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# Translation of ODC mRNA and Polyamine Transport Are Suppressed in *ras*-Transformed CREF Cells by Depleting Translation Initiation Factor 4E

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**Rapid tumor growth and metastasis require increased polyamine metabolism, which is coordinately regulated by ornithine decarboxylase (ODC) and the polyamine transporter. Both activities are stimulated by ras signalling and are dependent upon protein biosynthesis. T24$^{ras}$ oncogene expression in rat embryo fibroblasts (CREFT24) induces cellular transformation and malignancy, in part, by stimulating the rate-limiting translation initiation factor, eIF-4E. CREFT24 expressing antisense RNA to eIF-4E (AS4E) have markedly decreased tumor growth rates and metastatic capacity, without altered monolayer growth rates. Herein, we demonstrate that in AS4E, ODC is translationally suppressed resulting in decreased ODC activity. Additionally, exogenous polyamine uptake is suppressed in AS4E cells indicating that AS4E can neither generate nor import the polyamines necessary to support rapid tumor growth. These data provide evidence that eIF-4E is the link between ras-induced malignancy and increased polyamine metabolism and support the hypothesis that eIF-4E plays a pivotal role in mediating ras-induced malignancy.** 

The polyamines (putrescine, spermidine and spermine) are involved in cellular proliferation, tumorigenesis and metastasis (1). Intracellular polyamine concentrations are regulated by the activity of ornithine decarboxylase (ODC), the first and rate-limiting enzyme for polyamine biosynthesis, and by the uptake of exogenous polyamines by the polyamine transporter (1). Polyamine biosynthesis and the transport of exogenous polyamines are both regulated by fluctuations in protein synthesis. Both are upregulated in cells by growth factor (e.g. EGF) or hormonal stimuli (e.g. insulin) that activate the ras signal transduction cascade (1, 2). For instance, ras transformation of Rat-1 fibroblasts results in a 12-fold increase in ODC activity (3). ODC activity generally reflects ODC protein levels and ODC protein levels are largely governed by translation initiation (1). Indeed, the translational suppression of ODC mRNA in quiescent cells (4) can relieved by overexpression of the translation initiation factor, eIF-4E (5, 6).

The mRNA cap-binding protein, eukaryotic translation initiation factor 4E (eIF-4E), is the rate-limiting component of the eIF-4F translation initiation complex. As such, eIF-4E activity determines both the rate and spectrum of proteins translated within the cell. The translation of mRNAs containing lengthy, G+C-rich, 5′ untranslated leader sequences (non-competitive RNAs) is particularly sensitive to eIF-4E activity (7). By selectively upregulating the translation of non-competitive mRNAs, such as those coding for the protooncoproteins cyclin D1 (8), ODC (5, 6), c-*myc* (9) and FGF-2 (10), eIF-4E overexpression can drive tumorigenesis (9, 11). Furthermore, eIF-4E can be activated by phosphorylation and liberation from its inhibitory binding protein Phas-1, or 4E-BP-1, consequent to the activation of the ras signal transduction pathway (12, 13, 14, 15).

We have previously shown that the increased eIF-4E activity of *ras*-transformed CREF cells (CREF T24) is critical for *ras*-induced malignancy. Antisense RNA-mediated reduction of eIF-4E in CREF T24 (AS4E cells) results in significantly decreased tumor growth and

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diminished metastatic capacity, without marked differences in monolayer growth rates (16, 17). The expression of the malignancy-related proteins, matrix metalloprotease 9 (MMP-9) and the metastasis-specific variant of CD44 (CD44v6) are decreased in AS4E while the metastasis suppressor protein, nm23, is increased. Neither $\beta$-actin nor the matrix metalloprotease 2 are affected in AS4E, indicating that the decreased eIF-4E levels in AS4E affect only a very specific subset of proteins. Cell lines established from subcutaneous AS4E tumors (ASTDs) show levels of MMP-9, CD44v6 and nm23 protein similar to the parental CREF T24 demonstrating that AS4E cells underwent selection *in vivo* for increased MMP-9 and CD44v6 and decreased nm23 with only a minor increase in eIF-4E levels. Furthermore, these tumor-derived cell lines had regained the short tumor latency periods of CREF T24 but maintained the slower tumor growth rates of AS4E (17). We therefore sought to identify a protein, or proteins, involved in regulating tumor growth rates which may be regulated by eIF-4E activity. ODC activity and increased transport of exogenous polyamines are stimulated as a consequence of ras-signalling. Each requires *de novo* protein synthesis and both are intimately involved in regulating tumor growth rates. Therefore, we have explored the possibility that a deficiency in polyamine metabolism may explain, at least in part, the diminished tumor growth rates and malignancy of cells with reduced eIF-4E activity.

## MATERIALS AND METHODS

*Cell culture.* Cells were maintained in culture flasks or dishes as previously described (17). The generation of *ras*-transformed CREF cells (CREF T24), CREF T24 transfection controls (CREF T24Hygro and CREF T24PS/Hm), CREF T24 cells expressing antisense RNA to eIF-4E (AS4E and the A1 clone of AS4E, ASA1) and the establishment of tumor and lung nodule-derived cell lines (ASTD1, A1TD1, ASLN1 and A1LN1) has been described previously (16, 17).

*Northern blot analysis.* Cytoplasmic RNA was harvested from $1 \times 10^7$ exponentially growing cells by gentle lysis in NP-40 (18), separated by gel electrophoresis, transferred to a Magnacharge nylon membrane (Micron Separations, Inc., Westborough, MA) and probed with cDNA probes as prescribed by the manufacturer (MSI, Inc.). DNA probes were labeled with the random primer labeling system using $^{32}$P-dCTP (Boehringer- Mannheim, Indianapolis, IN) and purified by centrifugation through a Sephadex G-50 column (Pharmacia, Piscataway, NJ). Filters were exposed to X-ray film for at least 18 hours. The probe for Ornithine Decarboxylase (ODC) was generated by Pst 1 restriction of pCR3 (kindly provided by Dr. F. Berger, University of South Carolina). The probe for $\beta$-actin was obtained from the American Type Culture Collection (Bethesda, MD).

*Polysome analysis.* Cytosolic fractions were isolated from $1 \times 10^9$ cells treated with cycloheximide (100 $\mu$g/ml final concentration) and fractionated through a sucrose gradient as described (19). Cytosolic fractions (0.5 ml) were collected and 200 $\mu$l of each fraction were dot-blotted to a wet nitrocellulose membrane. After transfer, the membrane was soaked in 3 % formalin in PBS for 3 min. and rinsed with water. The membrane was baked in a vacuum oven for 1 hour at 80° C, soaked for 1 hour in 1 % SDS, rinsed in $ddH_2O$ and incubated 2 hours- overnight in prehybridization/ hybridization buffer (40 % formamide, 6× SSPE, 0.1 % SDS, 100 $\mu$g/ml nonfat dry milk) at 43°C. Denatured probes for either ODC or $\beta$-actin were added directly to the hybridization solution and incubated 24- 48 hours at 43°C. Filters were washed 2 × 30 min. at 65°C in 2× SSC/ 0.5 % SDS. Filters were exposed to X-ray film for a minimum of 2 days.

*Ornithine decarboxylase activity.* ODC activity was assessed by NaOH capture of $^{14}CO_2$ liberated from $^{14}$C-ornithine as previously described (20). Each point represents triplicate determinations.

*Spermidine transport assay.* Cells were plated at a density of $2 \times 10^5$ cells per well of a six well dish (Costar) and incubated overnight at 37° C /5 % $CO_2$. Uptake of $^{14}$C-spermidine was measured at time points ranging from 5 min. to 240 min. as previously described (20). Each data point was performed in triplicate.

*Polyamine pool analysis.* Intracellular polyamine polyamine pools were assessed by HPLC following cell lysis in 1× SSAT buffer as previously described (21).

## RESULTS

### *ODC Activity and Translation Are Diminished in Cells with Decreased eIF-4E Levels*

We have previously shown that the malignancy of *ras*-transformed CREF cells (CREF T24 cells) is diminished by antisense RNA-mediated reduction of eIF-4E protein levels (AS4E cells) (16, 17). Since the translational suppression of ODC is relieved in NIH 3T3 cells transformed by overexpression of eIF-4E (5, 6), we sought to determine whether ODC translation and activity were influenced by reduced expression of eIF-4E. In AS4E cells, in which eIF-4E expression and global protein synthesis rates were reduced 50-60% (16), ODC activity was decreased by nearly 50%. Similarly, the ASA1 clone of AS4E cells and the tumor-derived cell lines (ASTD1 and A1TD1) also showed this 50% reduction in ODC activity relative to the CREF T24 parental and transfection controls (Figure 1).

To evaluate whether reduced ODC activity could be attributed to reduced steady-state levels of ODC mRNA, we performed northern blot analysis on cells with reduced eIF-4E levels relative to the CREF T24 control cells. The steady-state levels of ODC mRNA are not reduced in AS4E, or the tumor and lung nodule-derived AS4E (ASTD1, ASLN1) relative to the CREF T24 control (Figure 2). Therefore, decreased ODC activity cannot be attributed to decreased steady-state levels of ODC mRNA.

Since ODC activity generally reflects ODC protein (1) and since ODC protein synthesis is primarily controlled at the level of translation initiation (4), we examined the translational efficiency of ODC mRNA by polysome analysis in cells with reduced eIF-4E activity. Inefficiently translated mRNAs (i.e., "non-competitive" mRNAs) will localize primarily in the lighter, monosomal and preribosomal fractions while efficiently translated mRNAs (i.e., "competitive" mRNAs) will localize to fractions throughout the gradient, including the heavier, polyribosomal fractions. Previous studies have demonstrated that ODC mRNA, a non-competitive

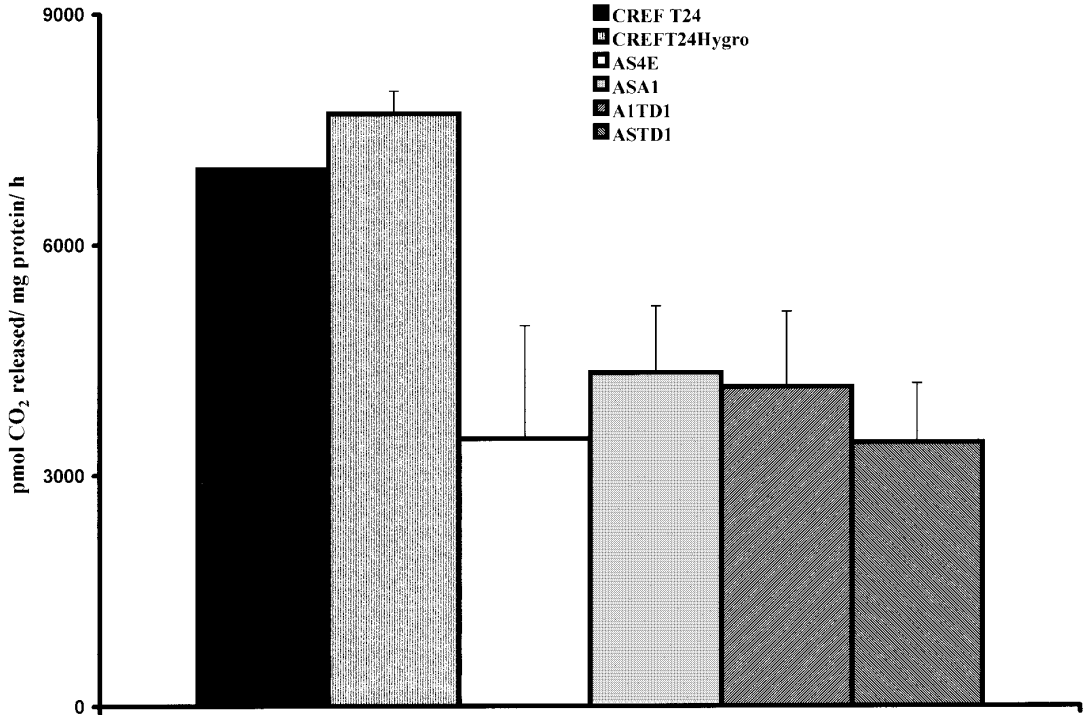


**FIG. 1.** ODC activity is decreased in CREF T24 cells with reduced levels of eIF-4E protein. ODC activity is expressed as the pmols of $^{14}CO_2$ released from $^{14}C$-ornithine per mg protein per hour. Error bars represent the standard error from the mean. All values shown are the mean of two independent experiments performed in triplicate (except those for CREF T24, which represent one experiment) representing a total of six similar experiments. CREF T24 and CREF T24Hygro are the parental and transfection controls. The legend is presented in the inset.

mRNA, shifts to the heavier, polyribosomal fractions in NIH3T3 cells overexpressing eIF-4E, while the control GAPDH mRNA, a competitive mRNA, localizes to all gradient fractions (6). In AS4E cells, the abundance of ODC mRNA in the heavier, polysomal fractions (pooled fractions 8 and 9) relative to the $\beta$-actin mRNA (a competitive mRNA) is markedly reduced compared to the CREF T24 control (Figure 3). ASTD1 cells showed a similar depletion of ODC mRNA in the heavier, polysomal fractions (data not shown). These data indicate that ODC translation is suppressed as a consequence of reduced eIF-4E protein in ras-transformed fibroblasts.

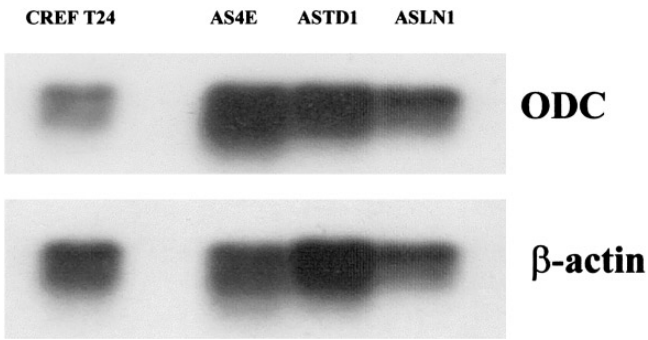


**FIG. 2.** Steady-state levels of ODC mRNA are unchanged in cells with reduced eIF-4E protein. Approximately 25$\mu$g cytoplasmic RNA were loaded for each lane. Panel A represents the northern blot for ODC. Panel B represents the same blot stripped and reprobed for $\beta$-actin to control for gel loading and transfer. Lane assignments are designated. Three additional northern blots revealed similar patterns for ODC expression relative to $\beta$-actin.

*Polyamine Transport Is Depleted as a Consequence of Reduced eIF-4E Activity*

Tumor cells often display increased uptake of exogenous polyamines, as compared to their normal counterparts (1, 22). When ODC activity is inhibited, cells often compensate for decreased polyamine biosynthesis by increasing the transport of exogenous polyamines in a protein synthesis-dependent manner (1, 23). We therefore examined the uptake of exogenous spermidine in AS4E and ASTD1 cells relative to the CREF T24 parental control cells. Surprisingly, despite decreased ODC activity, AS4E and ASTD1 cells showed nearly 75% reduced uptake of spermidine relative to CREF T24 (Figure 4A). Furthermore, this reduction in spermidine transport persisted throughout a 240 minute timecourse (Figure 4B) indicating that cells with reduced levels of eIF-4E protein did not, or were unable to, compensate for reduced ODC activity by increasing the transport of exogenous spermidine.

Since both ODC activity and spermidine uptake were

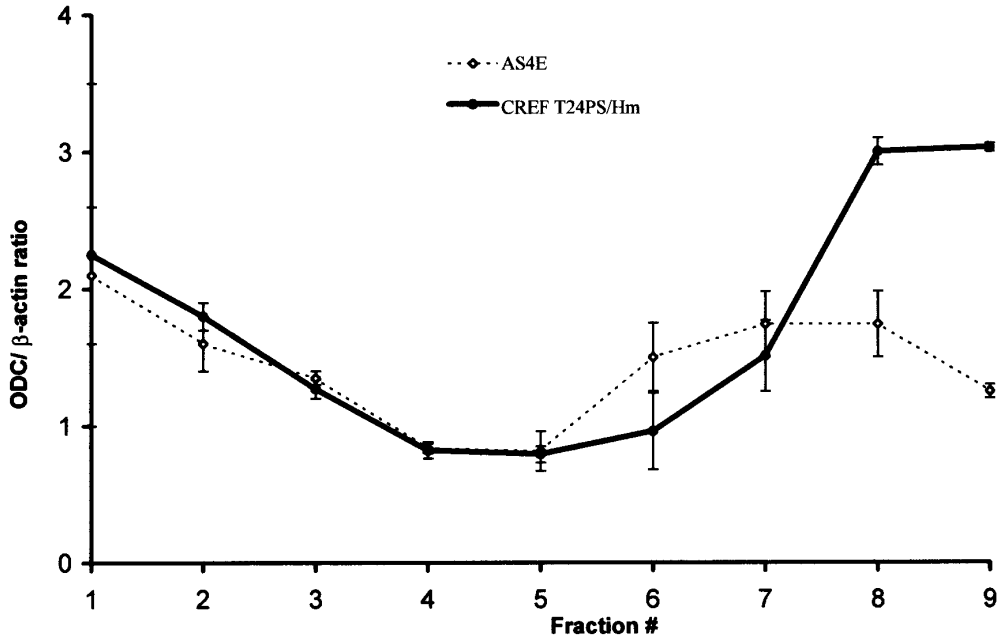


**FIG. 3.** Polysome fractionation reveals that ODC is translationally suppressed in AS4E. Cytosolic fractions were separated by sucrose gradient density centrifugation and hybridized to a cDNA probe for ODC and then stripped and hybridized to a cDNA probe for $\beta$-actin. $\beta$-actin was used as a control for fraction loading and as a model "competitive" mRNA that should localize efficiently in all gradient fractions, including the heavier, polysomal fractions. Data are expressed graphically as the ratio of the percent total ODC signal divided by the percent total $\beta$-actin signal for each fraction. Error bars represent the maximum range of signal from 2 fractions. The legend is inset. These data are representative of two independent experiments.

suppressed as a consequence of reduced levels of eIF-4E, we examined whether these changes altered intracellular polyamine pools over the course of 5 days in culture. Polyamine pools were unaffected in monolayer culture (Table 1). Similarly, intracellular polyamine pools were unchanged in Rat-1 fibroblasts transfected with the $EJ^{ras}$ oncogene despite 12-fold higher ODC activity (3). Previous reports have demonstrated that cells in culture may have a diminished requirement for polyamines since intracellular polyamine pools are often in excess in culture, especially in the presence of 10% fetal calf serum (21, 24).

## DISCUSSION

In the current study, we have demonstrated that reduction of eIF-4E in ras-transformed CREF cells resulted in decreased ODC activity. This decrease in ODC activity was not a result of decreased steady-state levels of ODC mRNA, but rather, a suppression of ODC translation. In this regard, our data support and extend earlier reports indicating that ODC translation is dependent upon the activity of the mRNA cap-binding protein, eIF-4E (4, 5, 6). However, our data are the first to link tumor-associated increases in ODC translation and activity to the activation of eIF-4E in ras-transformed cells.

Furthermore, the data in this report are the first to demonstrate that transport of exogenous polyamines is governed by eIF-4E. That exogenous polyamine transport may be governed by protein synthesis has been reported (23). Data from this report suggest that expression of the protein(s) that comprise the polyamine transporter may be translationally regulated and, therefore, sensitive to fluctuations in eIF-4E activity. The link between eIF-4E activity and exogenous polyamine uptake is further supported by the observations that both exogenous polyamine transport and eIF-4E activity can be upregulated by serum, epidermal growth factor and insulin treatments (1, 7, 22). Additionally, since all of the key enzymes that regulate intracellular polyamine levels (ODC, S-adenosyl methionine decarboxylase and Spermidine/ Spermine $N^1$- acetyltransferase) are regulated translationally (1, 25), it is plausible that the proteins comprising the polyamine transporter(s) may be translationally regulated as well. However, direct translational control of the transporter protein(s) cannot be examined as the mammalian genes for these proteins have not been cloned.

Increased ODC activity and exogenous polyamine transport are frequently associated with rapid tumor growth rates (1). We have previously demonstrated that antisense RNA-mediated reduction of eIF-4E protein levels diminishes the malignancy of *ras*-transformed CREF cells, particularly tumor growth rates (16, 17). After an extended latency period, subcutane-

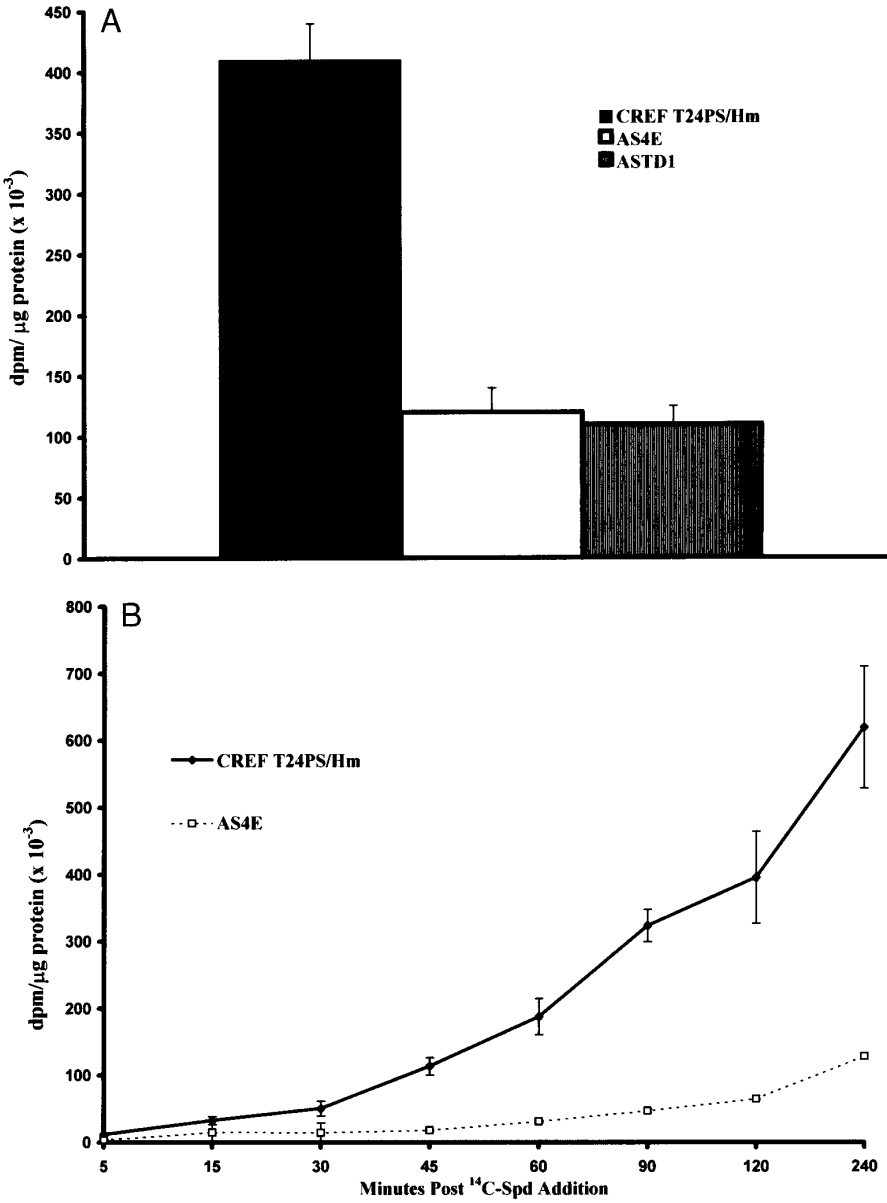


**FIG. 4.** Polyamine transport is depressed in cells with reduced eIF-4E levels. Panel A depicts transport of $^{14}$C-spermidine at 30 min. post addition of $^{14}$C-spermidine. The data presented are the mean of two independent experiments representing a total of 6 similar experiments. Panel B depicts a timecourse for $^{14}$C-spermidine ranging from 5 min. to 240 min. post addition of $^{14}$C-spermidine. The data presented are the mean values from two independent experiments representing a total of 4 similar experiments. The Y axis for both panels depicts the $^{14}$C-spermidine uptake in disintegrations per minute per $\mu$g protein. Error bars for both panels represent the standard error from the mean.

ously injected AS4E cells eventually formed tumors. These tumors had regained many of the malignant properties of the parental CREF T24- efficient soft agar colonization, short tumor latency periods, and full metastatic capacity as well as increased expression of MMP-9 and CD44v6 and decreased nm23 expression with only minimal recovery of eIF-4E levels. However, when reinjected into nude mice, these tumor-derived AS4E cells (ASTDs) maintained the slower tumor growth rates of the AS4E cells (17). Since increased polyamine biosynthesis and transport have both been associated with rapid tumor growth rates (1), it seems plausible that the decrement in ODC activity coupled with the diminished uptake of exogenous polyamines may contribute, at least in part, to the slower tumor growth rates of AS4E and ASTD cells, although other molecules such as FGF-2 (10) may also be involved.

In conclusion, these data provide the first evidence that ras upregulates polyamine metabolism via the activation of eIF-4E. These data also support the hypoth-

esis that the activation of eIF-4E, consequent to ras signalling, is a critical determinant of ras-induced malignancy. Persistent activation of eIF-4E by oncogenic ras would increase translation initiation rates resulting in disproportionately increased translation of "non-competitive mRNAs" (such as those for the protooncoproteins c-*myc*, c- *sis*, c- *fos*, FGF-2 and ODC; 4, 7, 9, 10). Coupled to ras-induced transcriptional alterations, the increased translation of a specific subset of proteins involved in tumor growth and malignancy (e.g. ODC and the polyamine transporter) may be driving ras-induced tumor formation and malignant progression (17). Recent reports have now demonstrated overexpression of eIF-4E in rat tumors (26) and in human breast cancer (27, 28), further supporting the emerging role of eIF-4E in malignancy.

## ACKNOWLEDGMENT

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