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S-Adenosylmethionine Decarboxylase Gene Expression is Regulated by the cAMP Signal Transduction Pathway in H-ras Transformed Fibrosarcoma Cells Capable of Malignant Progression

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Abstract The hypothesis that H-ras transformed cells contain alterations in signalling pathways important in controlling the expression of S-adenosylmethionine decarboxylase, (SAMDC) a highly regulated activity in the biosynthesis of polyamines was tested. Mouse 10 T1/2 fibroblasts and H-ras transformed cell lines of varying degrees of malignant potential were treated with agents which affect cAMP levels within cells. Elevations in SAMDC expression were noted in H-ras transformed metastatic C3 cells, which were not observed in either parental, non-transformed 10 T1/2 fibroblast cells, or in ras transformed NR3 cells, which are only capable of benign tumour formation. Forskolin, a stimulator of cAMP synthesis, was able to increase SAMDC enzyme activity but the response which occurred was dependent upon the cellular phenotype expressed. Actinomycin D pre-treatment of C3 cells prior to exposure to forskolin did not abrogate the elevation observed in SAMDC gene expression suggesting that this was not a transcriptional process mediated event. Forskolin pre-treatment of C3 cells did result in a marked increase in the half-life of SAMDC mRNA transcripts suggesting a role for post-transcriptional stabilization. Furthermore, cycloheximide treatment of malignant C3 cells resulted in elevated SAMDC mRNA levels. Treatment of malignant C3 cells with both cycloheximide and forskolin together resulted in a further additive elevation in SAMDC message levels. Cycloheximide treatment alone was found to affect the half-life of SAMDC mRNA through a mechanism of post-transcriptional stabilization. Additionally, altered SAMDC gene expression in C3 cells which occurred in response to cAMP alterations, was enhanced by stimulation of a protein kinase C pathway suggesting possible interactions between protein kinase C- and cAMP-mediated pathways which affect the regulation of SAMDC expression in highly malignant C3 cells. These results demonstrate aberrant regulation of signalling pathways involved in controlling SAMDC gene expression in H-ras transformed cells capable of malignant progression and provide further insight into the altered growth regulatory program associated with H-ras mediated cellular transformation and malignant progression. J. Cell. Biochem. Suppl. 36:209–221, 2001. © 2001 Wiley-Liss, Inc.

Key words: S-adenosylmethionine decarboxylase; H-ras; cAMP; malignant progression

Cyclic AMP (cAMP) is a key regulator of proliferation and differentiation in many cellular systems [Roger et al., 1995; Withers, 1997]. In previous studies, a link between cAMP regulation of malignant cell behaviour and alterations in the expression of ribonucleotide reductase [Hurta and Wright, 1994 a], a highly controlled rate limiting step in DNA synthesis and cell proliferation [Wright et al., 1990, 1993] was demonstrated. The results of this study suggested that other important growth altering activities may also be aberrantly regulated through the cAMP-dependent signal transduction during H-ras mediated-malignant progression. In this regard, a relationship between cAMP regulation and ornithine decarboxylase (ODC) gene expression and regulation was also previously demonstrated [Hurta and Wright, 1994b]. ODC is the first and rate limiting enzyme in the synthesis of polyamines, which are necessary for animal, plant, and microbial cell proliferation and survival [Tabor and

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Tabor, 1984; Pegg, 1988]. In the present study, this hypothesis was further tested by investigating the possible relationship between cAMP regulation and SAMDC gene expression. There are two key enzymes involved in polyamine biosynthesis, namely ODC and S-adenosylmethionine decarboxylase (SAMDC). ODC catalyzes the formation of putrescine from ornithine, whereas, SAMDC is responsible for the decarboxylation of S-adenosylmethionine and the subsequent donation of aminopropyl groups for spermidine and spermine synthesis [Heby and Persson, 1990]. SAMDC, like ODC, can also be a limiting factor in polyamine biosynthesis [Heby and Persson, 1990]. The study presented herein was performed with a series of well-characterized H-ras transformed cell lines exhibiting increasing malignant potential [Egan et al., 1987; Hurta et al., 1996].

MATERIALS AND METHODS

Cell Culture Growth Conditions

Mouse 10 T1/2 cell lines were routinely cultured at 37°C. on plastic tissue culture dishes (Life Technologies) in alpha minimal essential medium (MEM) supplemented with antibiotics and 7% fetal bovine serum (Hyclone Laboratories) (complete medium). In experiments wherein the effects of alterations in cAMP were investigated, a serum free medium was used, which contained 4 μg/ml of transferrin (Sigma) and 2 μg/ml of insulin (Sigma) [Hurta et al., 1996]. Cells were grown to confluence in complete medium then switched to a serum free (defined) medium for 24 h prior to exposure to cAMP altering agents (forskolin) (Sigma) for pre-determined times.

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 was 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 a ^{32}P labelled fragment generated from clone H2, which encodes for mouse SAMDC cDNA (kindly provided by Dr. A. E. I. Pajunen, University of Oulu, Finland). Probes were labelled using an oligo-labelling kit (Amersham Pharmacia Biotech) 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 transfer of the gels to the Nytran nylon membranes or by monitoring loading with a cDNA probe specific for rodent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (kindly provided by Dr. L. J. Z. Penn, Ontario Cancer Institute, Toronto, Ontario, Canada). Densitometric analysis of appropriately exposed autoradiograms was performed using a GS 700 Imaging Densitometer (Biorad) and the Molecular Analyst (Biorad) software program.

Nuclear Run-on Assays

Nuclei were prepared according to methods of deBustros et al. [1985] as described by Patel et al. [1998]. The cDNAs for SAMDC, GAPDH, and pGEM were linearized by digestion and were subsequently blotted (20 μg of cDNA per blot) onto nitrocellulose membranes. The GAPDH served as a positive control and pGEM served as a negative control. Membranes were dried and then were pre-hybridized for 24 h at 42°C. and then hybridized further for 24 h at 42°C. in the presence of [alpha-^{32}P]-UTP labelled RNA isolated from nuclear transcription experiments. Equal amounts of ^{32}P labelled RNA were hybridized to filters which contained the immobilized plasmids. Evaluation of SAMDC gene transcription was obtained following densitometric scanning and the results are expressed with reference to the signals obtained for GAPDH.

Assay for SAMDC Activity

SAMDC enzyme activity was measured essentially as previously described [Wang et al., 1992]. SAMDC activity was assayed monitoring the liberation of $^{14}CO_2$ from S-[carboxyl-^{14}C] adenosyl-L-methionine (Amersham) [Pegg and Williams-Ashman, 1969]. Radioactivity was determined by liquid scintillation spectroscopy using a LS6500 multipurpose scintillation counter (Beckman). SAMDC enzyme activity was calculated and expressed as nmoles CO_2/h/mg protein.

Protein content was determined using a BioRad protein assay kit, with bovine serum albumin as a standard.

RESULTS

Properties of T-24 H-Ras Transfected 10 T1/2 Mouse Fibroblasts

The isolation and characterization of the various cell lines used in this study have been described [Egan et al., 1987; Schwarz et al., 1988; Hurta et al., 1996]. Briefly, 10 T1/2 mouse cells were transfected with the plasmid pAL8A, which contains T-24 H-ras and the neo ^{R} gene. After transfection, cell lines were isolated which were either morphologically transformed (C3) or morphologically non-transformed (NR3). A summary of some of the relevant biological properties of the cell lines is presented in Table I. It is important to note that the 10 T1/2 parental cell line is not tumourigenic, and the NR3 cell line forms benign tumours, whereas, the C3 cell line exhibits malignant potential.

Selective Induction of SAMDC Gene Expression by Forskolin

cAMP has been shown to be an important regulator of cell growth and proliferation [Boyton and Whitfield, 1983; Roger et al., 1995; Withers, 1997]. To investigate the regulation of SAMDC in these cells, the effect of increased intracellular cAMP levels on SAMDC gene expression in the series of cell lines described in Table I was determined. Forskolin, which is a naturally occurring diterpene, directly stimulates adenylate cyclase and has been used extensively to elevate cAMP levels and to elicit cAMP-dependent physiological responses [Laurenza et al., 1989; Hurta and Wright, 1994a, 1994b]. To determine the effect of forskolin on the expression of the SAMDC gene in these cell lines, forskolin was added to cell cultures at a final concentration of 12 μM for 1, 3, 5, 24, and 48 h, respectively. Induction of SAMDC gene expression was not observed in either 10 T1/2 cells, which are non-transformed, or in NR3 cells, which are capable of forming benign tumours (Fig. 1). However, an obvious induction of SAMDC mRNA levels was noted in the malignant C3 line (Fig. 1). Densitometric evaluation of appropriately exposed autoradiograms revealed 3.5-, 6.5-, 4.8-, and 2.3- fold elevations of SAMDC mRNA levels in C3 cells following exposure of these cells to forskolin for 1, 3, 5, and 24 h respectively. The expression of SAMDC mRNA, which occurred following exposure to forskolin for 48 h, approximated that seen in control cells (Fig. 1). The forskolin analogue, 1, 9 dideoxyforskolin, is a naturally occurring compound that does not activate adenylate cyclase [Boyton and Whitfield, 1983]. The specificity of the cAMP effect observed with forskolin was tested by treating C3 cells with 1, 9 dideoxyforskolin for 1, 3, 5, 24, and 48 h, respectively. Northern blot analysis indicated that 1, 9 dideoxyforskolin (at a final concentration of 12 μM) did not modify SAMDC mRNA levels in C3 cells (Fig. 1). This finding is consistent with the view that the forskolin induced alterations of SAMDC message levels in C3 cells were due to a cAMP pathway involving activation of adenylate cyclase. It is possible that SAMDC gene expression may be induced in 10 T1/2 cells and in NR3 cells at higher doses of forskolin. Therefore, to test this possibility, 10 T1/2 cells and NR3 cells were exposed to 25 μM and to 50 μM forskolin for 1 and 5 h, respectively, and SAMDC mRNA expression was examined. No appreciable change in SAMDC mRNA expression was noted in either 10 T1/2 cells or in NR3 cells at these higher doses of forskolin (Fig. 2). However, a dose dependent increase in SAMDC mRNA expression was noted in C3 cells exposed to these doses of forskolin (Fig. 2). Increased SAMDC mRNA levels of about 2.6- and 6.0- fold and of about 5.5- and 8.5 -fold were noted in C3 cells in response to forskolin treatment (25 μM and 50 μM) for 1 h and in response to forskolin treatment (25 μM and 50 μM) for 5 h, respectively. To investigate further the relationship between SAMDC gene expression and alterations in cAMP levels, C3 cells were exposed to 8-bromo-cAMP, a slowly hydrolyzed analogue of cAMP [Boyton and Whitfield, 1983], and SAMDC mRNA levels determined by Northern blot analysis. As indicated in Table III, SAMDC mRNA expression levels were elevated in C3 cells grown in the presence of the cAMP analogue. 3.5-, 4.5-, and 6.0- fold increases in SAMDC mRNA expression levels were noted in C3 cells following exposure to 8-bromo-cAMP (0.8 mM) for 1, 3, and 5 h, respectively.

TABLE I. Tumourigenic and Metastatic Properties of the Mouse 10T1/2 Cell Lines

Cell line	Activated ras expression	Degree of malignancy
10 T1/2	—	normal
NR3	low	benign
C3	high	metastatic

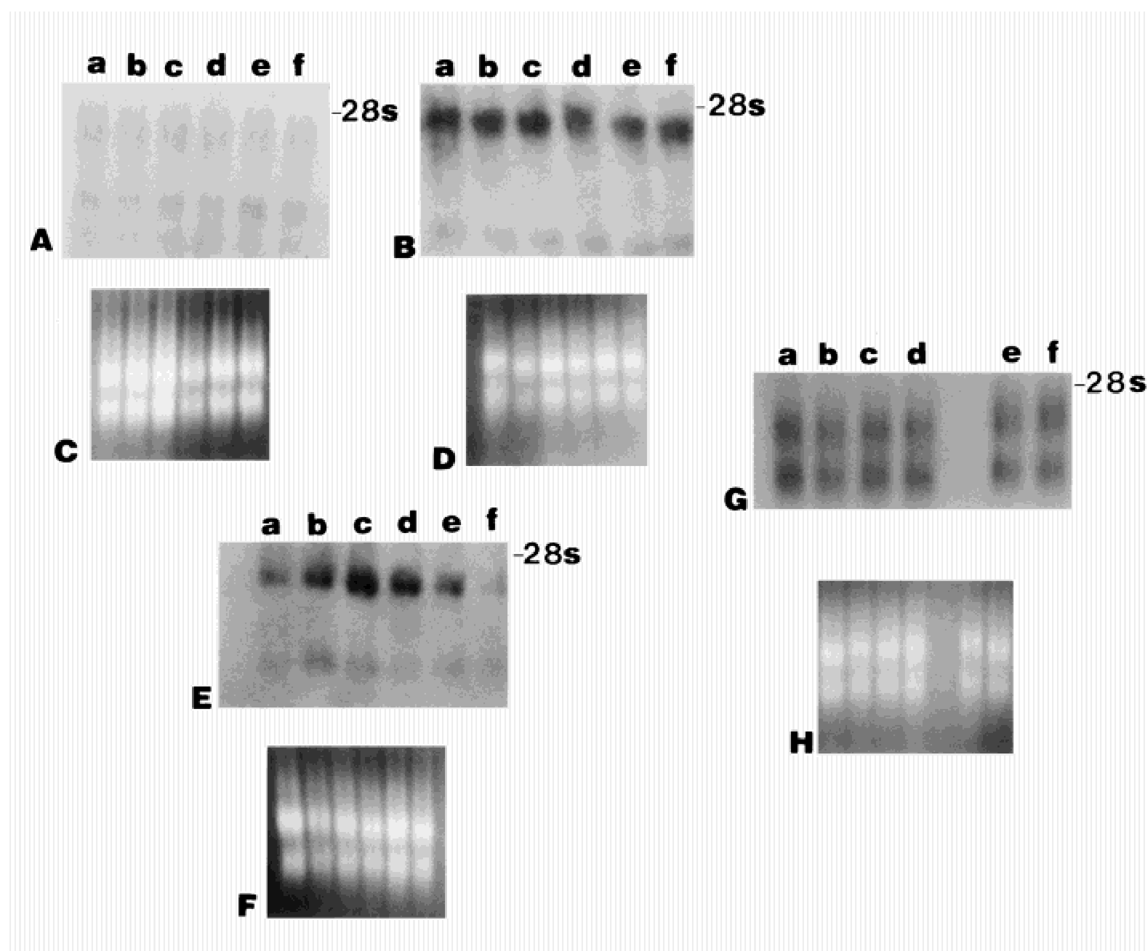

Fig. 1. SAMDC mRNA levels in 10 T1/2, NR3, and C3 cells in response to forskolin treatment. Cells were grown to confluence and then placed on a defined medium for 24 h prior to exposure to forskolin, (12 μM). Northern blot analysis of SAMDC mRNA levels in 10 T1/2 cells in the absence of forskolin (a) (control cells), and following exposure to forskolin for 1, (b), 3, (c), 5, (d), 24, (e) and 48 (f) h, respectively. **(B):** Northern blot analysis of SAMDC mRNA levels in NR3 cells in the absence of forskolin (a) (control cells), and following exposure to forskolin for 1, (b), 3, (c), 5, (d), 24, (e) and 48 (f) h, respectively. **(C)** and **(D):** Ethidium bromide stained ribosomal RNA bands are presented as a loading control for **(A)** and for (B), respectively. **(E):** Northern blot analysis of SAMDC mRNA levels in C3 cells in the absence of forskolin (a) (control cells), and following exposure to forskolin for 1, (b), 3, (c), 5, (d), 24, (e), and 48 (f) h, respectively. **(F):** Ethidium bromide stained ribosomal RNA bands are presented as a loading control. (Control cells were exposed to dimethylsulfoxide (DMSO), the vehicle in which the forskolin was dissolved). The SAMDC autoradiograms shown in (A), (B) and (E) were exposed for 168, 120, and 72 h, respectively, at $-70\,^0$C with intensifying screens. **(G):** Northern blot analysis of SAMDC mRNA levels in C3 cells in the absence of (a) and in the presence of 1,9 dideoxyforskolin for 1 (b), 3 (c), 5 (d), 24 (e) and 48 (f) h, respectively. **(H):** Ethidium bromide stained ribosomal RNA bands are presented as a loading control. The SAMDC autoradiogram shown in (G) was exposed for 120 h at −70°C with intensifying screens.

SAMDC Activity in Cells Treated with Forskolin

To determine if the observed increases in SAMDC mRNA lead to differences in enzymatic activity, SAMDC enzyme activity was examined in the highly malignant C3 cell line. SAMDC enzyme activity was also determined in the parental 10 T1/2 and in the NR3 cell lines. Assays of SAMDC activity at various times following exposure of these cells to forskolin (12 μM) revealed that interesting alterations in SAMDC enzyme activity occurred. No readily apparent increase in SAMDC activity was noted in 10 T1/2 cells (Table II). No change in SAMDC mRNA expression was noted in response to forskolin treatment in 10 T1/2 cells (as shown in Fig. 1). Increased SAMDC enzyme activity occurred in NR3 cells, following exposure to forskolin. Increased SAMDC enzyme activity of about 2.1-, 3.3- and 2.9- fold was noted in response to exposure to forskolin for 5, 24, and 48 h, respectively (Table II). Statistical analysis

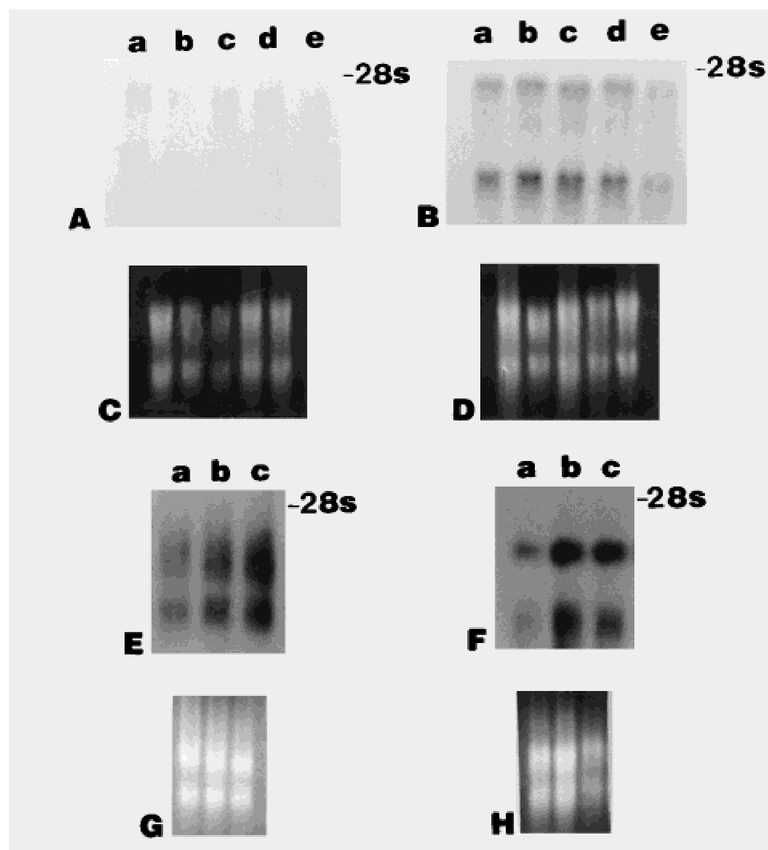

Fig. 2. SAMDC mRNA levels in 10 T1/2, NR3 and C3 cells in response to forskolin treatment. Cells were grown to confluence and then placed on a defined medium for 48 h prior to exposure to forskolin (25 μM and 50 μM) for 1 and 5 h, respectively. **(A):** Northern blot analysis of SAMDC mRNA levels in 10 T1/2 cells in the absence of forskolin (a) (control cells), and following exposure to forskolin (25 μM) for 1 (b) and 5 h (c), respectively, and following exposure to forskolin (50 μM) for 1 (d) and 5 h (e), respectively. **(B):** Northern blot analysis of SAMDC mRNA levels in NR3 cells as described above. **(C)** and **(D):** Ethidium bromide stained ribosomal RNA bands are indicated as loading controls. **(E):** Northern blot analysis of SAMDC mRNA levels in C3 cells in the absence of forskolin (a) and in the presence of forskolin (25 μM) (b) and in the presence of forskolin (50 μM) for 1 h (c), respectively. **(F):** Northern blot analysis of SAMDC mRNA levels in C3 cells in the absence of forskolin (a) and in the presence of forskolin (50 μM) (b) and in the presence of forskolin (25 μM) (c) for 5 h, respectively. **(G)** and **(H):** Ethidium bromide stained ribosomal bands are presented as loading controls for the Northern blot analysis results shown in (E) and (F). The autoradiograms shown in (A), (B), (E) and (F) were exposed at −70°C with intensifying screens for 168, 168, 48, and 24 h, respectively.

(ANOVA) revealed that these increases in SAMDC activity were statistically significant ($P < 0.01$). No change in SAMDC mRNA expression was noted in NR3 cells in response to forskolin (as shown in Fig. 1). SAMDC enzyme activity, in malignant C3 cells, unlike the responses observed with 10 T1/2 and NR3 cells, increased markedly in response to forskolin treatment and this increase occurred as early as 3 h post exposure to forskolin. SAMDC enzyme activity increased 2.5-, 5.4-, 9.8-, and 6.8- fold in response to forskolin treatment for 3, 5, 24, and 48 h, respectively (Table II). ANOVA revealed that these increases in SAMDC enzyme activity were statistically significant ($P < 0.01$). Notably, increased SAMDC mRNA expression was noted in C3 cells in response to forskolin (Fig. 1). The changes in SAMDC enzyme activity in response to forskolin treatment, which occurred in both NR3 cells and in C3 cells, relative to the response noted in 10 T1/2 cells, were also determined to be statistically significant ($P < 0.01$). Increased SAMDC enzyme activity was also determined to occur in C3 cells in response to 8-bromo-cAMP (0.8 mM). Increases in SAMDC enzyme activity in the order of 1.9-, 2.2-, and 4.9- fold occurred in C3 cells following exposure to 8-bromo-cAMP (0.8 mM) for 1, 3, and 5 h, respectively (Table III). ANOVA revealed that these alterations in SAMDC enzyme activity were also statistically significant ($P < 0.01$).

TABLE II. S-Adenosylmethionine Decarboxylase Enzyme Activity in H-ras Transformed Cells Following Exposure to Forskolin (12 μM)

Cell line	Exposure time (h)	SAMDC enzyme activity[1]	Fold increase
10T1/2	0	0.15 ± 0.04	—
	3	0.17 ± 0.06	1.1
	5	0.19 ± 0.03	1.2
	24	0.21 ± 0.04	1.4
	48	0.20 ± 0.03	1.3
NR3	0	0.30 ± 0.03	—
	3	0.33 ± 0.03	1.1
	5	0.63 ± 0.06	2.1
	24	0.98 ± 0.09	3.3
	48	0.88 ± 0.07	2.9
C3	0	0.61 ± 0.07	—
	3	1.52 ± 0.09	2.5
	5	3.30 ± 0.09	5.4
	24	5.98 ± 0.15	9.8
	48	4.14 ± 0.11	6.8

[1]The SAMDC enzyme activity is expressed as nmoles CO_2 /h/mg protein. The results presented are from duplicate experiments done in triplicate.

Effect of Forskolin Treatment on the Transcription of the SAMDC Gene

The possibility that the increase in SAMDC mRNA levels observed following exposure of C3 cells to forskolin was due to changes in gene transcription rates was investigated. C3 cells were pre-treated with 5 μg/ml of the transcription blocker, actinomycin D, [Phillips and Crothers, 1986; Hurta et al., 1996] prior to exposure of the cells to forskolin, (12 μM). As shown in Figure 3, actinomycin D treatment did not reduce the increase in SAMDC mRNA expression seen in C3 cells in response to treatment with forskolin. In the absence of actinomycin D, a 6.5- fold increase in SAMDC mRNA expression was noted, whereas, in C3 cells, in the presence of actinomycin D, the increase in SAMDC mRNA levels due to forskolin treatment was still evident (Fig. 3). A 7.8- fold increase in SAMDC mRNA expression was noted. Treatment of C3 cells with actinomycin D alone, showed levels of SAMDC mRNA expression comparable to those seen in control cells. These observations suggest that the forskolin effects on SAMDC gene expression which occur in C3 cells, do not apparently occur through alterations in the transcriptional process. To verify this possibility, nuclear run -on experiments were performed with C3 cells in the absence and in the presence of forskolin (12 μM). Nuclear run-on analysis of the transcription of the SAMDC gene in C3 cells revealed that following exposure to forskolin, no significant change in the transcriptional activity of the SAMDC gene occurred in response to forskolin treatment (Fig. 3). Based on these observations, the regulation of the expression of this gene by forskolin (and the alterations in cAMP) in C3 cells likely does not occur by a transcriptional mechanism but occurs possibly through post-transcriptional mechanism(s).

Effect of Forskolin on the Stability of SAMDC Gene Expression in Malignant C3 Cells

Since forskolin mediated alterations in SAMDC mRNA transcripts levels observed in C3 cells were not abrogated in the presence of actinomycin D (and no increase in SAMDC gene transcription in response to forskolin exposure was evident in nuclear run-on studies), the possibility that changes at the post-transcriptional level in response to forskolin treatment occur was investigated. 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., 1995] the rate of decay of the mature SAMDC message was evaluated in untreated and in forskolin treated C3 cells after exposure to an inhibitor of transcriptional initiation, 5, 6 dichloro-1-beta-D ribofuranosylbenzimidazole (DRB), [Mukherjee and Molloy, 1987; Hurta et al., 1996]. As shown in Figure 4, SAMDC mRNA transcripts in C3 cells are markedly more stable following

TABLE III. S-Adenosylmethionine Decarboxylase Expression in H-ras Transformed C3 Cells Following Exposure to 8-Bromo-cAMP (0.8 mM)

Cell line	Exposure time (h)	SAMDC m RNA (fold-increase)	SAMDC enzyme activity[1] (nmoles CO_2 /h/mg protein)
C3	0	—	0.54 ± 0.09
	1	3.5	1.02 ± 0.08
	3	4.5	1.21 ± 0.11
	5	6.0	2.63 ± 0.11

[1]The SAMDC enzyme activity results presented are from duplicate experiments done in triplicate.

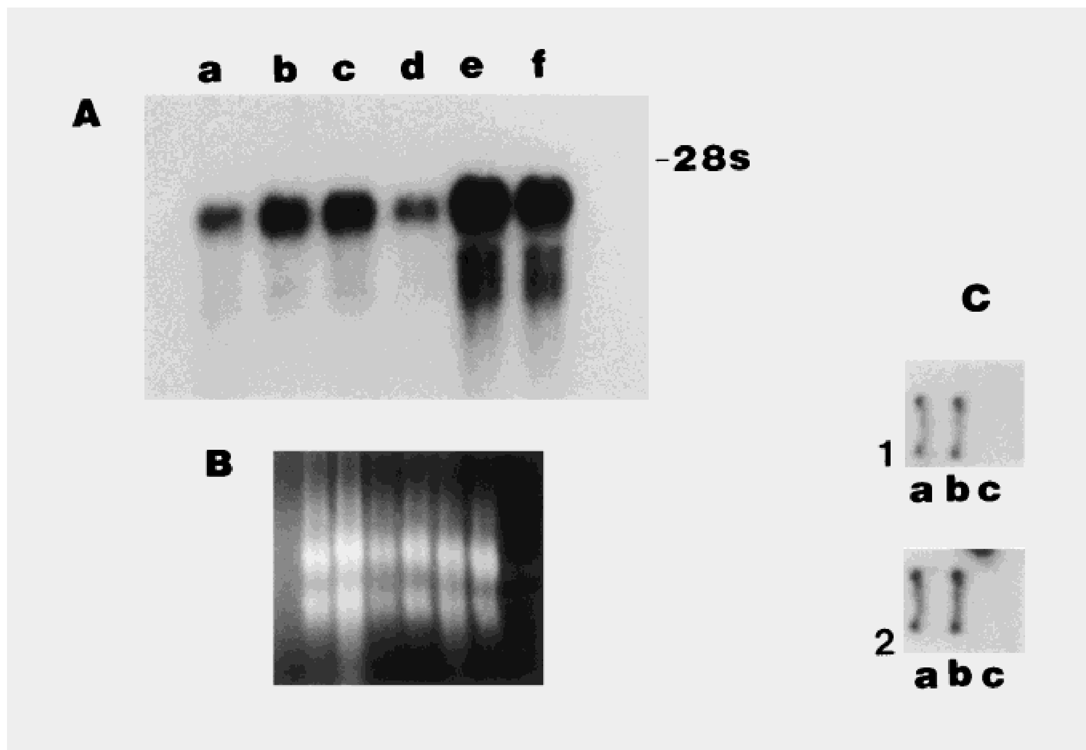

Fig. 3. Northern blot analysis of SAMDC mRNA levels. **(A):** C3 cells (control cells) (lane a); C3 cells exposed to 12 μM forskolin for 5 h (lane b); in the presence of 5 μg/ml actinomycin D with forskolin for 5 h, (lane c); C3 cells in the presence of 5 μg/ml of actinomycin D without forskolin (lane d); C3 cells in the presence of cycloheximide (10 μg/ml) and forskolin for 5 h, (lane e), and C3 cells in the presence of cycloheximide and in the absence of forskolin, (lane f). **(B):** Ethidium bromide stained ribosomal RNA bands are shown as a loading control. The autoradiogram shown in (A) was exposed for 48 h at −70°C with intensifying screens. Control cells were exposed only to DMSO alone, in the absence of forskolin, actinomycin D and cycloheximide. **(C):** Rate of SAMDC gene transcription in C3 cells in response to forskolin treatment. C3 cells were grown in control cultures (1) and in forskolin treated cultures (2). Nuclei were isolated and the rate of transcription of the SAMDC gene was measured. Equal amounts of labelled RNA were hybridized to filters containing immobilized plasmids of SAMDC (a), GAPDH (b) and pGEM (c).

forskolin treatment. Assuming that the decay of SAMDC mRNA which occurs after blocking RNA synthesis follows first order kinetics, the half-life of SAMDC mRNA in forskolin treated C3 cells was increased approximately 8-fold over that found in C3 cells not treated with forskolin. The half-life of SAMDC message in untreated C3 cells was estimated to be about 2.5 h, whereas, the half-life of this message in forskolin treated C3 cells was markedly altered and increased to approximately 20 h. These findings indicate that forskolin is capable of regulating SAMDC mRNA levels in C3 cells through a mechanism of post-transcriptional stabilization.

Effect of Protein Synthesis Inhibition on SAMDC Gene Expression Following Forskolin Treatment

To determine whether or not the forskolin mediated elevations in SAMDC mRNA required protein synthesis, C3 cells were exposed to 12 μM forskolin for 5 h in the presence or absence of 10 μg/ml cycloheximide, an inhibitor of eukaryotic protein synthesis, [Edwards and Mahadevan, 1992]. Figure 3 shows that cycloheximide treatment alone, substantially elevated SAMDC message levels in C3 cells. A 10 -fold increase was noted. Following exposure to cycloheximide and forskolin together, a 15.5-fold increase in SAMDC message levels was noted (Fig. 3). In the absence of cycloheximide treatment, a 6.5 -fold elevation in SAMDC mRNA levels was noted in C3 cells following exposure to forskolin for five hours. These results suggest that forskolin and cycloheximide regulate SAMDC mRNA levels in an approximately additive manner. These results suggest that SAMDC gene expression in the highly malignant cell line, C3, is controlled in a positive and cooperative manner by this inhi-

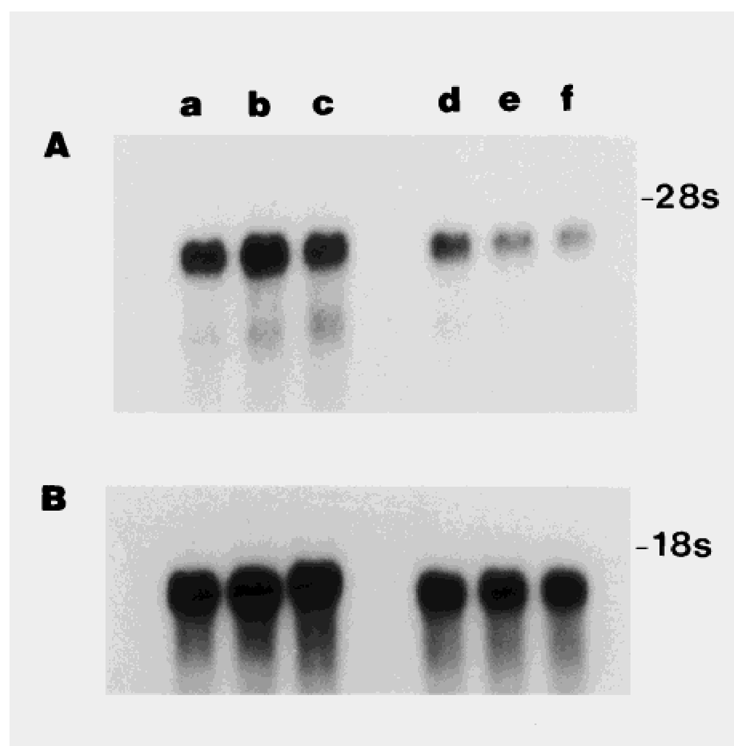

Fig. 4. Stability of SAMDC mRNA in response to forskolin exposure. **(A):** Northern blot analysis of SAMDC mRNA in C3 cells untreated and treated with forskolin (12 μM). C3 cells were either cultured in the presence of forskolin or in the presence of DMSO (the vehicle within which the forskolin was dissolved) for 5 h, and following this treatment were subsequently treated with DRB (75 μM). Total RNA was isolated and analyzed. (A): Northern blot analysis of SAMDC mRNA levels in C3 cells exposed to forskolin for 5 h prior to exposure to DRB for 2.5 (lane b) and for 5 h (lane c), respectively. Lane (a) indicates the SAMDC mRNA levels in forskolin -treated cells (control cells); SAMDC mRNA levels in C3 cells exposed to DMSO alone (in the absence of forskolin) prior to exposure to DRB for 2.5 (lane e) and 5 h (lane f), respectively. Lane (d) indicates the SAMDC mRNA levels in DMSO-treated C3 cells (control cells). **(B):** GAPDH autoradiogram as a loading control. The SAMDC and the GAPDH autoradiograms were exposed at −70°C with intensifying screens for 48 and for 24 h respectively.

bitor of protein synthesis. As illustrated in Figure 5, an aspect of the regulation associated with increased SAMDC mRNA levels in response to cycloheximide treatment is an increase in the stability of the SAMDC mRNA transcript. In the presence of cycloheximide, the half-life of the SAMDC message was estimated to be about 6 h, whereas, in the absence of cycloheximide exposure, the SAMDC mRNA half-life was determined to be about 2.1 h. This represents a stabilization of the SAMDC transcript of about 3-fold.

Effect of the Phosphodiesterase Inhibitor IBMX on SAMDC Gene Expression

If the forskolin and 8-bromo-cAMP induced increased SAMDC mRNA expression is in response to elevated cAMP levels as the results presented would suggest, then inhibition of the phosphodiesterase activity which converts cAMP to AMP might enhance the induction of SAMDC gene expression by preventing the turnover of cAMP produced in response to forskolin or 8-bromo-cAMP. If the elevations observed in SAMDC mRNA gene expression are cAMP independent, then the phosphodiesterase inhibitor, 3-isobutyl -1-methylxanthine (IBMX) should not appreciably enhance SAMDC gene expression in the presence of either forskolin or 8-bromo-cAMP. To test this possibility, the effect of 0.5 mM IBMX on the forskolin-and 8-bromo-cAMP mediated elevations in SAMDC gene expression in C3 cells was investigated. IBMX treatment amplified the forskolin- and 8-bromo-cAMP -mediated elevations in SAMDC gene expression in malignant C3 cells (Fig. 6). IBMX treatment alone, actually increased SAMDC mRNA levels slightly, increasing the levels by about 2- fold. Interestingly, IBMX is capable of acting in cooperation with the other cAMP altering agents, namely, forskolin and 8-bromo-cAMP to augment SAMDC mRNA

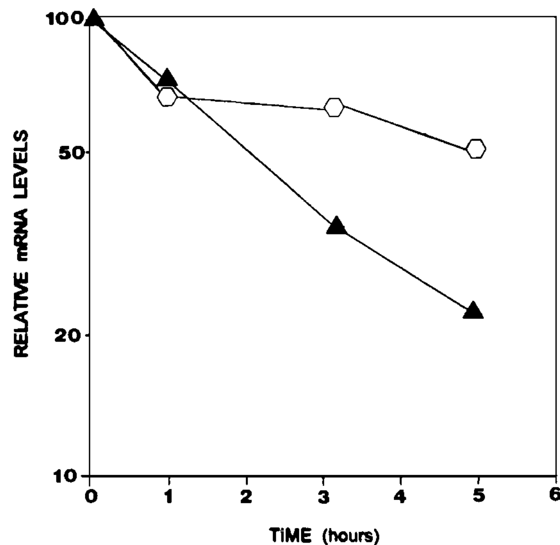

Fig. 5. Stability of SAMDC mRNA in untreated (▲) and in cycloheximide- treated (⬡) C3 cells. C3 cells were exposed to cycloheximide (10 μg/ml) for 1 h and subsequently treated with DRB (75 μM). Total RNA was isolated at the times indicated and subjected to Northern blot analysis as described. The relative levels of SAMDC mRNA were determined by densitometric analysis of autoradiograms exposed in the linear range for each set of samples. The results presented are from duplicate experiments.

expression levels in C3 cells. In C3 cells, 6.0- and 5.2 -fold increases in SAMDC mRNA expression were observed due to forskolin and 8-bromo-cAMP exposure, respectively (Fig. 6). However, with IBMX present, 12.0- and 11.0-fold increases in SAMDC mRNA expression were noted, following exposure of C3 cells to forskolin and 8-bromo-cAMP, respectively (Fig. 6). Similar interactions were also noted between IBMX, forskolin, and 8-bromo-cAMP effects when SAMDC enzyme activity was evaluated (Table IV). Forskolin and 8-bromo- cAMP treatment increased SAMDC enzyme activity by about 3.1- and 2.3- fold, respectively. Interestingly, these increases in enzyme activity were amplified when C3 cells were challenged with either forskolin or 8-bromo- cAMP in the presence of IBMX. As indicated in Table IV, increases of 6.9- and 5.1- fold in SAMDC enzyme activity were noted under these conditions. IBMX alone also increased SAMDC enzyme activity (about a 2-fold increase was found) (Table IV). ANOVA revealed that the alterations in SAMDC enzyme activity noted in Table IV were statistically significant ($P < 0.05$). The interactions between IBMX and these cAMP altering agents resulted in an alteration of SAMDC enzyme activity which appeared to result in a synergistic augmentation of SAMDC enzyme activity expression. These data further suggest and support the contention that the induction of SAMDC gene expression by forskolin and by 8-bromo-cAMP in these ras-transformed C3 cells occurs via a cAMP dependent signal transduction pathway.

Increased SAMDC Gene Expression by a cAMP Altering Agent, Forskolin, is Enhanced by Stimulation of a Protein Kinase Pathway

The effects of the phorbol ester tumour promoter, phorbol 12-myristate-13-acetate (PMA), (an activator of protein kinase C-mediated events) and forskolin (an activator of cAMP -dependent events) alone, and in combination, on SAMDC gene expression was investigated in highly, metastatic C3 cells. These cells were treated with either 0.1 μM PMA or with 12 μM forskolin or with PMA and forskolin together for 1, 3, and 5 h, respectively. Figure 7 indicates that in C3 cells, PMA and forskolin when exposed to C3 cells separately increased SAMDC mRNA levels 6.2-, 4.0-, and 4.5-fold, and 2.5-, 3.5-, and 4.0 -fold following exposure of these cells to these agents for 1, 3, and 5 h, respectively, whereas, in combination, PMA and forskolin increased SAMDC mRNA levels 8.5-, 7.0-, and 6.8 -fold following exposure of C3 cells to these agents for 1, 3, and 5 h, respec-

TABLE IV. S-Adenosylmethionine Decarboxylase Enzyme Activity in C3 Cells

Cell line	Conditions[1]	SAMDC enzyme activity[2]	Fold increase
C3	no additions	0.50 ± 0.07	—
	IBMX	0.99 ± 0.06	2.0
	forskolin	1.54 ± 0.11	3.1
	8-bromo-cAMP	1.13 ± 0.08	2.3
	IBMX + forskolin	3.46 ± 0.12	6.9
	IBMX + 8-bromo-cAMP	2.55 ± 0.12	5.1

[1]Exposure time was 2 h. The concentrations used were: IBMX, 0.5 mM; forskolin, 12 μM , and 8-Bromo-cAMP, 0.8 mM.
[2]SAMDC enzyme activity is expressed as nmoles CO_2/h/mg protein. The results presented are from duplicate experiment done in duplicate.

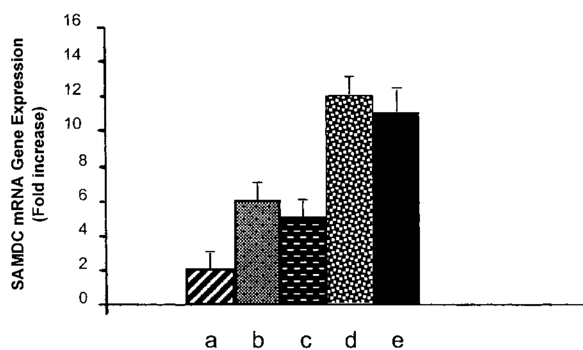

Fig. 6. Effects of the phosphodiesterase inhibitor, IBMX, on SAMDC mRNA expression in C3 cells. Marked elevations in SAMDC mRNA were found in C3 cells in response to forskolin and 8-bromo-cAMP alone and these elevations were enhanced in combination with IBMX. SAMDC mRNA levels noted in C3 cells exposed to IBMX (0.5 mM) alone (a); to forskolin (12 μM) (b); to 8-bromo-cAMP (0.8 mM) (c); to forskolin (12 μM) and IBMX (0.5 mM), (d); and to 8-bromo-cAMP (0.8 mM) and IBMX (0.5 mM), (e). SAMDC mRNA levels in C3 cells were determined following 2 h exposures to the above mentioned agents. The results are expressed as fold increases relative to SAMDC mRNA levels noted in control C3 cells.

tively (Fig. 7). The increases seen when both protein kinase-C and protein kinase-A mediated events were induced appeared to be augmented when both pathways were stimulated when compared to the increases observed when protein kinase-C and protein kinase-A mediated events were stimulated separately (Fig. 7). An approximately additive response occurred. These observed alterations in SAMDC expression in C3 cells suggest that protein kinase C- and cAMP- mediated pathways can regulate SAMDC expression in highly malignant C3 cells. These results further suggest that these alterations in SAMDC gene expression are the result of the influences of pathways that are most likely independent of each other.

DISCUSSION

This study demonstrated alterations in the expression of SAMDC in the presence of stimulators of cAMP synthesis, in the presence of an inhibitor of cAMP degradation, and following exposure to a stable analogue of cAMP. The marked changes in SAMDC mRNA expression occurred in H-ras transformed cells capable of malignant progression and were not evident in non-transformed parental 10 T1/2 cells or in H-ras transformed cells capable of only benign tumour formation. This observation demonstrates a link between alterations in cAMP regulation during malignant progression and the expression of SAMDC in this series of H-ras transformed cell lines. These findings are, in general, consistent with previous studies of cAMP mediated regulation of ribonucleotide reductase gene expression and DNA synthesis in normal and in malignant cells [Hurta and Wright, 1994a], which showed that malignant H-ras transformed cells also contain alterations in signal transduction pathways that lead to novel modifications of this proliferation related activity. Furthermore, and perhaps, more importantly, these findings are, in general, consistent with previous studies of cAMP-mediated regulation of ODC gene expression in H-ras transformed cells [Hurta and Wright, 1994b], which showed that ODC gene expression was aberrantly regulated by the cAMP signal transduction pathway in malignant H-ras transformed cell lines. There are some notable differences, in particular, the fact that alterations in ODC mRNA expression occur in response to alterations in cAMP levels in low ras, NR3 cells, capable of only benign tumour formation [Hurta and Wright, 1994b] but alterations in SAMDC mRNA expression apparently do not occur. However, in C3 cells, which have high levels of ras expression and exhibit malignant characteristics, alterations in both activities responsible for the regulation of polyamine biosynthesis, namely ODC and SAMDC, occur in response to alterations in cAMP levels within these C3 cells. The results of this study are in keeping with reports which have demonstrated direct connections between cAMP regulation and the transmission of growth signals through ras-mediated pathways [Cook and McCormack, 1993; Sevetson et al., 1993; Hurta and Wright, 1994a, 1994b]. These reports suggest that alterations in ras expression through the H-ras transfection of 10 T1/2 cells [Egan et al., 1987] would likely influence cross-talk between these two major pathways. The results presented in this report suggest that these changes in regulatory interactions between the ras and cAMP pathways have produced a novel mechanism(s) for regulating SAMDC gene expression relevant to malignant progression.

The cAMP induced changes in SAMDC message levels in malignant cells were apparently not due to alterations in the transcriptional efficiency of the SAMDC gene but were

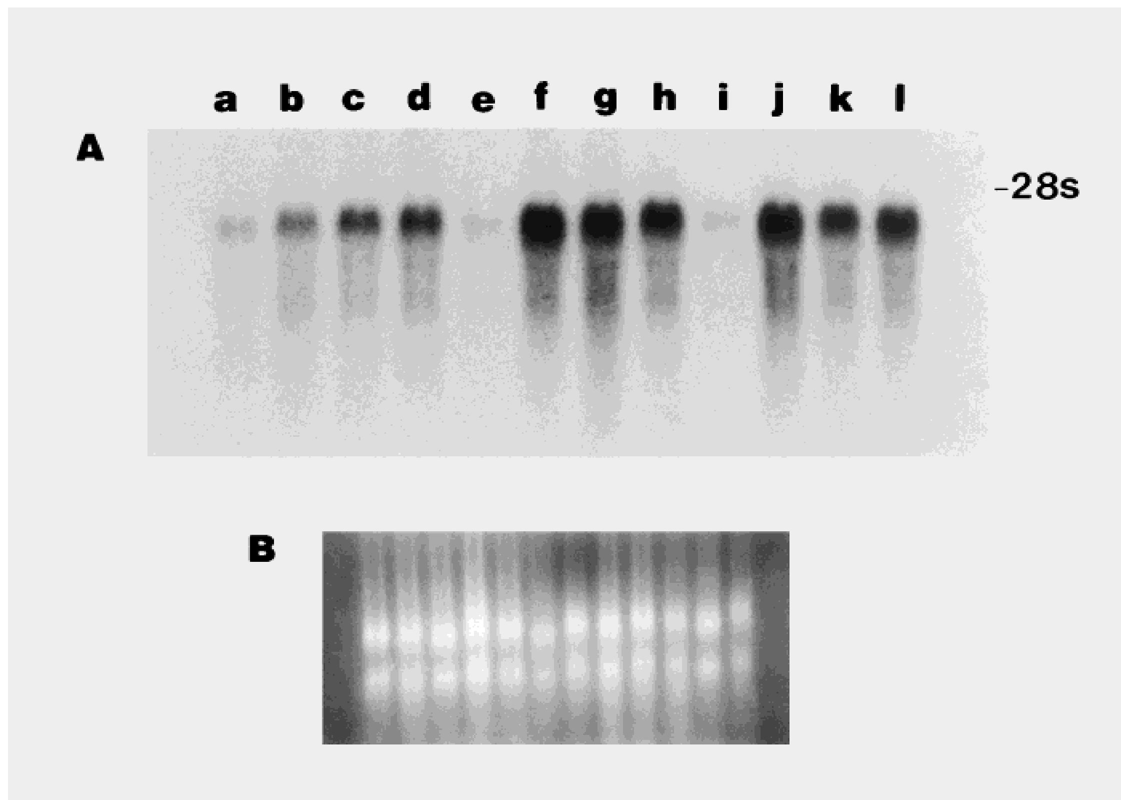

Fig. 7. Protein kinase C- and protein kinase A- mediated pathways interact to regulate SAMDC mRNA levels in C3 cells. **(A):** Northern blot analysis of SAMDC mRNA levels in control cells, (a), in cells exposed to forskolin (12 μM) alone for 1 (b), 3 (c), and 5 h (d) respectively, in control cells (e), in cells exposed to forskolin (12 μM) and PMA (0.1 μM) for 1 (f), 3 (g), and 5 (h) h, respectively, in control cells (i), in cells exposed to PMA (0.1 μM) alone for 1 (j), 3 (k), and 5 (l) h, respectively. Control cells received appropriate vehicle alone for 5 h. **(B):** Ethidium bromide stained ribosomal RNA bands as a loading control. The SAMDC autoradiogram was exposed for 48 h at −70°C with intensifying screens.

due to post-transcriptional regulation of the SAMDC gene. Previously, cAMP induced changes in ODC message levels in malignant C3 cells were demonstrated to be due to modifications in the transcriptional apparatus [Hurta and Wright, 1994b] and to be due to post-transcriptional stabilization of the ODC message (unpublished observations). The specific nature of the factors involved in the post-transcriptional stabilization of SAMDC and ODC in malignant C3 cells remain to be investigated and elucidated.

The finding that cycloheximide rapidly induced SAMDC expression in malignant C3 cells indicates, that in addition to positive signals elicited by altered cAMP levels, the expression of the SAMDC gene can also be altered by an inhibitor of protein synthesis. This observation is consistent with previous observations which showed that changes in cAMP levels coupled with inhibition of protein synthesis following exposure to cycloheximide exposure resulted in a dramatic increase in ODC mRNA levels which occurred in an additive manner [Hurta and Wright, 1994b]. These observations suggest the possible existence of a cycloheximide sensitive regulator of cAMP expression and ODC expression and also SAMDC expression in the highly malignant H-ras transformed C3 cells. The exact nature of the mechanisms and factors involved in this regulation of SAMDC and of ODC remains to be determined. However, a part of this regulation involves the possible post-transcriptional stabilization of the SAMDC mRNA transcript. Post-transcriptional stabilization of the ODC mRNA transcript in response to protein synthesis inhibition due to cycloheximide treatment also occurs in C3 cells. (unpublished observations). It is interesting to note that in H-ras transformed NR3 cells, cycloheximide abolished the observed induction of ODC expression following the modifications of the cAMP pathway [Hurta and Wright, 1994b] and no induction of SAMDC mRNA

occurred in NR3 cells in response to alterations in the cAMP pathway. NR3 cells are tumourigenic but not metastatic (Table I). These observations suggest a demarcation in the regulation of SAMDC gene expression between tumourigenic but non-metastatic cells and cells which possess a highly malignant phenotype. These observations are consistent with similar observations previously demonstrated regarding the regulation of ODC expression [Hurta and Wright, 1994b]. The altered coordinated upregulation of both activities, which play critical roles in the regulation of polyamine biosynthesis, namely ODC and SAMDC, constitutes an important part of the altered growth regulatory program associated with H-ras mediated cellular transformation which results in malignant progression.

This study also demonstrated that SAMDC gene expression can be modulated by signal transduction pathways involving both protein kinase C [Nishizuka, 1988] and protein kinase A [Boyton and Whitfield, 1983; Roger et al., 1995; Withers, 1997], which determine separate, although, at times interactive, signal transduction pathways [Pelech et al., 1990]. Studies with PMA and with forskolin alone and in combination indicated that the effects of including these two components together result in an additive induction of SAMDC mRNA expression. Synergistic interactions between protein kinase C and cAMP mediated pathways have been demonstrated in the regulation of ODC gene expression in highly malignant C3 cells [Hurta and Wright, 1994b]. These observations suggest that these two pathways are capable of modulating both SAMDC and ODC gene expression and that alterations in the two pathways are potentially important in cellular regulation affecting malignant cell proliferation. In addition to the above, it is possible that differences exist between the expression and the regulation of protein kinase A in response to alterations in cAMP levels in these cells and that these alterations in the expression and regulation of protein kinase A expression may also contribute, in part, to the mechanisms responsible for the alterations in SAMDC (and other genes) gene expression and regulation observed in H-ras transformed cells capable of malignant progression. It is further possible that the regulation of the expression of protein kinase A may change as cells progress from a non-transformed phenotype (10 T1/2 cells) to one where cells are capable of benign tumour formation (NR3 cells) to one where cells are capable of malignant progression (C3 cells). Such studies are in progress.

Determining how regulatory pathways interact to control SAMDC (and ODC) expression in "normal", non-transformed cells and how this regulation is abrogated or altered in malignant cells is a major but important challenge. SAMDC (and ODC) plays an important role in normal and in abnormal cell proliferation [Pegg, 1988; Hurta et al., 1996; Hurta, 1999] and through altered expression, SAMDC and ODC are capable of modifying cell transformation events (including oncogene mediated events) [Auvinenn et al., 1992, 1997; Manni et al., 1995a, 1995b; Auvinen, 1997; Kubota et al., 1997; Tabib and Bachrach, 1998; Hurta, 1999]. The present study emphasizes the importance of SAMDC regulation, in addition to alterations in ODC expression and regulation, in the process of H-ras -mediated cellular transformation and malignant progression and also describes a role for several signal pathways including the cAMP -dependent pathway in this process, and provides a focus for further study and investigation into the precise nature of the mechanisms involved.

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REFERENCES

Amara FM, Hurta RAR, Huang A, Wright JA. 1995. Altered regulation of message stability and tumour promoter responsive cis-trans interactions of ribonucleotide reductase R1 and R2 RNAs in hydroxyurea resistant cells. Cancer Res 55:4503–4506.

Auvinen M. 1997. Cell transformation, invasion and angiogenesis: a regulatory role for ornithine decarboxylase and polyamines? J Natl Can Inst 89:533–537.

Auvinen M, Laine A, Paasinen-Sohns A, Kangas A, Kangas L, Saksela O, Andersson LC, Holtta E. 1997. Human ornithine decarboxylase-overproducing NIH-3T3 cells induce rapidly growing, highly vascularized tumours in nude mice. Cancer Res 57:3016–3025.

Auvinen M, Paasinen A, Andersson LC, Holtta E. 1992. Ornithine decarboxylase activity is critical for cell transformation. Nature 360:355–358.

Boyton AL, Whitfield JF. 1983. The role of cyclic AMP in cell proliferation: a critical assessment of the evidence. In: Greegard P, Robison G.A, editors. Advances of cyclic nucleotide research. NY: Raven Press. pp. 193–294.
deBustros A, Baylin SB, Berger CL, Roos BA, Leong SS, Nelkin BD. 1985. Phorbol esters increase calcitonin gene transcription and decrease c-myc RNA levels in cultured human medullary thyroid carcinoma. J Biol Chem 260: 98–104.
Cook SJ, McCormack F. 1993. Inhibition by cAMP of ras dependent activation of raf. Science 262:1069–1072.
Edwards DL, Mahadevan LC. 1992. Protein synthesis inhibitors differentially superinduce c-fos and c-jun by three distinct mechanisms: lack of evidence for labile repressors. EMBO J 11, 7:2415–2424.
Egan SE, McClarty GA, Jarolim L, Wright JA, Spiro J, Hager G, Greenberg AH. 1987. Expression of H-ras correlates with metastatic potential: evidence for direct regulation of the metastatic phenotype in 10 T1/2 and NIH-3T3 cells. Mol Cell Biol 7:830–837.
Heby O, Persson L. 1990. Molecular genetics of polyamine biosynthesis in eukaryotic cells. Trends Biochem Sci 15: 153–158.
Hurta RAR. 1999. Altered expression/regulation of ornithine decarboxylase and S-adenosylmethionine decarboxylase correlates with malignant progression in H-ras transformed fibroblasts. In: Experimental Biology,99. Washington, D.C: abstract
Hurta RAR, Wright JA. 1994a. Alterations in the cyclic AMP signal transduction pathway regulating ribonucleotide reductase gene expression in malignant H-ras transformed cell lines. J Cell Physiol 158:187–197.
Hurta RAR, Wright JA. 1994b. Ornithine decarboxylase gene expression is aberrantly regulated via the cAMP signal transduction pathway in malignant H-ras transformed cell lines J Cell Physiol 161:383–391.
Hurta RAR, Huang A, Wright JA. 1996. Basic fibroblast growth factor selectively regulates ornithine decarboxylase gene expression in malignant H-ras transformed cells. J Cell Biochem 60:572–583.
Kubota S, Kiyosawa H, Nomura Y, Yamada T, Seyama Y. 1997. Ornithine decarboxylase overexpression in mouse 10 T $\frac{1}{2}$ fibroblasts: cellular transformation and invasion. J Natl Can Inst 89, 8:567–571.
Laurenza A, Sutkowski EM, Seamon KB. 1989. Forskolin: A specific stimulator of adenylyl cyclase or a diterpene with multiple sites of action? Trends Pharmacol Sci 10:442–447.
Manni A, Badger B, Grove R, Kunselman S, Demers L. 1995a. Isolation and characterization of human breast cancer cells over-expressing S-adenosylmethionine decarboxylase. Cancer Lett 95:23–28.
Manni A, Wechter R, Wei L, Heitjan D, Demers L. 1995b. Phenotypic features of breast cancer cells over- expressing ornithine decarboxylase. J Cell Physiol 163:129–136.
Mukherjee R, Molloy GR. 1987. 5,6, dichloro-1-beta- D-ribofuranosylbenz-imidazole inhibits transcription of the beta-hemoglobin gene in vivo at initiation. J Biol Chem 262:13697–13705.
Nishizuka Y. 1988. The molecular heterogeneity of protein kinase C and its implications for cellular regulation. Nature 334:661–665.
Patel AR, Li J, Bass BL, Wang J-Y. 1998. Expression of the transforming growth factor-beta gene during growth inhibition following polyamine depletion. Am J Physiol C590–C597.
Pegg AE. 1988. Polyamine metabolism and its importance in neoplastic growth and as a target for chemotherapy. Cancer Res 48:759–774.
Pegg AE, Williams-Ashman HG. 1969. On the role of S-adenosyl-L- methionine in the biosynthesis of spermidine by rat prostate. J Biol Chem 244:682–693.
Pelech SL, Sanghera JS, Daya-Makin M. 1990. Protein kinase cascades in meiotic and mitotic cell cycle control. Biochem Cell Biol 68:1297–1330.
Phillips DR, Crothers DM. 1986. Kinetics and sequence specificity of drug-DNA interactions: an in vitro transcription assay. Biochemistry 25:7355–7362.
Roger PP, Reuse S, Maenhaut C, Dumont JE. 1995. Multiple facets of the modulation of growth by cAMP. Vitam Hormon 51:159–191.
Schwarz LC, Gingras M-C, Goldberg G, Greenberg AH, Wright JA. 1988. Loss of growth factor dependence and conversion of TGF-beta$_1$ inhibition to stimulation in metastatic H-ras transformed murine fibroblasts. Cancer Res 48:6999–7003.
Sevetson BR, Kong X, Laurence JC, Jr. 1993. Increasing cAMP attenuates activation of mitogen activated protein kinase. Proc Natl Acad Sci USA 90:10305–10309.
Tabib A, Bachrach U. 1998. Polyamines induce malignant transformation in cultured NIH-3T3 fibroblasts. Intl J Biochem Cell Biol 30:135–146.
Tabor CW, Tabor H. 1984. In: Richardson CH, Boyer PD, Meister A, editors. Ann review of biochemistry. Palo Alto, CA: Annual Reviews, Inc. pp. 749–790.
Wang J-Y, Viar MJ, McCormack SA, Johnson LR. 1992. Effect of putrescine on S-adenosylmethionine decarboxylase in a small intestinal crypt cell line. Am J Physiol G494–G501.
Withers DJ. 1997. Signalling pathways involved in the mitogenic effects of cAMP. Clin Sci 445–451.
Wright JA, Chan AK, Choy BK, Hurta RAR, McClarty GA, Tagger AY. 1990. Regulation and drug resistance mechanisms of mammalian ribonucleotide reductase and the significance of DNA synthesis. Biochem Cell Biol 68:1364–1371.
Wright JA, Turley EA, Greenberg AH. 1993. Transforming growth factor beta and fibroblast growth factor as promoters of tumour progression to malignancy. Crit Rev Oncog 4:473–492.