Amino Acids (1995) 8: 59–68

Amino
Acids

Polyamine acetylations in normal and neoplastic growth processes

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Accepted November 15, 1993

Summary. The expression patterns of cytosolic and nuclear polyamine acetyltransferases were studied in normal and neoplastic growth processes *in vivo* and *in vitro* to evidentiate the roles played by these enzymes in cell proliferation. In regenerating liver, cytosolic spermidine/spermine N^1-acetyltransferase showed similar augments of mRNA level and enzymatic activity during the prereplicative period (4–8 h), whereas spermidine N^8-acetyltransferase activity increased later (24 h) when DNA synthesis was maximally enhanced. In fibroblasts continuously dividing, the messenger for spermidine/spermine N^1-acetyltransferase rapidly accumulated after serum-stimulation. In cultured Morris hepatoma cells stimulated to logarithmic growth, spermidine N^8-acetyltransferase activity remained at plateau for 1 day declining thereafter, while spermidine/spermine N^1-acetyltransferase activity immediately decreased. In Yoshida AH-130 hepatoma cells transplanted in rat peritoneum, spermidine N^8-acetyltransferase and spermidine/spermine N^1-acetyltransferase activities rose, respectively, in concomitance with elevated proliferation-rate and quasi-stationary phase of growth. Since the expression of cytosolic and nuclear acetyltransferases underwent different temporal activation, an involvement of these enzymes in separate metabolic processes controlling normal and neoplastic growth may be suggested.

Keywords: Amino acids – Liver regeneration – Hepatocellular carcinoma – Polyamines – Acetylation

Introduction

Naturally occurring polyamines (putrescine, spermidine and spermine) are widely distributed organic cations that play various and fundamental roles in the cells (Marton and Morris, 1987; Schuber, 1989). Polyamine metabolism and physiological functions are strictly controlled (Pegg, 1988; Sjöholm, 1993). These polycations are increasingly being recognized as regulators of normal and neo-

plastic cell proliferation (Pegg, 1988; Jänne et al., 1991). The concentration of polyamines is low in resting cells, and increases rapidly when the cells are stimulated to divide in response to growth factors, hormones and tumor promoters (Pegg, 1988; Scalabrino and Lorenzini, 1991). The importance of polyamines for cell proliferation has been indicated by studies carried out with inhibitors of the rate-limiting enzymes of polyamine biosynthesis, i.e. ornithine decarboxylase (EC 4.1.1.17, ODC) and *S*-adenosylmethionine decarboxylase (EC 4.1.1.50, AdoMetDC), or mutants lacking ODC (Pegg, 1988; Jänne et al., 1991)

Various types of cells show precise time-dependent increases in ODC and AdoMetDC expression as well as in polyamine levels when proliferative stimuli are given during quiescence (G0), or at G1 phase of normal cycle. Early enhancements of the activities and mRNA levels of the biosynthetic decarboxylases take place during the transition from G0 to G1 phase. Later peaks of these enzyme activities and polyamine levels occur in normally cycling cells, i.e. at the G1/S phase boundary, indicating that polyamine formation may be related to ongoing DNA synthesis (Stimac and Morris 1987; Sjöholm, 1993). In eukaryotes, endogenous polyamines are associated with highly condensed chromatin *in vivo* (Marton and Morris, 1987), affecting the activities of enzymes involved in nucleic acid metabolism, like histone acetyltransferase, thymidine kinase and restriction enzymes (Estepa and Pestaña, 1981; Conrad and Topal, 1989; Desiderio, 1992; Desiderio et al., 1993).

Acetyltransferases which catalyze the acetylation of polyamines, using acetylCoA as a substrate, are detected and isolated from cytosol and nuclei of mammalian cells (Pegg, 1988; Desiderio et al., 1991; Casero et al., 1993). The cytosolic spermidine/spermine N^1-acetyltransferase (cSAT) catalyzes the acetylation of the aminopropyl termini of spermidine and spermine. This enzyme is part of the two-step catabolic reaction by which the FAD-dependent polyamine oxidase (PAO) leads to back conversion of acetylpolyamines ultimately to putrescine (Casero et al., 1993). cSAT activity seems to play a major role in the regulation of polyamine levels in proliferating cells being its expression growth-controlled (Desiderio et al., 1993). Nuclear acetyltransferase(s) catalize(s) the acetylation of the aminobutyl group of spermidine, and the fate for this N^8-acetylderivative is to be hydrolized in the cytosol (Desiderio et al., 1992). Polyamine acetylation, while lowering the stability and weakening the conformational change of the nucleosome core caused by underivatized polyamines, seems to favour DNA transcription and/or replication (Xiao et al., 1991).

The pattern of cSAT activity during normal and neoplastic growth has recently generated considerable interest. cSAT activity induction has been shown in different tissues and cells after a variety of growth stimuli, like carbon tetrachloride, thioacetamide, growth hormone, partial hepatectomy, folic acid, phytohemagglutinin, dialkylnitrosamines (Pegg, 1988). A regulatory control of cSAT activity and mRNA levels is exerted by spermidine and polyamine analogues (Pegg, 1988; Desiderio et al., 1993; Shappell et al., 1993). The interconversion pathway of polyamines is also involved in the carcinogenesis of colon (Halline et al., 1990) and liver (Sessa et al., 1988; Sessa and Perin, 1991) as

well as in the proliferation of lung cancer cells (Casero et al., 1989) and melanoma cells (Shappell et al., 1993). In the last years, our group began to investigate spermidine N^8-acetyltransferase (nSAT) activity in growth processes to explore its inducibility (Desiderio, 1992; Desiderio et al., 1992, 1993).

In the present paper, cSAT and nSAT expression patterns have been examined during normal and neoplastic growth using *in vitro* and *in vivo* systems. The enzyme activities have been studied in regenerating liver, in normally cycling fibroblasts, and during the growth of Morris 7288C hepatoma cells in culture and Yoshida AH-130 ascites hepatoma cells in the rat. Recent cloning of a human cSAT gene (Casero et al., 1993) led us to investigate cSAT mRNA expression besides the enzymatic activity.

Materials and methods

Chemicals

[^{32}P] Deoxycytidine triphosphate (dCTP) (3000 Ci/mmol), [1-^{14}C] acetylCoA (50 Ci/mol) and Hybond-C nitrocellulose filters were purchased from Amersham International (Amersham, Bucks, U.K.). (2R,5R)-6-Heptyne-2,5-diamine hydrochloride [(MDL 72.175); (2R,5R)MAP] and 5'{[(Z)-4-amino-2-butenyl]methylamino-5'-deoxyadenosine} [MDL 73.811; (Z)AbeAdo] were generously given by Marion Merrell Dow Research Institute (Strasbourg, France).

Animal treatments

Male Wistar rats (150–160 g) were subjected to partial hepatectomy (PH) under light ether anaesthesia. The liver, exteriorized through a midabdominal incision, was resected by 70%. Sham-operated animals were treated in an identical manner except that no liver tissue was removed.

An other group of male Wistar rats (250–300 g) was intraperitoneally injected with 1×10^7 Yoshida AH-130 hepatoma cells, that have been collected from rat peritoneum at 7–8 days of tumor growth, i.e. before the entry in the quasi-stationary phase of growth.

Cell cultures

NIH 3T3 fibroblasts were seeded (2×10^6 cells/10 cm diameter dish) and cultured in Dulbecco's-modified Eagle's medium in the presence of 10% horse serum (HS) (Desiderio et al., 1993). To obtain continously dividing cells, as soon as fibroblasts had reached confluence they were diluted (1 : 8, v : v) with fresh medium containing 10% HS and replated (3.3×10^6 cells/dish). After 24 h, part of these plated cells were treated with 100 μM (2R,5R)MAP plus 25 μM (Z)AbeAdo for 48 h. At this time the inhibitor-treated cells were used in part for the biochemical analyses while the remaining were added fresh medium containing 20 μM spermidine plus the two inhibitors, and were harvested to be tested 6 h later.

Morris 7288C rat hepatoma tissue culture (HTC) cells were grown as previously reported (Desiderio et al., 1992).

RNA extraction and Northern blot analysis

Total RNA was extracted from liver and fibroblasts by the method of Chomczynski and Sacchi (1987). Thirty microgram samples of total RNA were fractionated on 1.2% agarose gel containing 6% formaldehyde, and transferred overnight to the nitrocellulose filters. Hybridization was carried out with nick-translated cDNA probes labelled with ^{32}P-dCTP to a specific activity of 5×10^8 cpm/μg DNA (Desiderio et al., 1990).

The following cDNA probes were used: p9.3 for cSAT (Casero et al., 1993) and pHcGAP for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Tso et al., 1985). The autoradiograms were scanned with an LKB Ultroscan laser densitometer (LKB Instruments, Gaithersburg, MD) to determine the relative SAT mRNA amounts, which were normalized against GAPDH values.

Preparation of cytosol and nuclei

As previously reported, cytosolic fractions (Matsui and Pegg, 1980; Erwin et al., 1986) and nuclei (Desiderio, 1992; Desiderio et al., 1992) were prepared from liver tissue and cells.

Assays of acetyltransferase activities

cSAT and nSAT activities were assayed in cytosol and nuclei, respectively, by measuring the conversion of [1-^{14}C]acetylCoA into [^{14}C]acetylspermidine (Matsui and Pegg, 1980; Desiderio et al., 1992a, b). The formation of the acetylderivatives at N^1- and N^8- positions was controlled by HPLC (Desiderio, 1992).

Protein determination

Protein content was determined by the method of Lowry et al. (1951).

Statistical analysis

Data were analyzed by ANOVA at the $p < 0.05$ level of significance.

Results and discussion

The objective of the present study was to determine the expression patterns of cytosolic and nuclear polyamine acetyltransferases in normal and neoplastic growth processes, and to evaluate their relevance, if any, in regulating metabolic events implicated in cell proliferation.

As an *in vivo* model for normal growth stimulation, we chose regenerating liver following PH, since the remaining hepatocytes are synchronously triggered to enter the cell cycle from a resting G0 state. The hypertrophic prereplicative period lasts 12–16 h, the peaks of DNA replication and cell division occurring at 24 h and 30 h respectively (Thompson et al., 1986).

Hepatic cSAT activity increased 4 h after PH (2-fold), peaked at 8 h (2.5-fold) and returned to control value at 16 h remaining, thereafter, largely unchanged until 24 h (Fig. 1A). cSAT activity in the liver of sham-operated rats was similar to that of controls (data not shown). In order to investigate the molecular mechanisms responsible for the increases in cSAT activity, relative cSAT mRNA levels were measured. Figure 1B shows that hepatic cSAT mRNA level rapidly augmented after PH, being in 4 h-regenerating liver (lane 3) 3-fold higher than those of control (lane 1) and sham-operated (lane 2) rat livers. SAT mRNA level declined thereafter, remaining still 1.5-fold higher than the control value at 8 h (lane 4), while diminishing of about 60% under the control at 24 h (lane 5). It is under investigation whether the changes in cSAT mRNA levels observed at early times post-PH depended on increases in trascription-rate of the gene and/or in the stability of the transcript.

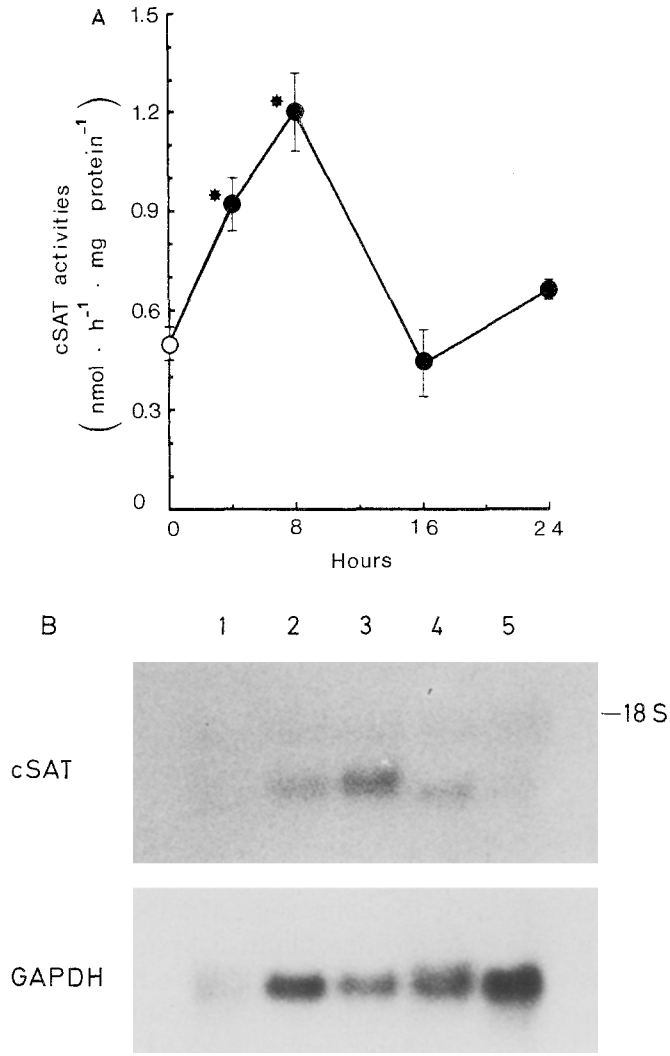

Fig. 1. cSAT activity (**A**) and cSAT mRNA levels (**B**) in regenerating rat liver. Enzyme activity assay, total RNA extraction and Northern blot analysis were performed as reported in Material and methods. Autoradiographic mRNA signals were quantitated by scanning densitometry, and corrected relative to GAPDH mRNA levels. **A** (○), liver of intact animals; (●), regenerating liver. Data are the mean ± S.E. of 5 rats. $*p < 0.01$ vs. control. **B** Lanes: *1*, liver of intact animals; *2*, liver of sham-operated animals; *3*, regenerating liver at 4 h; *4*, regenerating liver at 8 h; *5*, regenerating liver at 24 h

The present results together with previously published data (Desiderio et al., 1990) provide evidence that an interesting parallel occurs between cSAT and ODC inductions during the prereplicative period of liver regeneration, when enhanced expression of the early growth-controlled genes c-*fos* and c-*myc* as well as of p53 antioncogene occurs (Thompson et al., 1986). At variance, nSAT activity increased at later times (24 h post-PH) (Fig. 2) in concomitance with the enhancements of DNA synthesis (Desiderio, 1992), as well as of c-*Ha-ras* and c-*myc* mRNA levels (Thompson et al., 1986). The time-coordinate increases in the expression of polyamine metabolic enzymes and of protooncogenes observed in

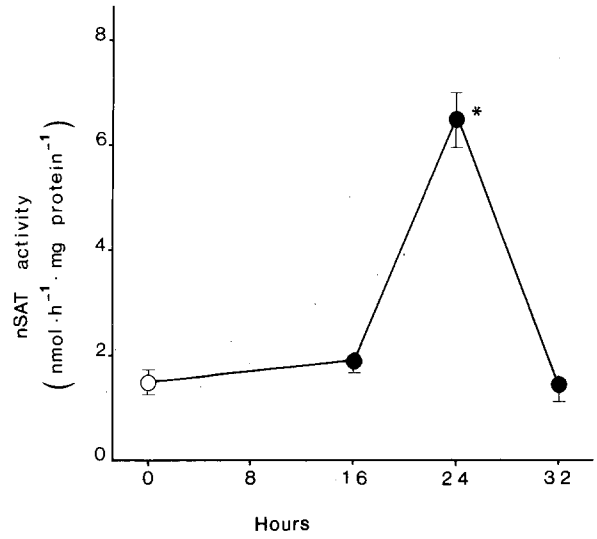

Fig. 2. Time-course of nSAT activity during liver regeneration. Enzyme activity assay was performed as reported in Material and methods. (o), Liver of intact rats; (•) regenerating liver. Data are the mean ± S.E. of 5 rats. *$p < 0.01$ vs. control

regenerating liver lead to hypothesize that polyamines might be involved in the transcription of genes important for cell proliferation.

Similar kinetics of protooncogene expression have been observed in regenerating liver *in vivo* and in 3T3 fibroblasts growth-activated from quiescence *in vitro* (Thompson et al., 1986; Stimac and Morris, 1987). These two systems are comparable also for the expression of adherens junction proteins, which link extracellular matrix to microfilament system (Glück et al., 1992). It has been previously shown using 3T3 fibroblasts rescued from G0 phase that cSAT as well as ODC are growth-controlled (Stimac and Morris, 1987; Desiderio et al., 1993). Rapid enhancements of cSAT expression take place, therefore, when cells are activated from quiescence both *in vivo* and *in vitro*. However, a wide difference exists in the response of nSAT in regenerating hepatocytes versus fibroblasts growth-activated from quiescence, since the acetylation at N^8-position increased in post-PH liver while remaining unchanged after serum-stimulation of quiescent fibroblasts (Desiderio et al., 1993). Separate regulatory signals may be triggered *in vivo* and *in vitro* and/or different forms of nuclear acetyltransferase(s) may exist.

The events occurring during emergence from G0 phase do not seem necessarily identical to those in the G1 phase of the continously dividing cells. ODC activity increases 4-5 h before DNA synthesis in both these experimental conditions (Cress and Gerner, 1980), but different biochemical mechanisms seem likely to be involved. ODC mRNA level also augments (4.5-fold) 6 h after serum-stimulation of confluent resting cells (data not shown), a time that may correspond to the mid G1 phase (Sjöholm, 1993). However, ODC mRNA level is not regulated during the traverse of normal cell cycle (Stimac and Morris, 1987).

Here, we thought interesting to study the expression of cSAT mRNA in continuously dividing fibroblasts to make a comparison with that in fibroblasts

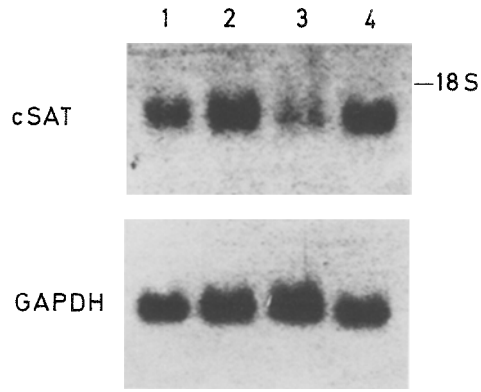

Fig. 3. cSAT mRNA levels in normally cycling fibroblasts. Northern blot analysis of total RNA was performed as reported in Material and methods. Autoradiographic mRNA signals were quantitated by scanning densitometry, and corrected relative to GAPDH mRNA levels. Lanes: *1*, confluent cells; *2*, serum-stimulated cells (6 h); *3*, inhibitor-treated cells; *4*, inhibitor-treated cells added with spermidine (6 h)

stimulated from quiescence. As reported in Fig. 3, when confluent cells (lane 1) were stimulated to proliferate by dilution with fresh medium, cSAT mRNA level doubled within 6 h (lane 2). Similar increases in cSAT mRNA levels were, therefore, observed during the G1 phase both in continuously dividing cells (Fig. 3) and in cells stimulated out from quiescence (Desiderio et al., 1993). These data do not exclude that different regulatory mechanisms are involved. Depletion of polyamine content by treatment of growing cells with 100 μM (2R,5R)MAP plus 25 μM (Z)AbeAdo (Desiderio et al., 1993), caused a strong reduction of cSAT mRNA level (Fig. 3, lane 3). To better understand the role of polyamines in normal cell growth through the control of cSAT expression, spermidine was added to inhibitor-treated polyamine-depleted cells to replenish polyamine pool (Pegg, 1988; Desiderio et al., 1993). Since SAT mRNA level triplicated 6 h after spermidine treatment (Fig. 3, lane 4), it can be suggested that polyamines exert a control on SAT expression in cells rescued from resting state (Desiderio et al., 1993).

The data obtained by examining normal proliferation processes suggested us to investigate the patterns of the two acetyltransferases in tumors, to asses whether these enzymes may have a role in neoplastic growth. We examined two rapidly proliferating and poorly differentiated hepatocellular carcinomas, such as the Yoshida AH-130 growing *in vivo* and the Morris 7288C cultured *in vitro*.

Yoshida AH-130 hepatoma cells grow exponentially after transplantation in rat peritoneum almost until day 5. The cell growth-rate diminishes thereafter, reaching a quasi-stationary phase of growth between days 10 and 14. As reported in Fig. 4, nSAT activity was elevated (2-fold) in Yoshida AH-130 hepatoma cells at day 5, and declined to the value of normal liver between days 10 and 14. In host liver nSAT activity was unchanged during all the observation period relative to control liver. Preliminary data indicate that cSAT activity remarkably

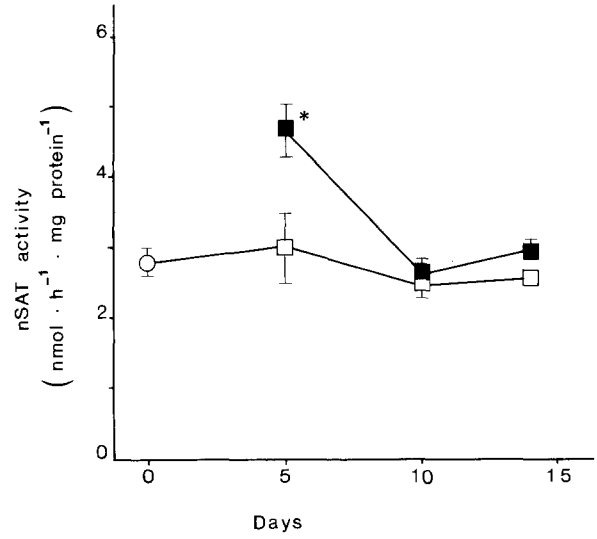

Fig. 4. Time-course of nSAT activity during intraperitoneal growth of Yoshida AH-130 hepatoma cells. Enzyme activity assay was performed as reported in Material and methods. (○) Liver of intact rats; (□) host liver; (■) Yoshida AH-130 hepatoma cells. Data are the mean ± S.E. of 5 rats. $*p < 0.01$ vs. control

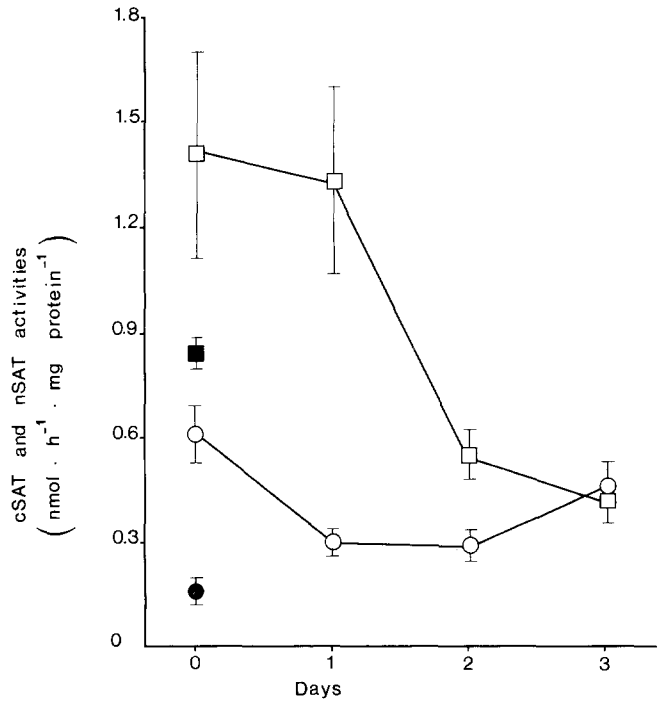

Fig. 5. Pattern of cSAT and nSAT activities in HTC cells growing *in vitro*. Enzyme activity assays were performed as reported in Material and methods. cSAT activity of control liver (●) and of hepatoma cells (○); nSAT activity of control liver (■) and of hepatoma cells (□). Data are the mean ± S.E. of 3 experiments

augmented in hepatoma cells between 10 and 14 days of tumor growth (data not shown).

Figure 5 shows cSAT activity pattern during the 3 day-logarithmic growth period of HTC cells, which was induced by serum-treatment of cells at confluence. cSAT activity sharply declined during the first day of logarithmic growth, compared with that of confluent cells, and returned toward starting values only at the third day of growth. nSAT activity behaved differently, since it remained at plateau for 1 day rapidly declining thereafter (Desiderio et al., 1992). The two SAT activities in tumor cells were higher than in normal liver (Fig. 5).

The different patterns of cytosolic and nuclear polyamine acetyltransferases during growth of hepatoma cells suggest that nSAT may be implicated in early biochemical events responsible for rapid neoplastic cell proliferation, whereas cSAT enhancement may partecipate in maintaining optimal levels of polyamines when the cells reach high density *in vivo* or confluence *in vitro*. It remains to investigate whether the induction of cSAT favours polyamine excretion under acetylated form, preventing a deleterious effect of these polycations on the growth of neoplastic cells likely affected by the cachectic state established in the host.

Acknowledgements

We are grateful to Dr. A. E. Pegg (University Hospital the Milton S. Hershey Medical Center, Hershey, Pennsylvania) for the p9.3 probe. This paper was supported by grants of the Ministero dell' Università e della Ricerca Scientifica e Tecnologica (MURST), and of Consiglio Nazionale delle Ricerche (CNR), Finalized Project ACRO 1993, sub-project 2, Contract no. 133323, Rome, Italy.

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Received August 25, 1993