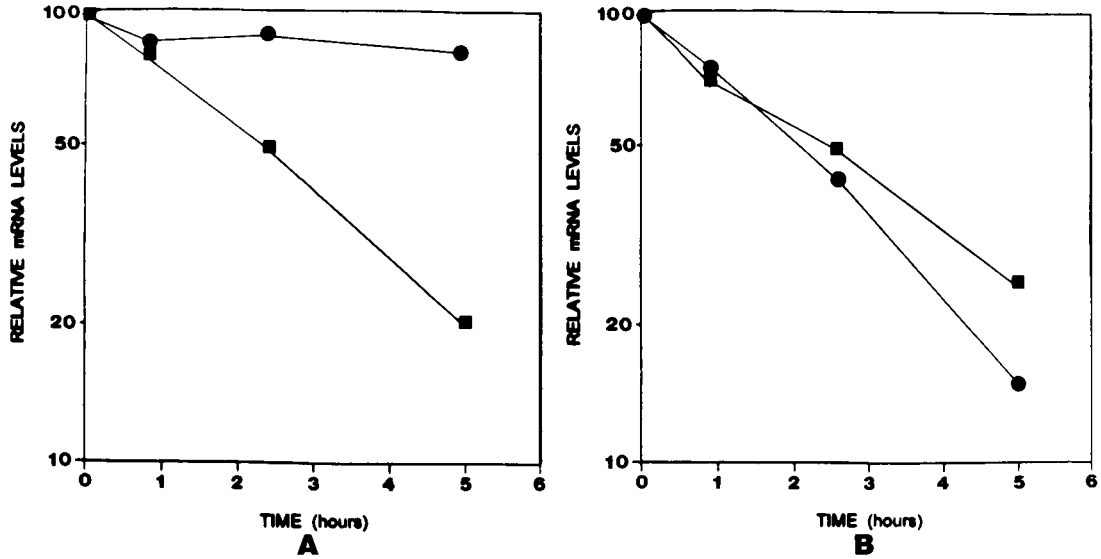

Fig. 3. Stability of ODC mRNA in untreated and in TGF-β_1 treated C2 cells (**A**) and in TGF-β_1 treated NR3 cells (**B**). Cells at confluence exposed to TGF-β_1 (10 ng/ml) (●) or to BSA/HCl solution (the vehicle within which the TGF-β_1 is dissolved/activated) (■) for 4 h were subsequently treated with DRB (75 μM). Total cellular RNA was isolated at the times indicated and subjected to Northern blot analysis as described. The relative levels of ODC mRNA were determined by densitometric evaluation of autoradiograms exposed in the linear range for each set of samples. The results presented are from duplicate experiments.

mRNA in C2 cells is more stable in the presence of TGF-β_1. Assuming that the decay of ODC mRNA after blocking RNA synthesis follows first-order kinetics, the half-life of ODC in TGF-β_1 treated C2 cells, estimated by extrapolation, was increased approximately by 8-fold over that found in C2 cells not treated with TGF-β_1. The half-life of ODC message in untreated C2 cells was estimated to be 2.3 h whereas the half-life of this message in TGF-β_1 treated C2 cells was altered and increased to approximately 18.5 h. The half-life of ODC message transcript in NR3 cells was determined to be approximately 2.5 and 2.2 h in untreated and in TGF-β_1 treated NR3 cells, respectively (Fig. 3). These findings indicate that TGF-β_1 is capable of regulating ODC mRNA levels through a mechanism of post-transcriptional stabilization in highly malignant C2 cells, however, TGF-β_1 mediated post-transcriptional stabilization of ODC message apparently does not occur in NR3 cells, which are non-metastatic.

Possible Role for Protein Kinase C-Mediated Events and Protein Phosphatases in the TGF-β_1 Mediated Regulation of Ornithine Decarboxylase

The involvement of a protein kinase cascade in the action of some mitogens has been proposed [Pelech et al., 1990; Ralph et al., 1990]. Phorbol ester tumour promoters, such as, phorbol 12-myristate-13-acetate (PMA) activate protein kinase C (PKC) [Nishizuka, 1986], although the precise pathway responsible for transducing the signal generated through PKC activation is unknown. We hypothesized that the PMA-mediated and the TGF-β_1-mediated effects on ODC gene expression in C2 cells may involve some components of the same pathway. To test this hypothesis, C2 cells were pre-treated with PMA (0.1 μM) for 48 h prior to exposure to TGF-β_1. Extended treatment with PMA results in the down regulation of PKC activity [Young et al., 1987]. Following this 48 h PMA treatment, cells were exposed to TGF-β_1 for 6 h. Total cellular RNA was prepared and Northern analysis was performed. In C2 cells, unexposed to PMA, ODC gene expression was increased 7.7-fold in response to TGF-β_1 for 6 h (Fig. 4). However, in PMA pretreated C2 cells, the increase in ODC mRNA expression was 2.1 fold (Fig. 4). This observation implies a role for PKC mediated events in the TGF-β_1 mediated alterations in ODC mRNA in C2 cells. To further explore this possibility and to confirm this observation, the effect of calphostin C on the TGF-β_1 mediated alterations in ODC mRNA levels in C2 cells

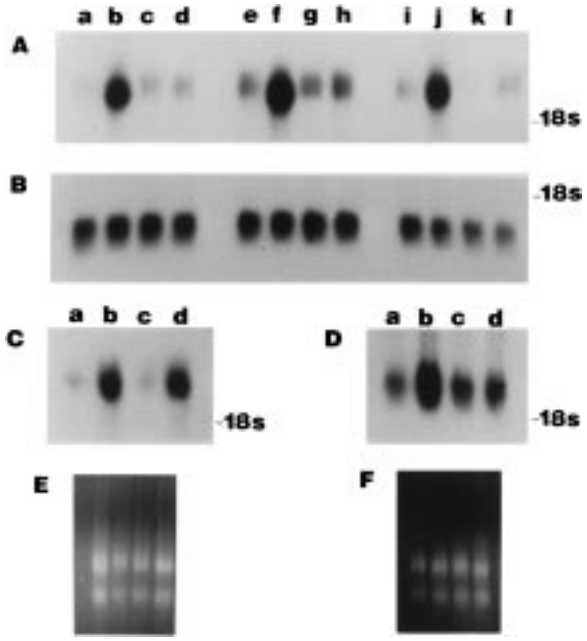

Fig. 4. Northern blot analysis of ODC gene expression in C2 cells: (**A**) ODC mRNA levels in C2 cells not exposed to either TGF-β_1 or to PMA, (a), in C2 cells exposed to TGF-β_1 for 6 h, (b), in C2 cells pre-treated with PMA (0.1 μM) for 48 h, (c), and in C2 cells pre-treated with PMA for 48 h, and then exposed to TGF-β_1 for 6 h, (d), in C2 cells (control cells), (e), in C2 cells exposed to TGF-β_1 for 6 h, (f), in C2 cells in the presence of calphostin C (0.5 μM), (g), in C2 cells in the presence of calphostin C and TGF-β_1 (h), in C2 cells (control cells) (i), in C2 cells in the presence of TGF-β_1 for 6 h, (j), in C2 cells pre-treated with okadaic acid (10 nM) for 48 h, (k), and in okadaic acid-pre-treated C2 cells exposed to TGF-β_1 for 6 h, (l). (**B**) GAPDH mRNA levels as loading controls are shown. (**C**) Northern blot analysis of ODC gene expression in NR3 cells: (a) NR3 control cells, (b) in NR3 cells exposed to TGF-β_1 for 3 h; (c) in NR3 cells exposed to pertussis toxin (100 ng/ml) for 2 h; and (d) in pertussis toxin treated NR3 cells exposed to TGF-β_1 for 3 h. (**D**) Northern blot analysis of ODC in C2 cells is presented as described above. (**E**) and (**F**) are ethidium bromide stained ribosomal RNA bands as loading controls. The autoradiograms shown in (A), (B), (C) and (D) were exposed for 24, 24, 24 and 12 h, respectively at −70°C with intensifying screens.

was determined. Calphostin C is a specific inhibitor of PKC. Calphostin C, at an effective concentration of 0.5 μM, inhibits PKC activity 100% [Kobayashi et al., 1989]. C2 cells were grown to confluence, placed on a defined medium for 24 h, then exposed to calphostin C for 1 h prior to exposure to TGF-β_1 for 6 h. Figure 4 shows that in the absence of exposure to calphostin C, a 6.9-fold increase in ODC mRNA levels occurred in response to TGF-β_1, whereas, in C2 cells pre-treated with calphostin C this increase in ODC mRNA level in response to TGF-β_1 treatment was abrogated (Fig. 4). These observations suggest that the TGF-β_1 mediated up-regulation of ODC mRNA levels in C2 cells can be abrogated when PKC-mediated events are inhibited. (Similar observations were noted in NR3 cells (data not shown)). These results suggest that TGF-β_1 may be using, to some extent, a PKC-dependent pathway to modulate ODC expression in NR3 cells and in C2 cells.

Previously, we have demonstrated a possible role for protein phosphatases in the TGF- β_1-mediated regulation of ribonucleotide reductase expression in malignant H-ras transformed fibrosarcomas [Hurta and Wright, 1995]. Okadaic acid specifically inhibits protein phosphatases 1 and 2A/2B; it has been suggested that prolonged exposure to okadaic acid results in the inhibition of protein phosphatase activity and that this prolonged protein phosphatase inhibition reduces the tumourigenic properties of cells [Cohen et al., 1990]. To test the possibility that protein phosphatases may also play a role in the TGF-β_1 regulation of ODC expression, C2 cells were pre-treated with okadaic acid for 48 h prior to exposure to TGF-β_1 for 6 h. In cells pre-treated with okadaic acid for 48 h, TGF-β_1 was unable to elevate ODC mRNA levels in C2 cells (Fig. 4). In control C2 cells, that is C2 cells without okadaic acid pretreatment, but exposed to TGF-β_1 for 6 h, a 6.3-fold increase in ODC mRNA levels was noted (Fig. 4), suggesting that the TGF-β_1 was indeed functional in these experiments (Fig. 4). These findings suggest a possible role for protein phosphatases in the TGF-β_1 mediated modulation of ODC expression in malignant fibrosarcomas (C2 cells). (The role of protein phosphatases in the TGF-β_1 mediated alterations in NR3 cells was not determined.)

Possible Involvement of G-Proteins in TGF-β_1 Regulated Ornithine Decarboxylase Gene Expression

Previously, it has been suggested that both G-protein-dependent and G-protein independent signalling may be involved in TGF-β_1-regulated events [Howe et al., 1989]. To test the possibility of G-protein involvement in the TGF-β_1 modulation of ODC gene expression, NR3 and C2 cells were pre-treated with pertussis toxin alone and in combination with TGF-β_1. Total cellular RNA was isolated, and ODC mRNA expression was evaluated by

Northern blot analysis. Treatment of NR3 and C2 cells with TGF-β_1 for 3 h resulted in 6.5- and 9.5-fold elevation of ODC mRNA levels, respectively (Fig. 4). Treatment of NR3 and C2 cells with pertussis toxin alone did not affect ODC mRNA expression. However, the TGF-β_1 mediated elevation in ODC mRNA expression in C2 cells was abrogated in C2 cells exposed to pertussis toxin (Fig. 4). Elevations of ODC mRNA in response to TGF-β_1 treatment were still evident in pertussis toxin pre-treated NR3 cells (a 5.9-fold increase in ODC mRNA levels was noted) (Fig. 4). These observations suggest a possible role(s) for a pertussis toxin sensitive G-protein(s) involvement in the TGF-β_1-modulated up-regulation of ODC gene expression in malignant C2 cells, and further suggest that the TGF-β_1 modulated increases in ODC gene expression in benign tumour forming NR3 cells does not apparently involve pertussis toxin sensitive G-protein(s).

TGF-β_1 Mediated Regulation of ODC Expression in Non-Transformed Versus H-ras Transformed Cells is Concentration Dependent

Previously, it was demonstrated that TGF-β_1 could stimulate cell locomotion in these H-ras transformed cells [Samuel et al., 1993]. In this regard, Postlethwaite and co-workers [1987] demonstrated that picogram levels of TGF-β_1 are sufficient to enhance chemokinesis and chemotaxis of normal fibroblasts. These observations prompted an investigation into whether or not lower levels of TGF-β_1 could influence ODC expression in normal versus transformed cells. To test this question, the effect of TGF-β_1 at concentrations of 10 pg/ml, 100 pg/ml, and 1 ng/ml on ODC mRNA expression and ODC enzyme activity in normal, non-transformed $10T\frac{1}{2}$ cells and in malignant H-ras transformed C2 cells was determined. ODC mRNA levels in the H-transformed malignant C2 cells did not change in response to exposure to these concentrations of TGF-β_1 (Fig. 5). Interestingly, however, the level of ODC mRNA expression did change in the normal, non-transformed $10T\frac{1}{2}$ cells. Elevations of ODC mRNA levels of 2.6-, 6.8- and 4.7-fold were noted in $10T\frac{1}{2}$ cells in response to exposure to 10 pg/ml, 100 pg/ml, and 1 ng/ml of TGF-β_1, respectively (Fig. 5). No change in ODC enzyme activity was detected in response to TGF-β_1 at these concentrations either in $10T\frac{1}{2}$ or in C2 cells (Table III). (The effect(s) of these lower concentrations of TGF-β_1 on ODC expression in NR3 cells was not determined).

A Role for Cellular Polyamine Levels in the TGF-β_1 Mediated Regulation of Ornithine Decarboxylase Expression in H-ras Transformed Cells

To test the hypothesis that the TGF-β_1 mediated alterations in ODC expression in H-

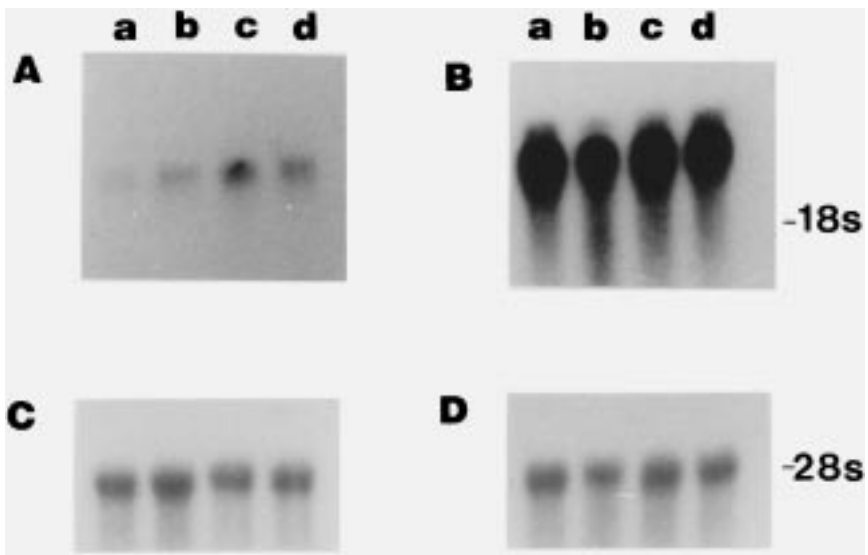

Fig. 5. ODC gene expression in $10T\frac{1}{2}$ cells (**A**) and in C2 cells (**B**) following exposure (for 4 h) to TGF-β_1 at the following concentrations, 10 pg/ml (b), 100 pg/ml (c), and 1 ng/ml (d). ODC message expression in controls cells are shown in (a). Control cells received vehicle only. (**C**) and (**D**) are 28 S rRNA bands as loading controls. Autoradiograms shown in (A) and in (B) were both exposed for 168 h, the autoradiograms shown in (C) and (D) were exposed for 1 h, at −70°C with intensifying screens, respectively.

TABLE III. Ornithine Decarboxylase Activity in Nontransformed Parental $10T\frac{1}{2}$ Cells and in H-ras Transformed Malignant C2 Cells in Response to Different Concentrations of TGF-β_1

Cell line	TGF-β_1 (concentration)	Ornithine decarboxylase activity (nmoles CO_2/ h/mg protein)
$10T\frac{1}{2}$	Control	0.16 ± 0.07
	10 pg/ml	0.19 ± 0.07
	100 pg/ml	0.22 ± 0.07
	1 ng/ml	0.18 ± 0.09
C2	Control	0.69 ± 0.09
	10 pg/ml	0.72 ± 0.06
	100 pg/ml	0.76 ± 0.04
	1 ng/ml	0.80 ± 0.05

TABLE IV. Ornithine Decarboxylase Enzyme Activity in DFMO-Treated H-ras Transformed Cells

Cell line	Additions	Ornithine decarboxylase enzyme activity (nmoles CO_2 / h/mg protein)
NR3	None	0.34 ± 0.09
	TGF-β_1	2.31 ± 0.44
	DFMO	0.17 ± 0.07
	DFMO and TGF-β_1	0.21 ± 0.07
C2	None	0.57 ± 0.17
	TGF-β_1	19.30 ± 1.12
	DFMO	0.42 ± 0.20
	DFMO and TGF-β_1	5.64 ± 0.93

ras transformed cells required/involved the participation of cellular polyamines, the effect of TGF-β_1 on the expression of ODC in NR3 cells and in C2 cells was determined in cells wherein the intracellular polyamine levels were depleted in response to difluoromethylornithine (DFMO). DFMO is a specific irreversible inhibitor of ODC and this inhibition of ODC causes a depletion of polyamine pools [Pegg and McCann, 1988]. Cellular polyamines, (putrescine, spermidine and spermine), are ubiquitous components of mammalian cells [Janne et al., 1991; Pegg and McCann, 1988]. Treatment of cells with DFMO (5 mM) results in a greater than 90% depletion of putrescine and spermidine with an accompanying decrease in spermine levels of about 50% (data not shown). H-ras transformed NR3 and C2 cells were cultured in the presence of DFMO (5 mM) for 96 h (which resulted in depletion of intracellular polyamine pools), prior to the exposure to TGF-β_1 (10 ng/ml) for 6 h. No increase in ODC mRNA levels occurred in response to TGF-β_1 exposure in DFMO-treated NR3 cells (data not shown) and no increase in ODC enzyme activity occurred in response to TGF-β_1 exposure in DFMO-treated NR3 cells (Table IV). This was not the case in the H-ras transformed C2 cells. As shown in Figure 6, TGF-β_1 can apparently interact with alterations in intracellular polyamine levels to up-regulate ODC mRNA expression. As shown in Figure 6, in the absence of DFMO treatment, TGF-β_1 increased ODC mRNA levels by about 7.1-fold. Interestingly, in DFMO-treated cultures, TGF-β_1 was still able to induce ODC mRNA levels. In such DFMO-treated C2 cells, TGF-β_1 treatment resulted in a 15.5-fold increase in ODC mRNA levels. Interestingly, DFMO treatment alone results in 7.2-fold increase in ODC mRNA levels in these cells (Fig. 6). These changes in ODC mRNA expression in response to TGF-β_1 and to DFMO treatment are not paralleled by the response seen when ODC enzyme activity is measured. In the absence of DFMO treatment, TGF-β_1 increases ODC enzyme activity by about 34-fold (Table IV). In DFMO-treated C2 cultures, in the absence of TGF-β_1 exposure, no increase in ODC enzyme activity is noted, in fact, an inhibition of ODC enzyme activity is found (Table 4). In DFMO- treated C2 cells which are then exposed to TGF-β_1, although an increase in ODC activity was still evident (about 9.9-fold increase) (Table IV), this is markedly reduced when compared to the increases in ODC activity which occur in response to TGF-β_1 in cells where cellular polyamine levels have not been perturbed. Based on these observations, it would appear that in H-ras transformed C2 cells, the interactions which occur between TGF-β_1 and cellular polyamine levels with respect to the regulation of ODC mRNA levels and ODC enzyme activity levels are not linked events. To further explore possible mechanisms which could account for increased ODC mRNA levels in C2 cells in response to DFMO treatment, the effect of DFMO on the transcription and the post-transcriptional regulation of the ODC gene was investigated. DFMO apparently did

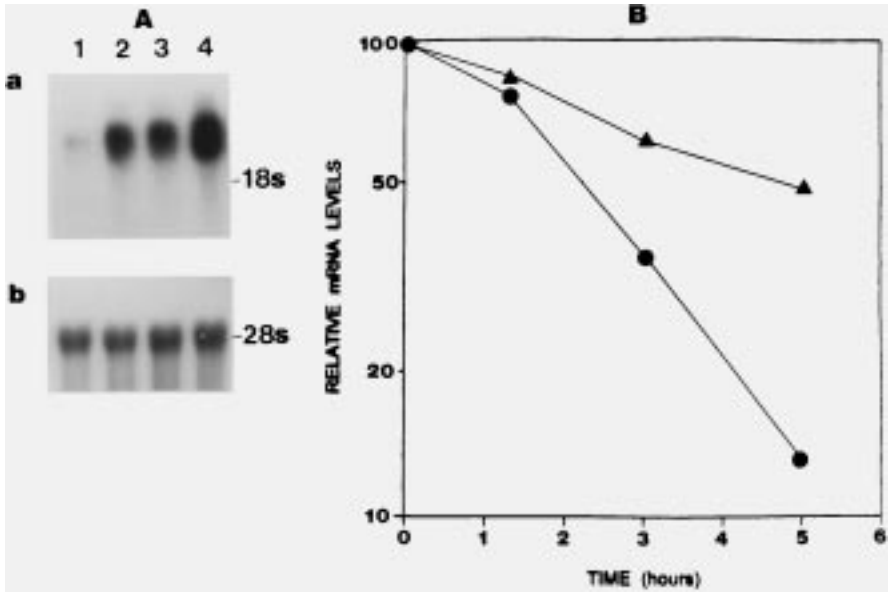

Fig. 6. (**A**): (a) Northern blot analysis of ODC mRNA expression in C2 cells: control cells, (1), in TGF-β_1 treated C2 cells (6 h exposure), (2); in DFMO (96 h)-treated C2 cells, (3); and in DFMO-pre-treated C2 cells exposed to TGF-β_1 for 6 h, (4). (b) 28 S rRNA expression as a loading control. Autoradiograms shown in (a) and in (b) were exposed at −70°C with intensifying screens for 24 and 2 h, respectively. (**B**): Stability of ODC mRNA transcript in untreated and in DFMO-treated C2 cells. C2 cells cultured in the presence of DFMO (5 mM) for 96 h (▲) or not (●) were subsequently treated with DRB (80 μM). Total cellular RNA was isolated at the times indicated and subjected to Northern blot analysis as described. The relative levels of ODC mRNA expression was determined by densitometric evaluation of appropriately exposed autoradiograms. The results presented are representative of observations noted in duplicate experiments.

not exert its effect by increasing the transcription of the ODC gene (data not shown) but by increasing the stability of the ODC mRNA transcript. The half-life of ODC mRNA was determined in C2 cells in the absence of DFMO and in C2 cells grown in the presence of DFMO (5 mM) for 96 h. As shown in Figure 6, the half-life of the ODC mRNA transcript in C2 cells was determined to be about 2.0 h, whereas, in DFMO-treated C2 cells, the half-life was estimated to be about 5 h (Fig. 6).

A Role for Cellular Polyamines in the Regulation of the Expression of the Transforming Growth Factor β_1 Gene in H-ras Transformed Cells

Since there appears to be a possible relationship between cellular polyamine levels and TGF-β_1 mediated modulation of ODC expression in these H-ras transformed cells, the possibility that a relationship between cellular polyamine levels and the expression of TGF-β_1 in these transformed cells was also explored. To determine if there was a link between cellular polyamine levels and TGF-β_1 expression, a time course analysis of TGF-β_1 mRNA was done in C2 cells cultured in the presence of DFMO (5 mM). As shown in Figure 7, TGF-β_1 mRNA expression increased in response to treatment of C2 cells with DFMO. Increases of 4.6-, 4.7-, and 2.3-fold in TGF-β_1 mRNA were found in C2 cells in response to exposure to DFMO for 48, 96 and 144 h, respectively (Fig. 7). Increases in TGF-β_1 mRNA also occurred in NR3 cells in response to DFMO treatment (data not shown). An aspect of this up-regulation of TGF-β_1 mRNA expression in NR3 and in C2 H-ras transformed cells in response to alterations in the cellular polyamine levels which occur in the presence of DFMO involves post-transcriptional stabilization of the TGF-β_1 mRNA transcript. As shown in Figure 7, the half-life of TGF-β_1 mRNA is increased in response to DFMO treatment in both NR3 cells and in C2 cells. The half-life of TGF-β_1 mRNA was determined to be 1.5 and 2.5 h in NR3 cells and in C2 cells, respectively (Fig. 7). However, in DFMO-treated NR3 cells and in DFMO-treated C2 cells, the half-life of TGF-β_1 mRNA transcripts was estimated to be about 5 and 15.5 h in NR3 cells and in C2 cells, respectively (Fig. 7). This represents about a 3- and 6.2-fold stabilization of the TGF-β_1 mRNA

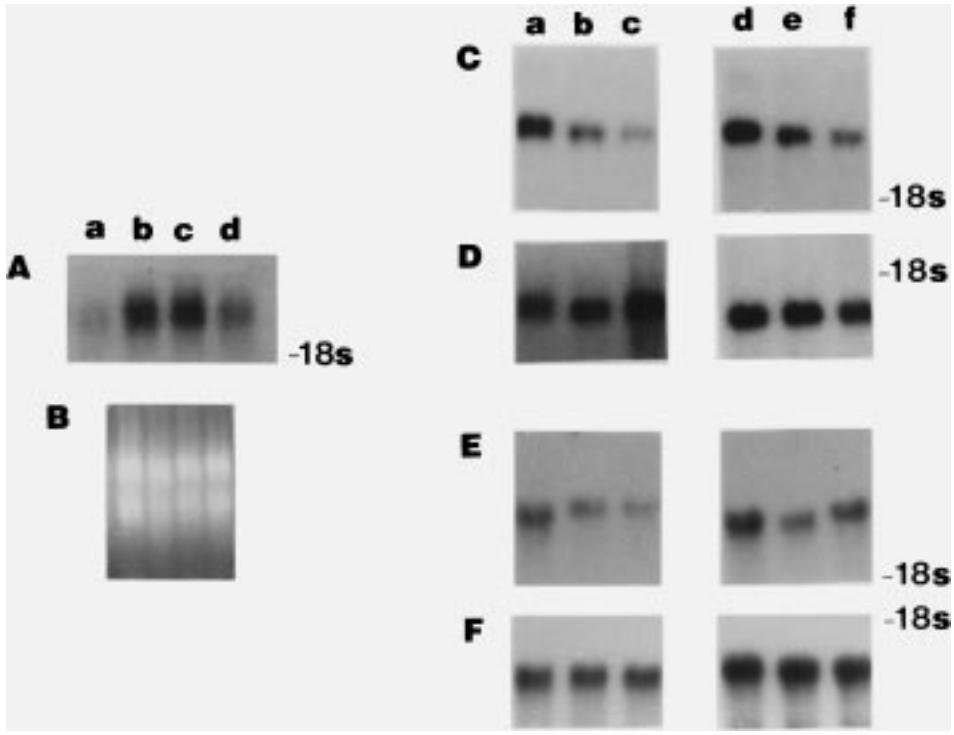

Fig. 7. TGF-β_1 mRNA levels in DFMO-treated ras transformed cells. Northern blot analysis of TGF-β_1 mRNA levels (**A**): in C2 cells, (control cells) (a), in C2 cells cultured in the presence of DFMO (5 mM) for 48 (b), 96 (c) and 144 (d) h, respectively. (**B**) Ethidium bromide stained ribosomal RNA bands as control. (**C**) TGF-β_1 mRNA expression in untreated and in DFMO-treated NR3 cells. NR3 cells were either grown in the absence (a–c) or in the presence of DFMO (5 mM) for 96 h (d–f) and then exposed to DRB (80 μM) for 2.5 (b and e) and for 5 (c and f) h, respectively. TGF-β_1 mRNA expression levels in untreated and in DFMO-treated NR3 cells (control cells) are shown in (a) and in (d), respectively. (**D**) GAPDH expression is shown as a loading control. (**E**) TGF-β_1 mRNA expression in untreated and in DFMO-treated C2 cells as described above. (**F**) GAPDH expression is shown as a loading control. The autoradiograms shown in (A), (C), (D), (E), and (F) were exposed at −70°C with intensifying screens for 24, 72, 24, 24, and 24 h, respectively.

transcript in NR3 cells and in C2 cells, respectively, in response to alterations in cellular polyamine levels in response to DFMO treatment. These observations, coupled with previous observations, suggest the existence of an interesting relationship between TGF-β_1 expression and TGF-β_1 mediated alterations in cellular signalling, and cellular polyamine levels and the regulation of ODC expression.

DISCUSSION

Obvious alterations in the expression of ODC were observed in response to TGF-β_1 exposure in H-ras transformed cells. These alterations were observed (in response to 10 ng/ml TGF-β_1) in both low ras expressing NR3 cells which are capable of forming benign tumours in syngeneic hosts, and in high ras expressing C2 cells capable of malignant progression. Normal, non-transformed 10T$\frac{1}{2}$ cells did not exhibit this change in response to this concentration of TGF-β_1. These observations are consistent with and substantiate previous studies of TGF-β_1-mediated regulation of ODC gene expression in non-transformed and in H-ras transformed cells [Hurta et al., 1993].

This present study also presented findings which suggest that the status of the cells' growth may be critical in determining the response which ensues, and this may be especially true for the NR3 cells. Indeed, NR3 cells are responsive to the regulatory influences of TGF-β_1 when the cells are at confluence but are non-responsive when they are actively growing (in logarithmic or exponential growth phase). These findings are in agreement with other studies which have shown that TGF-β_1 can have both growth promoting or growth inhibitory effects within the same cells depending on the status of the cells' growth [Fukami et al., 1995; Goodman and Majeck, 1989]. TGF-β_1 has

been shown to be a bi-functional response, density dependent regulator of vascular smooth muscle cells [Goodman and Majeck, 1989]. This study suggested that TGF-β_1 may exert opposite biological effects on the same cell type via an interaction with distinct, selectively expressed receptor subtypes [Goodman and Majeck, 1989]. It is possible that the response observed in NR3 cells may be due to the specific nature and type and number of distinct receptor subtypes expressed in NR3 cells during different phases of cell density. Such studies to investigate this possibility are in progress. Based on these observations, TGF-β_1 is a bi-functional, density dependent regulator of ODC expression in NR3 cells, and by extension, ODC is a bi-functional response gene to TGF-β_1 exposure.

Increased levels of ODC message expression in H-ras transformed cells following exposure to TGF-β_1 was, at least, partly due to modifications in the transcriptional efficiency of the ODC gene. Evidence was also presented that changes at the post-transcriptional level in response to TGF-β_1 can also occur in malignant H-ras transformed C2 cells. TGF-β_1 treatment increased the stability of ODC messages in malignant C2 cells following exposure to TGF-β_1 indicating that TGF-β_1 is capable of regulating ODC mRNA levels in these cells through a mechanism of post-transcriptional stabilization. This mechanism of regulating ODC mRNA expression did not occur (or at least, is not expressed) in ras-transformed NR3 cells, which are capable of only forming benign tumours. These observations are in agreement with previous studies [Hurta et al., 1993, 1996]. The specific nature of these post-transcriptional interactions and of the factors involved are not known but studies to investigate this are ongoing. The prevailing hypothesis is that growth factors such as TGF-β_1 exert their biological modifications by inducing an altered growth program of gene expression through transcriptional activation of genes. In this regard, we suggest that the TGF-β_1-mediated post-transcriptional stabilization of ODC may represent a component of an altered growth regulatory program associated with malignant transformation.

The cycloheximide-induced ODC gene expression observed in this study indicates that the ODC gene in malignant C2 cells can also be controlled in a positive manner by an inhibitor of protein synthesis. This behaviour is absent or non-expressed in normal 10T$\frac{1}{2}$ cells or tumourigenic but non-metastatic NR3 cells [Hurta et al., 1993, 1996]. This type of regulation may be caused by the presence of a labile protein repressor that disappears in the absence of protein synthesis or by the appearance of an activator which subsequently induces ODC gene transcription. The exact nature of this regulation of ODC in malignant C2 cells remains to be determined. TGF-β_1 exposure in conjunction with protein synthesis inhibition by cycloheximide resulted in a further marked elevation of ODC mRNA expression. Cycloheximide abrogated the TGF-β_1 mediated elevations in ODC mRNA levels in tumourigenic but non-metastatic NR3 cells. These observations confirm our previous observations in C3 cells [Hurta et al., 1993] and are in agreement with other studies including those investigating bFGF-mediated regulation of ODC expression in H-ras transformed cells, amongst others [Hurta and Wright, 1995; Hurta et al., 1993, 1996; Voskas et al., 1999]. The results presented in this study indicate the existence of a cycloheximide sensitive regulator of ODC expression in the highly malignant H-ras transformed cells.

Previously, we have demonstrated that bFGF-mediated alterations in ODC mRNA expression in malignant cells occur through a protein kinase C independent pathway [Hurta et al., 1996] and that TGF-β_1 mediated alterations in ribonucleotide reductase R2 and R1 gene expression in malignant H-ras transformed cells occurred through protein kinase C dependent events [Hurta and Wright, 1995]. Previously, we have suggested that both ribonucleotide reductase and ornithine decarboxylase gene expression (both growth-related activities) may be subject to similar regulatory constraints in these H-ras transformed cells [Hurta and Wright, 1995; Hurta et al., 1993, 1996]. Prolonged exposure of mammalian cells to phorbol ester tumour promoters (PMA) has been shown to result in a down regulation of PKC activity [Young et al., 1987]. PMA treatment of C2 cells causes a rapid but transient elevation of ODC gene expression with ODC mRNA levels returning to approximately unstimulated levels following prolonged exposure to PMA (for 24–48 h) (unpublished observations). These results are consistent with previous observations obtained with BALB c/3T3 cells [Choy et al., 1989]. The observation that the

TGF-β_1 induced increases in ODC message levels can be reduced or abrogated in cells pretreated with PMA (which results in the down regulation of PKC activity) or with calphostin C (a documented specific inhibitor of PKC), suggests that a PKC-dependent pathway may be involved in the TGF-β_1 modulation of ODC expression in NR3 cells and in C2 cells. It is possible that these treatments may have resulted in a down-regulation or inactivation of TGF-β_1 receptors in NR3 cells or in C2 cells. Whether this occurs was not evaluated, and it would be interesting to determine if alterations in PKC-mediated events also results in alterations in TGF-β_1 receptor number and expression, thereby contributing to the abrogation of the TGF-β_1 induced increases in ODC gene expression observed in this study. Previously we have demonstrated, in these H-ras transformed cells, a role for cellular protein phosphatases in the regulation of ribonucleotide reductase gene expression in response to TGF-β_1 [Hurta and Wright, 1992, 1995]. Inhibition of protein phosphatases allows the unopposed activity of protein kinases constitutively present in the cell and leads to enhanced phosphorylation of many of the substrates of protein kinases [Sassa et al., 1989]. Down-regulation of protein phosphatases by prolonged exposure to okadaic acid resulted in an abrogation of the TGF-β_1 induced elevations of ODC mRNA levels in C2 cells, suggesting a possible role(s) for protein phosphatase mediated events in the signalling pathway affecting TGF-β_1-mediated induction of ODC gene expression in malignant ras-transformed cells. In this regard, in human keratinocytes, growth arrest induced by TGF-β_1 is accompanied by protein phosphatase activation [Gruppuso et al., 1991]. The specifics of the intracellular signalling pathways responsible for the TGF-β_1 directed modulation of ODC gene expression and the possible role of protein phosphatases in this regulatory process are unknown and remain to be further elucidated.

Several studies have indicated that G-proteins may participate in transducing the mitogenic signal of polypeptide growth factors [Chambard et al., 1987; Fisher and Schonbrunn, 1987; Hurta et al., 1996; Luttrel et al., 1988; Hurta and Wright, 1995]. Furthermore, multiple G-protein dependent and independent transducing pathways are responsible for mediating the biological signals of TGF-β_1 [Howe et al., 1989]. In keeping with this idea, possible pertussis-toxin sensitive G-proteins may play a role in the TGF-β_1-mediated alterations of ODC gene expression in highly malignant H-ras transformed C2 cells. However, in tumourigenic, non-metastatic NR3 cells, the TGF-β_1-mediated elevations in ODC expression occur independent of pertussis toxin sensitive G-protein involvement. In keeping with this idea, possible pertussis toxin sensitive G-protein mediated events have been suggested to play a role in the TGF-β_1-mediated alterations in ribonucleotide reductase gene expression and in the bFGF-mediated alterations in ODC gene expression in highly malignant H-ras transformed cells [Hurta and Wright, 1995; Hurta et al., 1996]. The nature of the specific G-proteins and the signalling mechanisms involved are unknown and subject to further investigation.

This present investigation further suggested a critical role for cellular polyamine levels in the TGF-β_1-mediated alterations in ODC gene expression in both tumourigenic but non-metastatic NR3 cells and in malignant, metastatic C2 cells. The alterations in ODC mRNA and enzyme activity seen in NR3 cells in response to TGF-β_1 were dependent upon intact cellular polyamine levels. The relationship between cellular polyamine levels and ODC expression and TGF-β_1 which occurs in malignant C2 cells is quite different. In fact, depletion of cellular polyamine levels resulted in an increase in ODC mRNA expression in C2 cells. Similar observations were noted previously in human colon carcinoma cells [Celano et al., 1989]. TGF-β_1 exposure and altered cellular polyamine levels can cooperate / interact to result in an additive up-regulation of ODC mRNA expression. However, this is not the case with ODC enzyme activity. Part of the mechanism responsible for the increased ODC mRNA levels in C2 cells is a post-transcriptional stabilization of the ODC gene transcript in response to depletion of cellular polyamine levels following treatment with DFMO. Post-transcriptional stabilization of ODC has been suggested to be one mechanism by which ODC mRNA levels increase in response to DFMO treatment in COLO 320 cells [Celano et al., 1989]. Others have suggested that increases in ODC gene amplification can occur in response to DFMO treatment [Alhonen-Hongisto et al., 1985], whether this occurs in C2 cells in response to DFMO treatment is not known.

The increase in ODC mRNA levels in highly malignant C2 cells, in response to alterations in cellular polyamine levels, may represent a compensatory response on behalf of C2 cells to the stresses imposed by cellular polyamine depletion. This increase in ODC expression constitutes part of the altered growth regulatory program found in H-ras transformed malignant (metastatic) cells and is absent or non-expressed in tumourigenic but non-metastatic H-ras trasformed cells. A role for cellular polyamines in the regulation of TGF-β_1 expression in H-ras transformed cells was also presented. Increased TGF-β_1 expression occurred in H-ras transformed cells in response to DFMO mediated depletion of cellular polyamine levels. Post-transcriptional stabilization of the TGF-β_1 mRNA transcript in response to DFMO-mediated cellular polyamine depletion occurred in both NR3 and in C2 H-ras transformed cells. These observations are consistent with other studies which have suggested a link between cellular polyamine levels and TGF-β_1 expression [Patel et al., 1998; Wang et al., 1997]. Further study is warranted. It is possible that polyamine depletion may be affecting the nature and number of TGF-β_1 receptor and receptor subtypes being expressed in these cells and this may also be playing a role. Whether this occurs remains to be determined. The induction of TGF-β_1 and ODC in H-ras transformed malignant C2 cells in response to alterations in cellular polyamine levels suggests that cellular polyamine levels are critical regulators of growth related gene expressions in these cells. In this regard, in addition to up-regulation of ODC and TGF-β_1 expression, DFMO-mediated alterations in cellular polyamine levels results in an up-regulation of the expression of a number of genes in these C2 cells, including the R2 subunit of ribonucleotide reductase, cytoskeletal genes, extracellular matrix genes and matrix metalloproteinase genes (unpublished observations). The precise nature of the interactions which occur in gene expression(s) in response to cellular polyamine depletion (and polyamine supplementation) and the specifics of the temporal mechanisms, and intracellular signalling pathways involved in this process remain to be further elucidated.

In summary, this study has demonstrated a novel relationship between TGF-β_1 signalling and ODC expression in H-ras transformed cells capable of benign tumour formation and cells capable of malignant progression. Further studies are required to determine the nature of specific regulatory systems and the factors involved and how they interact to control ODC in normal and in transformed cells, and how this regulation is abrogated or altered in transformed cells following exposure to TGF-β_1. Finally, this present report contributes to our understanding of the regulation of the transformed phenotype by TGF-β_1 and constitutes the basis for further investigations of the control mechanisms and regulatory factors involved.

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