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Pyrimidine Salvage Pathway and Their Effects on Breast
Cancer Therapy

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13. ABSTRACT (Maximum 200 Words) This research project has been focused on the elucidation of the mechanisms affecting the therapeutic efficacy of fluoropyrimidines to improve the clinical outcome of breast cancer patients. Based on the findings of the last scientific year that the nullification in ES cells of uridine phosphorylase (UPase) leads to a significantly increased cell resistance to fluoropyrimidines and that wild-type p53 protein down-regulates UPase expression, we have focused our research on the following two areas: 1) Elucidation of the p53 regulation mechanism(s) of UPase expression; In this investigation, we demonstrated that wild-type p53 protein represses UPase gene expression via sequence-specific DNA binding at promoter level, and that the mutations of p53 leads to loss of this regulatory function. 2) In vivo study of UPase function in fluoropyrimidine metabolism and uridine regulation. In order to better translate the basic research understanding to the clinical service, we have extended our first year's findings on UPase knockout ES cell to in vivo study through generation and use of UPase knockout mouse model. The research results defined the important role in vivo of UPase in fluoropyrimidine metabolism and uridine regulation in plasma and tissue.				
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Introduction

This research project has been aimed to improve the fluoropyrimidine therapeutic index through the investigation of the function and regulatory mechanisms of uridine phosphorylase (UPase), an enzyme induced in various human and rodent tumors. UPase can reversibly convert uridine to uracil, and therefore, may play an important role in the metabolism of pyrimidine and the activation of fluoropyrimidines. During past three years, we have reached considerable achievements in this project: 1) We have confirmed the important contribution of UPase in fluoropyrimidine activation in both UPase knockout ES cell and mouse models, 2) We have determined the important role of UPase in the regulation of uridine homeostasis in plasma and tissues (cells), 3) We have elucidated the p53 regulatory mechanism of UPase expression and the effect of this regulation on pyrimidine salvage pathway activity, and 4) We have initially characterized the regulatory mechanisms of cytokines on UPase expression. These findings provide the important information on the mechanism of fluoropyrimidine action, the modulation of host toxicity, and the development of new bio-modulating agents.

Body

A. Scientific accomplishments

1. UPase is an important enzyme in the activation of fluoropyrimidines

1.1. Disruption of UPase gene in mouse embryonic stem (ES) cells indicates its importance in fluoropyrimidine activation. Please see reprint in appendix 2.

1.2. The role of UPase in fluoropyrimidine activation is confirmed in the knockout mouse model.

In vivo studies will provide more useful information for the clinical management of patient, compared to the in vitro study in cultured cells. With the encouraging finding in murine ES cells, we further extended our research interest into the in vivo level by generating UPase knockout mice and evaluating the antiproliferative activity in this mouse model. A manuscript on this topic is in preparation.

i). Generation of UPase knockout mice

Selected UPase +/- ES cells have been introduced into C57BL/6J blastocysts, which then completed their development in pseudopregnant C57BL/6J host. The chimeric offsprings identified

by coat colors have been mated with C57BL/6J to generate the F1 UPase +/- mice. F2 UPase +/+ and UPase -/- littermates are produced from F1 UPase +/- males and females and used in the experiments. The genotype of mice has been defined by Southern blot analysis (Figure 1) and confirmed by detection of its products using reverse transcription polymerase chain reaction (RT-PCR) (Figure 2) and enzyme activity assay (Table I). Our data show that in the major organs investigated of UPase -/- mice the uridine phosphorolytic activity is completely abrogated. Uridine kinase (UK) is induced in spleen, liver, and kidney (30.6%, 60.9%, and 73.1% respectively), as shown in ES cells. However, no enzymatic activity changes in other organs we observed. OPRase activity was not altered (Table I).

ii). Resistance of UPase knockout mice to fluoropyrimidines

Due to its obvious clinical significance, it has been our main goal to determine the effect of UPase on fluoropyrimidine activity. Thus we have determined the response of UPase knockout mice to 5-fluorouracil (5-FU), capecitabine, and its intermediate metabolite 5'-deoxy-5-fluorouridine (5'DFUR), respectively. As showed in Figure 3, 85 mg/kg of 5-FU (i.p. weekly) represents the maximum tolerated dose (MTD) in UPase +/+ mice causing 15-20% weight loss within 3 weeks. A higher dose level at 100 mg/kg results in the rapid death of the animals after a single dose of 5-FU. In UPase -/- mice, however, 100 mg/kg of 5-FU did not cause any toxic effect even after the administration of the 5th dose. Higher doses up to 200 mg/kg of 5-FU caused toxicity comparable to the one observed for the 85 mg/kg in UPase +/- mice. The effect of UPase-knockout on activity of 5'DFUR and capecitabine is more significant (Figure 4-6). 500 mg/kg of 5'DFUR (oral daily, 5 days a week) resulted in the death of 3 in 6 mice on day 3 in UPase +/+ mice, due to severe gastrointestinal toxicity. In UPase -/- group, however, no significant toxicity and animal death occurred till day 7 when we ended our observation (Figure 4). The abrogation of UPase also provides a significant host protection from capecitabine toxicity. At dose of 1000 mg/kg (oral daily, 5 days a week), wild type mice showed 20% weight loss during a four weeks period while the weight of UPase knockout mice actually increased (Figure 5) and the histological examinations of small intestines clearly demonstrate the protection of UPase abrogation in these animal lacking UPase activity (Figure 6).

2. UPase plays a key role in the regulation of uridine homeostasis.

Uridine is an efficient bio-modulator of fluoropyrimidine-

induced host toxicity through the protection of RNA from the disturbance of fluorouridine-5'-triphosphate (FUTP), but the clinical utility has been negligible by its rapid clearance. In this study, we have elucidated the molecular mechanism of uridine homeostatic regulation.

We have analyzed the uridine metabolism in UPase knockout mice by HPLC. Our data show a 5 to 6-fold increase in the circulating uridine level in UPase $-/-$ mice and about 2-fold elevation in UPase $+/-$ mice, compared with UPase $+/+$ mice (Table II), indicating the importance of UPase in the regulation of uridine homeostasis. Under physiological condition, uridine degradation occurs through a series of enzymatic reactions initiated by UPase, finally forming β -alanine. To understand the compensate mechanisms of uridine homeostatic regulation in UPase $-/-$ mice, we further examined the uridine concentrations in urine and found a 24-fold increase in UPase $-/-$ mice and a 4-fold increase in UPase $+/-$ mice, compared with UPase $+/+$ mice (Table II). To understand the effect on tissue uridine pools of the uridine elevation in plasma and of the blockage of uridine degradation pathway in tissues, we have also measured the tissue uridine concentrations in brain, heart, lung, liver intestine, spleen, and kidney by HPLC, and the preliminary data have shown up to a 20-fold increase in these tissues (Table II).

To elucidate the metabolic fate of exogenous uridine in UPase $-/-$ mice, we injected (i.p.) a tracer dose (25 μ Ci/mouse) of [3 H]-uridine to UPase $-/-$ mice. The plasma [3 H] uridine was separated by HPLC and determined by liquid scintillation counter (Beckman). As showed in Figure 7, we have observed a very rapid disappearance of [3 H] uridine from the plasma in UPase $+/+$ mice, with a $T_{1/2} < 2$ min. On the contrary, [3 H] uridine $T_{1/2}$ was approximately 15-18 min in UPase $-/-$ mice and a significant amount of radioactivity is still present as [3 H] uridine at 60 min after the administration. To trace the metabolic fate of [3 H] uridine, we have examined the presence of [3 H] uracil by HPLC analysis. At 5 min after the administration, an obvious [3 H] uracil peak is present in UPase $+/+$ mice, but not in UPase $-/-$ mice (Figure 8), indicating the disappearance of [3 H] uridine in UPase $+/+$ mice by rapid phosphorolysis. These data have clearly indicated the important role of UPase in the regulation of uridine homeostasis.

3. UPase expression is down-regulated by wild type p53 and up-regulated by cytokines

i). p53 represses UPase gene expression by sequence-specific DNA binding

We have found and elucidated the mechanism of the negative regulation of p53 on UPase gene expression. Please see the included reprint for more detail (Appendix 2).

ii). Cytokine induction of UPase through IRF-1, NF-kB, AP-1, and SP-1 elements in promoter region

To elucidate the regulatory mechanisms of UPase by cytokines, we have further analyzed the motifs in UPase promoter region, and found some important cytokine-response elements present in this region. To verify the regulatory function of these elements on UPase expression, we have investigated the responses of these elements to the treatment with cytokines in murine cell lines, EMT 6 (a murine breast cancer cell line) and NIH3T3, using a series of promoter-luciferase reporter gene constructs. As shown in Figure 9, cytokines induce UPase gene expression via these elements. Gel shifting assays indicated the DNA specific binding of these elements with the nucleus-extracted proteins (Figure). Further confirmation of their regulatory mechanisms is under investigation.

B. Training accomplishments

In the past years I have attended the 62nd and 63rd Annual Meetings of American Association for Cancer Research (AACR) held in New Orleans (2001) and San Francisco (2002) and will attend the meeting held in Washington, DC in July 2003. I was awarded an AACR-Aventis Scholar-in-Training Award (2002), and an AACR-AstraZeneca Scholar-in-Training Awards in recognition of the promising researches and meritorious submissions. These meetings gave me the chances to meet many excellent scientists and pioneers in my research field. We had very constructive conversations and exchanges of our research experience, which are very helpful for my current research and future development in cancer research.

I attended several seminar series held by Yale Comprehensive Cancer Center. These seminars are usually given by excellent scientists who stand in the frontier in their research fields. I have also attended seminars of experimental therapeutic program held once every two weeks. Through these seminars, I have been exposed to new knowledge and technologies employed in cancer research, helpful for my current research and future scientific career.

Key research achievements

A. UPase is an important enzyme in fluoropyrimidines activation

UPase plays an important role in activation of 5-FU and 5' DFUR/capecitabine, which has been confirmed in both cultured UPase knockout cells and mice.

B. UPase is a key enzyme in the homeostatic regulation of uridine in plasma and tissues

The UPase knockout mice demonstrated severe abnormality in uridine metabolism and disruption in uridine homeostasis. This finding is of significance in the development and clinical use of "uridine rescue" strategy.

C. p53 down-regulates UPase expression via DNA-specific binding

1. Wild-type p53 protein represses UPase gene expression via sequence-specific DNA binding at the promoter level
2. p53 mutation or abrogation abolishes its repressive action on UPase gene expression.

D. Cytokines up-regulate UPase expression through IRF-1, NF-kB, AP-1, and SP-1 regulatory elements

Various cytokines induce UPase expression alone or synergistically. These cytokines include tumor necrosis factor- α (TNF- α), interleukin-1 α (IL-1 α) and interferon- α and γ (IFN- α & γ).

Reportable outcomes

1. Two Scholar-in-Training Awards (AACR, 2002 and 2003)
2. Five reprints
3. Seven abstracts

Bibliography of publications and meeting abstract

1. Publication

- a. Cao, D., Russell, R., Zhang, D., Leffert, J. J., and Pizzorno, G. Uridine phosphorylase (-/-) murine ES cells clarify the key role of this enzyme in the regulation of the pyrimidine salvage pathway and in the activation of fluoropyrimidines. *Cancer Res.* 62: 2313-2317, 2002

- b. Russell, R.L., Cao, D., Zhang, D., Handschumacher, R E., and Pizzorno, G. Uridine phosphorylase association with vimentin. Intracellular distribution and localization. J. Biol. Chem. 276(16): 13302-13307, 2001
- c. Zhang, D., Cao, D., Russell, R., and Pizzorno, G. P53-dependent suppression of uridine phosphorylase gene expression through direct promoter interaction. Cancer Res. 61:6899-6905, 2001
- d. Pizzorno, G., Cao, D., Leffert, J. J., Russell, R. L., Zhang, D., and Handschumacher, R. E. Homeostatic control of uridine and the role of uridine phosphorylase: a biological and clinical update. Biochim. Biophys. Acta. 1587(2-3): 133-144, 2002
- e. Cao, D. and Pizzorno, G. Uridine Phosphorylase: An Important Enzyme in Pyrimidine Metabolism and Fluoropyrimidine Activation. Drugs of Today, Prous Science. In Press.

2. Abstracts:

- a. Cao, D., Handschumacher, R. E., and Pizzorno, G. Uridine phosphorylase plays a crucial role in 5-flurouracil metabolisms and its anti-proliferative activity. In Proceeding of 92nd Annual Meeting of American Association for Cancer Research. New Orleans, LA. March 2001
- b. Zhang, D., Cao, D., Nimmakayalu, M. A., and Pizzorno, G. Expression and regulation of uridine phosphorylase (UPase) appears altered in human tumors: genomic structure, chromosome mapping, and promoter characterization of the human UPase gene. In Proceeding of 92nd Annual Meeting of American Association for Cancer Research. New Orleans, LA. March 2001
- c. Russell, R., Cao, D., and Pizzorno, G. Uridine phosphorylase is an actin binding protein that can alter actin polymerization dynamics in vitro. In Proceeding of 92nd Annual Meeting of American Association for Cancer Research. New Orleans, LA. March 2001
- d. Cao, D., and Pizzorno, G. Effect of uridine phosphorylase abrogation on uridine metabolism and fluoropyrimidine anti-proliferative activity. In Proceeding of 93rd Annual Meeting of American Association for Cancer Research. San Francisco CA. April 2002

- e. Cao, D., Zhang, D., Leffert, J. J., and Pizzorno, D. p53 suppression of uridine phosphorylase gene expression and the key role of this enzyme in the activation of fluoropyrimidines. ERA OF HOPE 2002. Department of Defense Cancer Research Program Meeting. Orlando, Florida. September 2002
- f. Cao, D., Leffert, J. J., and Pizzorno, G. Uridine phosphorylase deficient mice display disruption of the homeostatic uridine metabolism and resistance to fluoropyrimidines. In Proceeding of 94th Annual Meeting of American Association for Cancer Research. Washington DC. July 11-14 2003
- g. Wan, L., Cao, D., Leffert, J.J, Diasio, R., Johnson, M., and Pizzorno, G. Uridine phosphorylase activity in human tumor tissues, its correlation with Thymidine phosphorylase expression and its effect on patient prognosis. In Proceeding of Annual Meeting of American Association for Cancer Research. Washington DC. July 11-14 2003

Personnel receiving pay

Deliang Cao

Conclusion

During three years or the support by this award, we have elucidated the important role of UPase in fluoropyrimidine anticancer activity through the UPase knockout cell and mouse models. We have uncovered and confirmed the important function of UPase in the regulation of uridine homeostatic regulation and pyrimidine metabolism. We have elucidated the regulatory mechanism of p53 on UPase gene expression and confirmed that mutant p53 protein could lose its repressive function on UPase gene expression. We have also initially elucidated the mechanisms of cytokine induction of UPase gene expression. These molecular events significantly impact the fluoropyrimidine antitumor activity through the effect on UPase gene expression. Therefore, this study will benefit the management of clinical cancer patients.

Reference

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Appendices

1. Figures and tables
2. Reprint
3. Meeting abstract
4. Awards: AACR-Aventis Scholar-in-Training Award
AACR- AstraZeneca Scholar-in-Training Award

APPENDIX I: FIGURES AND TABLES

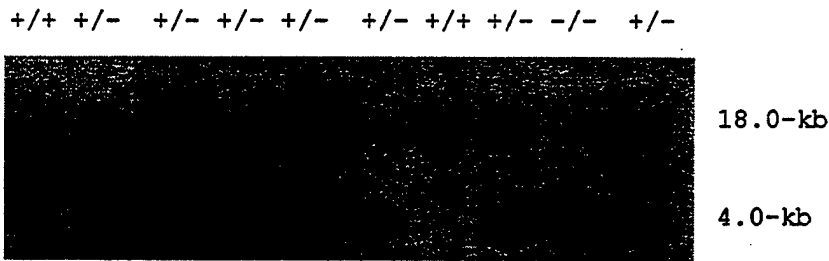


Figure 1 Genotyping analysis of one litter of F2 mice obtained from a cross between two UPase +/- mice. Tail genomic DNA was prepared and digested by *Bam*HI. The blot was hybridized with 0.6-kb genomic DNA probe. *Left*, molecular size marker.

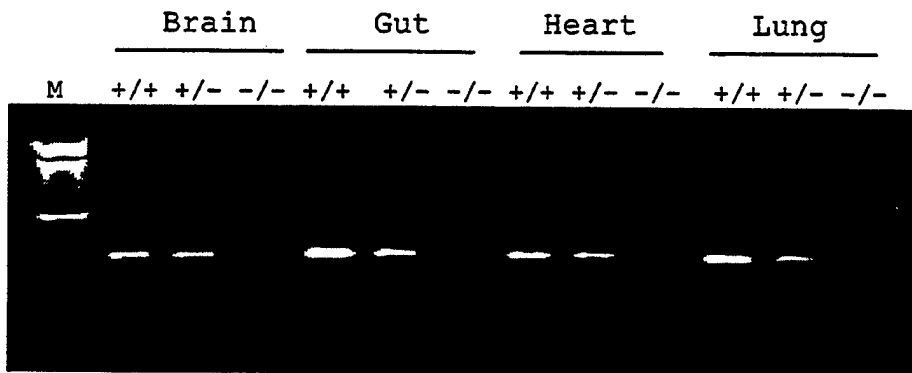


Figure 2 RT-PCR analysis of UPase gene mRNA isolated from organs of UPase +/+, UPase +/-, and UPase -/- mice. The forward primer (5'GCTCTTCCCGGATGAACACC) is located in exon 5, the targeted region, and the reverse primer (5'CGCCTGAAGTGCCAATGC) is in exon 6. There is no UPase mRNA present in the organs from UPase -/- mouse.

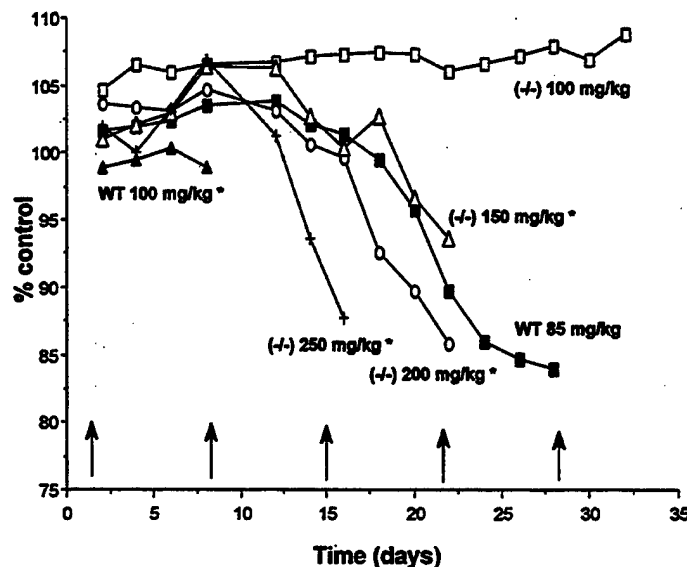


Figure 3 Response of UPase $-/-$ mice to 5-FU administrated weekly. The drug was administered once a week i.p. (arrows) at the indicated doses. The mice were weighed every other day, and the number represents the average of 6 mice in each treatment group. The observation was terminated in each group after the first animal death.

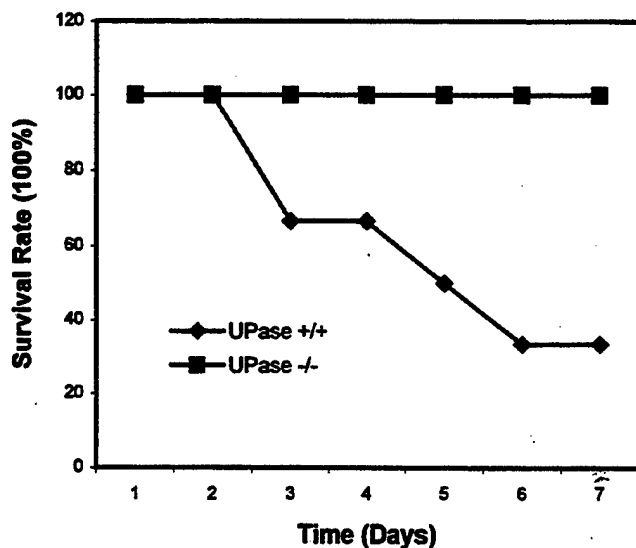
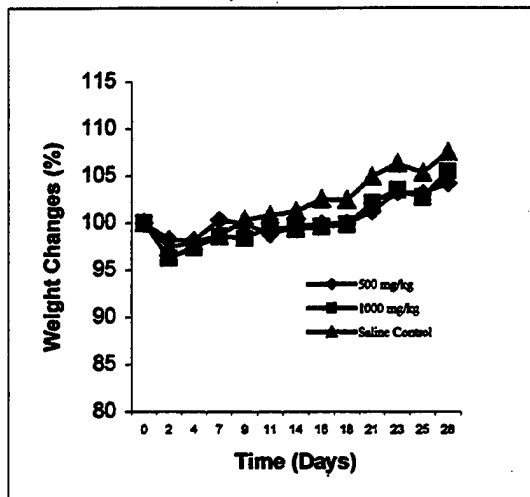
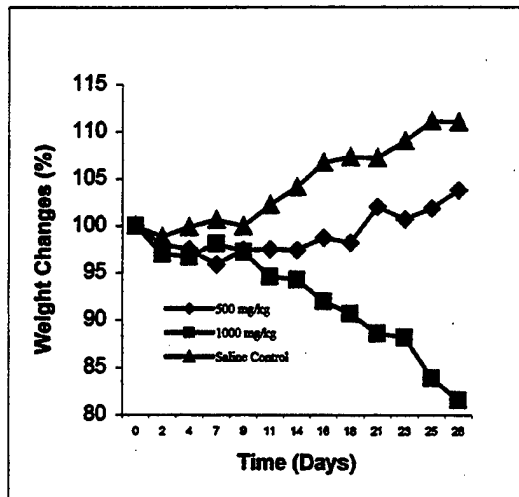


Figure 4 Effect of 5'-Deoxy-5-fluorouridine (5'DFUR) on UPase $-/-$ mouse survival. 500 mg/kg of 5'DFUR was orally delivered daily, and the mouse survival was checked every day. The wild type mouse death occurred after dose 3 while no toxicity appeared in UPase $-/-$ mice.



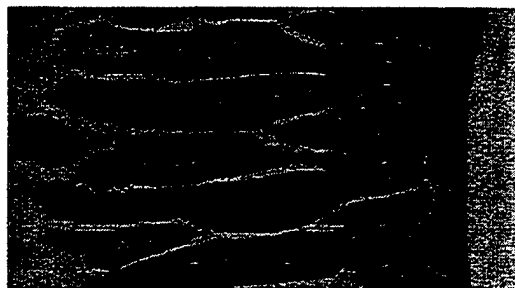
UPase -/- Mice



Wild Type Mice

Figure 5 The effect of capecitabine on UPase -/- mouse weight. 1000 mg/kg of capecitabine was orally administered daily, 5 days a week. The value represents the average of 6 mice.

UPase -/-

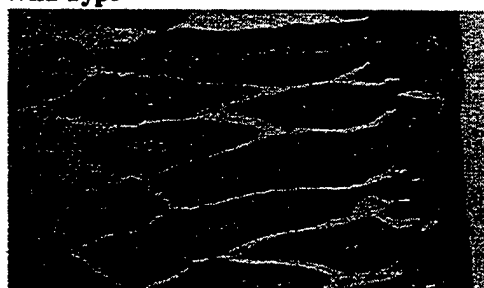


(Control)

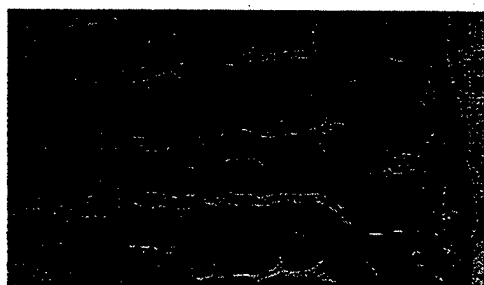


(1000 mg/kg Capecitabine)

Wild Type



(Control)



(1000 mg/kg Capecitabine)

Figure 6 The protection of UPase nullification on murine small intestine. In wild mice, 1000 mg/kg of capecitabine delivered orally daily led to cell death in the crypts and the damage of the regeneration layer, while no obvious changes seen in UPase -/- mice.

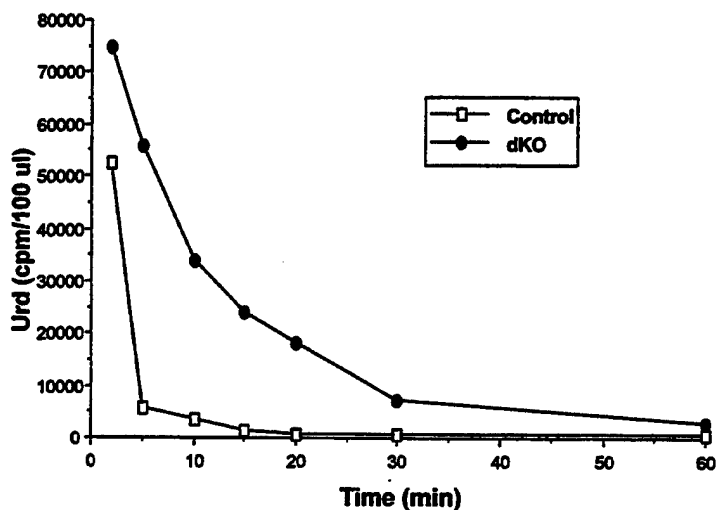


Figure 7 Clearance of exogenous [^3H]-Uridine. A trace dose (25 μCi) of [^3H] uridine was administered (i.p.) to UPase +/+ (control) and -/- (dKO) and the presence of plasma [^3H] uridine was determined by HPLC analysis at the indicated time points. The half-life of [^3H] uridine in UPase -/- is much longer than that in UPase +/+.

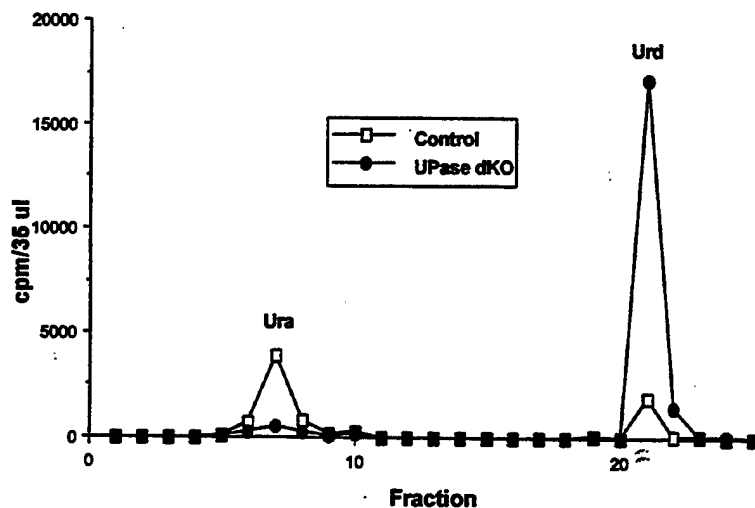


Figure 8 Metabolic fate of [^3H] Uridine. Blood samples were collected after [^3H] uridine administration by i.p. and analyzed by HPLC. An obvious uracil peak is shown in UPase +/+ mice at 5 min after administration, but not in UPase -/- ones.

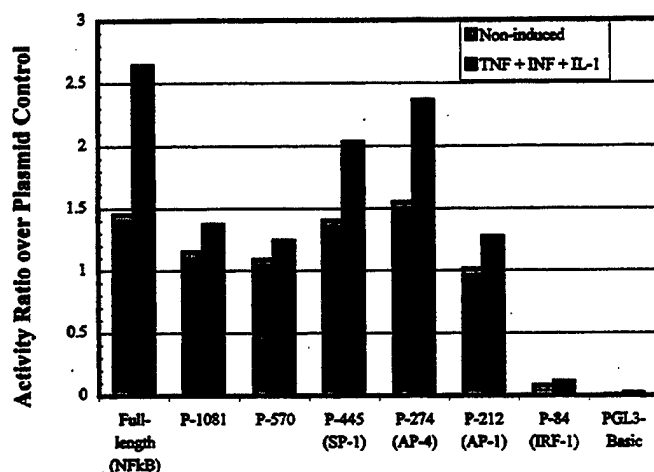


Figure 9 Cytokine induction of UPase promoter activity. Scheduled deletion of UPase promoter altered the promoter response to cytokines upon the presence of the regulatory elements as indicated.

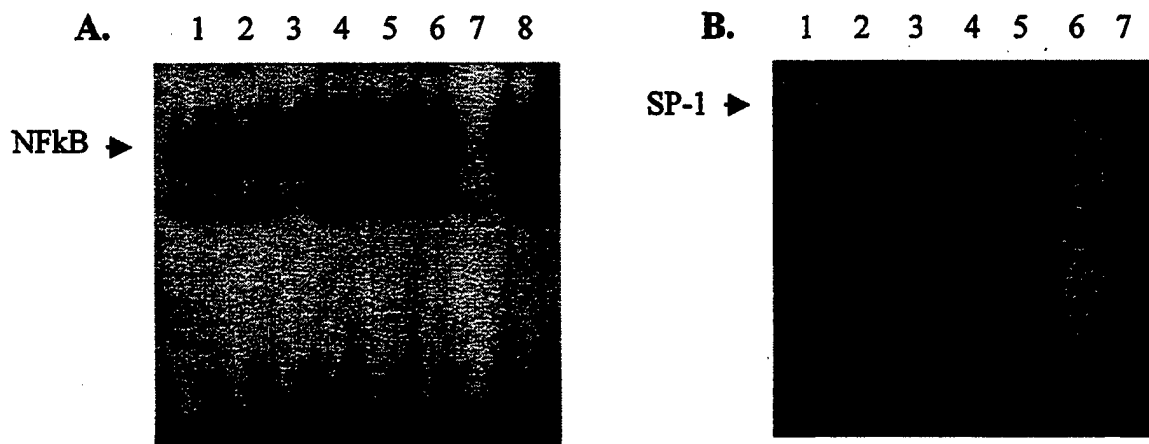


Figure 10 Electrophoretic mobility-shift assay (EMSA) of NFkB (A) and SP-1 (B). Two (NFkB) or five µg (SP-1) nuclear extract from EMT-6 cells were used, after treating with different cytokines for 24 hours: Panel A. 1. Control, 2. IL-1-alpha, 3. INF-gamma, 4. TNF-alpha, 5. TNF-alpha + IFN-gamma, 6. TNF+IFN+IL-1 (3T), 7. 3T + cold probe, and 8. 3T + non-specific competitor. Panel B. 1. Control, 2. TNF-alpha, 3. TNF+ cold probe, 4. TNF + non-specific competitor, 5. IL-1 alpha, 6. IL-1-alpha + cold probe, and 7. IL-1 alpha + non-specific competitor.

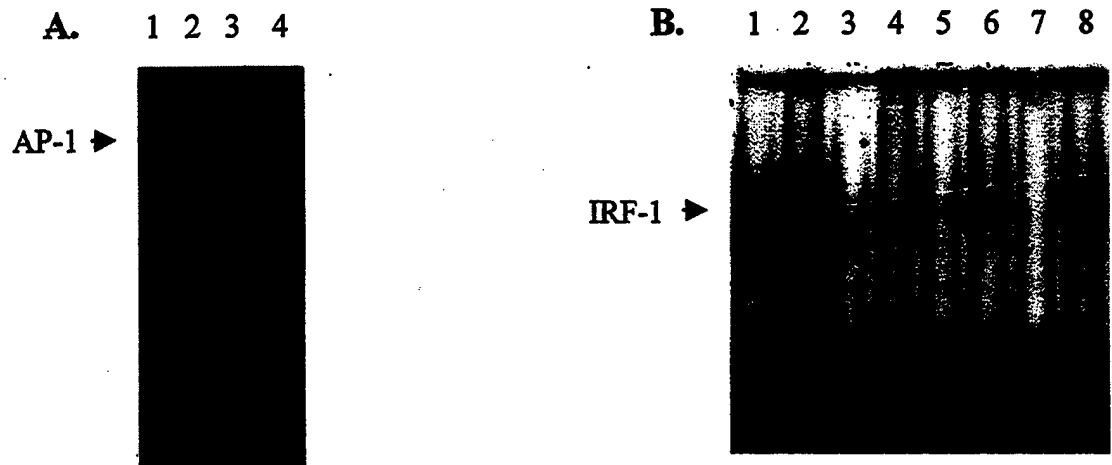


Figure 11 Electrophoretic mobility-shift assay (EMSA) of AP-1 (A) and IRF-1 (B). 5µg nuclear extract from EMT-6 cells was used, which was treated with different cytokines for 24 hours: Panel A. 1. Control, 2. IL-1-alpha, 3. IL-1-alpha + cold probe, 4. IL-1-alpha + non-specific competitor. Panel B. 1. Control, 2. IL-1-alpha, 3. IFN-gamma, 4. TNF-alpha, 5. TNF + IFN, 6. TNF + IFN + IL-1 (3T), 7. 3T + cold probe, and 8. 3T+ non-specific competitor.

Table I Activity of pyrimidine enzymes in UPase -/- mice (nmol/mg/h)

	-/-				+/+			
	UPase	TPase	UK	OPRTase	UPase	TPase	UK	OPRTase
Brain	DN	ND	3.83	1.51	6.52	ND	3.90	1.48
Lung	ND	ND	2.78	ND	37.36	ND	2.72	ND
Heart	ND	ND	ND	ND	0.12	ND	ND	ND
Liver	ND	18.72	1.48	1.34	4.64	16.64	0.92	1.72
Spleen	ND	ND	7.24	ND	5.12	ND	5.56	ND
Gut	ND	3.84	ND	ND	576.80	4.24	ND	ND
Kidney	ND	ND	2.77	ND	32.63	ND	1.60	ND

Table II. Uridine levels in plasma, urine, and tissues of UPase -/- mice

	Plasma	Urine	Brain	Lung	Heart	Liver	Spleen	Gut	Kidney
UPase +/+	5.5	10.1	84.0	2.9	17.6	143.6	75.2	185.7	232.0
UPase +/-	12.3	42.1	ND	ND	ND	ND	ND	ND	ND
UPase -/-	32.3	240.4	193.4	58.2	213.7	181.3	235.0	390.2	340.4

Uridine Phosphorylase (-/-) Murine Embryonic Stem Cells Clarify the Key Role of this Enzyme in the Regulation of the Pyrimidine Salvage Pathway and in the Activation of Fluoropyrimidines¹

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ABSTRACT

We have reported the elevation of uridine phosphorylase (UPase) in many solid tumors and the presence of a variant phosphorolytic activity in breast cancer tissues (M. Liu *et al.*, *Cancer Res.*, 58: 5418-5424, 1998). To better understand the biological and pharmacological significance of these findings, we have developed an UPase gene knockout embryonic stem (ES) cell model by specific gene targeting techniques. In this cellular model, we establish the critical role of UPase as an important anabolic enzyme in 5-fluorouracil (5-FU) activation and pyrimidine salvage pathway regulation. It has long been known that UPase regulates the plasma concentration of uridine; however, little is known of the role of UPase in the activation and metabolism of 5-FU and its derivatives, mainly because of the lack of an appropriate model system. The experimental data indicate that the disruption of UPase activity in murine ES cells leads to a 10-fold increase in 5-FU IC₅₀ and a 2-3-fold reduction in its incorporation into nucleic acids, whereas no differences in toxicity is seen with other pyrimidine nucleoside analogues such as 5-fluorouridine, 2'-deoxy-5-fluorouridine, and 1-β-D-arabinofuranosylcytosine compared with WT (wild-type) ES cells. Benzylacetyl-L-aspartic acid can specifically prevent the WT ES cells from the sensitivity of 5-FU. Our data also shows the effect of UPase on the cytotoxicity of 5'-deoxy-5-fluorouridine (5'DFUR), a 5-FU prodrug. The IC₅₀ is increased almost 16-fold in the knockout cells compared with the wild type cells, demonstrating the role of UPase in catalyzing the conversion of 5'DFUR to 5-FU. These findings additionally elucidate the tumor-specific selectivity of capecitabine, the oral fluoropyrimidine prodrug approved for the treatment of metastatic breast and colorectal cancers.

Not only do the knockout cells present a decreased incorporation of 5-FU into nucleic acids but also an increased reliance on the pyrimidine salvage pathway. The reduced dependence of UPase knockout cells on the pyrimidine *de novo* synthesis is reflected in the apparent resistance to phosphonacetyl-L-aspartic acid, a specific inhibitor of pyrimidine pathway, with a 5-fold elevation in its IC₅₀ in UPase-nullified cells compared with WT. In summary, we have successfully generated an UPase gene knockout cell model that presents reduced sensitivity to 5-FU, 5'DFUR, and phosphonacetyl-L-aspartic acid, although it does not affect the basic cellular physiology under normal tissue culture conditions. Considering the role of UPase in 5-FU metabolism and the elevated expression of this protein in cancer cells compared with paired normal tissues, additional investigation should be warranted to firmly establish the clinical role of UPase in the tumor selective activation of 5-FU and capecitabine.

INTRODUCTION

5-FU³ still represents one of the most active antitumor agents in the treatment of solid tumors such as breast, colon, and head and neck

cancers. Two main mechanisms of action contribute to the cytotoxic effect of 5-FU: (a) DNA-directed toxicity, where the formed 5-fluorodUMP tightly binds to thymidylate synthetase, resulting in inhibition of DNA synthesis and cell growth with a minor role played by DNA incorporation of the fluorodeoxynucleotides leading to the fragmentation of DNA and cell death; and (b) RNA-directed cytotoxicity with 5-FU incorporation into various RNA species, including polysomal RNA, nuclear RNA, and mRNA, thereby disrupting RNA maturation and functions (1-3).

Whereas the mechanisms of action have been well established, the contribution of the different pathways to 5-FU activation is still controversial because of the lack of an appropriate model system. 5-FU can be converted to 5-FUMP via the OPRTase pathway in the presence of phosphoribosyl PP_i or activated to 5-fluorouridine first and then to 5-FUMP via the UPase-kinase salvage pathway in the presence of R-1-P. 5-FU can also be converted to 5-fluorodeoxyuridine by TPase and then to 5-fluoro-dUMP by TK (1, 4). Some investigators have proposed that OPRTase plays a main role in the activation of 5-FU because of the limited pool of R-1-P available in the cells (5). However, Schwartz *et al.* (6) indicated that UPase is a critical enzyme in activation of 5-FU. This controversy arises from the presence of both UPase and OPRTase in the experimental models investigated thus far.

The clinical effectiveness of 5-FU is limited by its severe side effects such as myelosuppression, thrombocytopenia, and gastrointestinal lesions. Uridine has been used to reduce 5-FU toxicity leading to an increased therapeutic index (7). Several preclinical studies and clinical trials have demonstrated the ability of uridine to selectively protect normal tissues from 5-FU host toxicity. However, the clinical use of uridine rescue is hampered by its rapid clearance via degradation initiated by UPase in liver and dose-limiting toxicities resulting from high dose administration of uridine necessary to obtain the desired concentration for tissue protection. BAU, developed as an inhibitor of UPase, has been shown to be able to increase plasma uridine concentration by conserving endogenous uridine leading to similar protection of normal tissues (8-10). Finally, UPase was found to be elevated in most human tumors, and we have identified variant forms of UPase, particularly in breast tumors with various degrees of insensitivity to BAU providing the rationale for the increased selectivity of 5-FU-based therapy in these tumors (11). Currently, we have developed an UPase knockout ES cell model through gene targeting technology. Because of its clear genetic background, this model will provide a significant tool to elucidate the biological function of UPase and its role in 5-FU activation and uridine metabolism in cells. Here we report the effects of UPase disruption on pyrimidine metabolism and 5-FU antiproliferation.

MATERIALS AND METHODS

Cell Culture. Undifferentiated WT 129/JV ES murine cells and UPase knockout clones were maintained in gelatinized tissue culture flasks with high

phosphoribosyl-transferase; R-1-P, ribose-1-phosphate; TPase, thymidine phosphorylase; MUP, murine UPase; NEO, neomycin resistance; TK, thymidine kinase; WT, wild-type.

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³ The abbreviations used are: 5-FU, 5-fluorouracil; UPase, uridine phosphorylase; ES, embryonic stem; BAU, benzylacetyl-L-aspartic acid; 5'DFUR, 5'-deoxy-5-fluorouridine; PALA, phosphonacetyl-L-aspartic acid; FUMP, fluorouridine monophosphate; OPRTase, orotate

glucose DMEM supplemented with 15% heat-inactivated fetal bovine serum, 2 mM glutamine, 0.1 mM β -mercaptoethanol, and 1000 units/ml of recombinant leukemia inhibitory factors (Life Technology, Inc., Grand Island, NY) at 37°C and 5% CO₂.

Construction of MUP Gene Targeting Vector and Selection of UPase Mutants. A 8.5-kb *EcoRI-XhoI* genomic DNA fragment containing the whole MUP gene (12) was subcloned into pBluescript KS II vector (Stratagene, La Jolla, CA). To disrupt UPase gene, we replaced with a 1.6-kb NEO gene (a positive selection marker) expression cassette a 2.5-kb fragment of UPase gene, which includes the 3' part of intron 3, the whole exon 4 and intron 4, and the 5' part of exon 5. The MUP/NEO fragment was subsequently inserted into a vector containing herpes simplex virus TK gene cassette, a negative selection marker, to exclude the nonrecombination (nontargeted) mutants, generating the targeting construct (Fig. 1).

The linearized MUP/NEO/TK Bluescript targeting construct was introduced by electroporation into 129/JV ES cells and the clones doubly selected by G418 and ganciclovir for the presence of NEO gene and absence of TK gene. The ES clones carrying the mutant UPase gene were identified by PCR and Southern blot. Double knockout cells (two alleles disrupted) were generated by exposing the clones to high concentrations of G418 up to 5.5 mg/ml (13).

Western Blot Analysis. ES cells were solubilized in 2× SDS gel loading buffer [50 mM Tris-Cl (pH 6.8), 100 mM dithiothreitol, 2% (w/v) SDS, 0.1% bromophenol blue, and 10% (v/v) glycerol], and the lysate was separated on a 15% SDS-PAGE and then transferred to polyvinylidene difluoride membrane (Hybond P; Amersham). UPase was detected using a polyclonal anti-UPase antibody generated in our laboratory diluted 1:100 in casein buffer [5% casein, 0.05% Triton X-100, 0.3 M NaCl, 50 mM citric acid, 0.3 M Tris base (pH 7.6)]. The membrane was reprobed by commercial antiactin monoclonal antibody to evaluate the amount of the protein loaded.

Cell Growth and Drug Sensitivity Assay. Cell growth rate was measured using a Cell Proliferation kit (2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-2H-

tetrazolium-5-carboxanilide inner salt; Boehringer Mannheim, Indianapolis, IN) in 96-well plates and absorbance at 500 nm, indicating the viable cell number, was determined with a Titertek Multiskan MCC340 (Huntsville, AL) microplate reader, using A⁷⁵⁰ as an internal control. The drug sensitivity assays were performed similarly as above. Next, 3000 cells/well were plated in gelatinized 96-well tissue culture plates. After overnight incubation, cells were treated with different drugs and harvested at indicated time points. Each concentration point was replicated in six wells, and all of the experiments were repeated at least twice.

Enzyme Activity Assays. UPase activity was measured by uridine conversion to uracil using a Tris-HCl lysate (11) incubated with 200 μ M [³H]uridine and 1 mM potassium phosphate. The UPase product, [³H]uracil, was separated on silica TLC plates (Kieselgel 60; Merck) using an 85:15:5 mixture of chloroform, methanol, and acetic acid, respectively. Protein amounts were determined with protein assay dye (Bio-Rad Laboratories, Hercules, CA). TPase activity was similarly analyzed using 200 μ M [³H]thymidine as a substrate (11).

OPRTase activity was assayed by measuring the conversion of [¹⁴C]fluorouracil into FUMP in the presence of 200 μ M [¹⁴C]fluorouracil, 1 mM phosphoribosyl PP_i, and 100 μ M MgCl₂ (3).

Uridine kinase was determined by the conversion of [³H]uridine into [³H]UMP with 200 μ M [³H]uridine, 1 mM ATP, and 100 μ M MgCl₂ (3).

R-1-P concentration was measured in the presence of 50 μ M [¹⁴C]fluorouracil and 5 μ g pure recombinant UPase protein followed by TLC separation as indicated above (11).

Incorporation of [³H]5-FU and [³H]Uridine into Nucleic Acids and Measurement of Ribonucleotide Triphosphate Pools. Cells (5 × 10⁵/flask) were incubated for 24 h in medium containing [³H]5-FU (5 μ M) or [³H]uridine (2 μ M), washed twice with cold PBS, and the macromolecular precipitated with 15% trichloroacetic acid. The final precipitates were dissolved in tissue solubilizer (TS-1) and the radioactivity determined.

The cell supernatants obtained with the TCA precipitation were neutralized 1 N triethylamine in freon. The nucleoside triphosphates were eluted isocratically on an high-performance liquid chromatography anion exchange column (Partisil-10-SAX; Altex) using 0.4 M NH₄H₂PO₄ (pH 4.5) as mobile phase.

RESULTS

Targeted Disruption of the MUP Gene and Abrogation of UPase Expression in ES Cells. To disrupt the MUP gene and abrogate the expression of its functional product, we replaced a 2.5-kb fragment of the UPase gene, which contains the 3' part of intron 3, the whole exon 4 and intron 4, and the 5' part of exon 5, with a 1.6-kb NEO gene cassette, leading to a 92 and 1/3 amino acid deletion, and a shifting mutation of downstream codons (out of frame). After a TK gene expression cassette was flanked at the 3' end of the UPase gene fragment, the linearized targeting construct was electroporated into murine 129/JV ES cells. The transfected cells were then exposed to G418 and ganciclovir to select for the targeted UPase mutant clones. Analysis of 38 survival clones by PCR and Southern blot hybridization indicated that 13 of them underwent a correct homologous recombination, resulting in the targeted UPase gene disruption (data not shown). Clone DL362 containing the single allele UPase mutation was expanded and exposed at 5.5 mg/ml G418 for 2 weeks to select for UPase double allele knockout clones (13). The resultant 50 clones were subjected to Southern blot analysis, and 5 of them were found to have the targeted disruption at both alleles (Fig. 1).

The expression products of the disrupted UPase gene were evaluated in two double knockout clones, DL16 and 22, and their parental single knockout cell clone DL362. A WT parental 129/JV ES clone was also used as a control. A 600-bp UPase cDNA probe, corresponding to exons 3–7, identified a 1.4-kb UPase RNA in WT and single knockout cells. This RNA species was not detectable in the two double knockout clones DL16 and 22. The abundance of UPase mRNA in single knockout cells was ~50% of the WT control cells indicating that the UPase gene disruption results in the reduction of

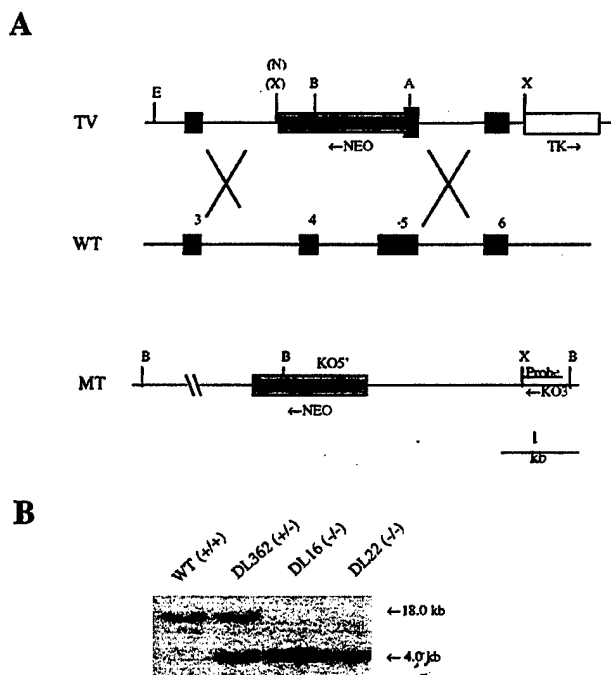


Fig. 1. Targeted disruption of UPase locus. **A**, partial restriction map of UPase locus in genome, targeting vector, and the targeted allele. A 2.5-kb genomic fragment of MUP (*NheI*-*Apal*) was replaced by a 1.6-kb NEO cassette. TK gene cassette was flanked at 3' end. Arrows indicate the orientations. TV, targeting vector; WT, wild type allele; and MT, mutant allele. A, *Apal*; B, *Bam*HI; E, *Eco*RI; (N), *Nhe*I and (X), *Xba*I, both were blunted; and X, *Xho*I. KOS' (5'CGGCCTTATACATGGCGTAGCG) and KOS3' (5'GTGATG-GTTTTCAAGGTCCTTGC) are primers for PCR screening of mutants. **B**, Southern blot analysis of the UPase gene locus. The genomic DNA was digested by *Bam*HI, and the blot was hybridized with the 600-bp PCR fragment immediately outside of *Xho*I cloning site. The WT band is ~18 kb, and the length of the disrupted allele is 4 kb, because of the introduction of a *Bam*HI site in NEO cassette. Left, molecular size markers. Lane 1, WT; Lane 2 single knockout clone; and Lanes 3 and 4, two double knockout clones.

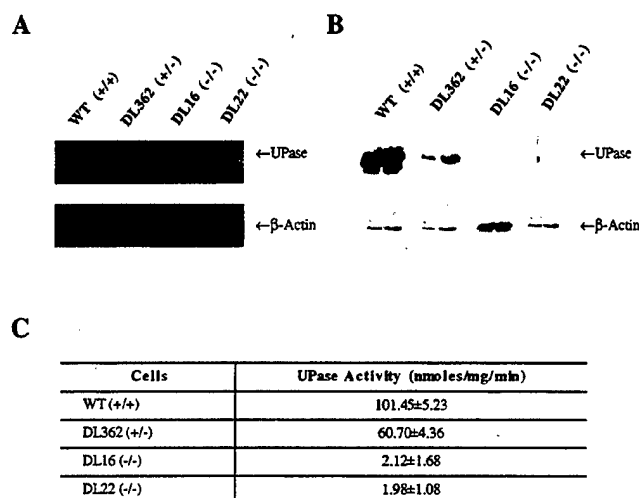


Fig. 2. UPase gene expression in the knockout clones. A, Northern blot analysis of UPase gene transcripts. Total RNA (10 µg) was used in each lane, and the blot was sequentially probed with mouse UPase and β-actin cDNA random-labeled with ³²P. A 1.4-kb RNA band is seen in WT and single knockout (halved in density) cells but not detectable in double knockout cells. Lane 1, WT; Lane 2, single knockout clone; and Lanes 3 and 4, two double knockout clones. B, Western blot analysis of UPase protein. Total cell lysates were separated on 15% SDS-PAGE, and the protein blot was hybridized sequentially with UPase polyclonal and β-actin monoclonal antibodies. A *M_r* 36,000 protein band is seen in WT and single knockout cell lysates but not in the double knockout cell lysate. Lanes 1 and 2, WT; Lanes 3 and 4 single knockout clone; Lanes 5, and 6, double knockout clone DL16; and Lanes 7 and 8, double knockout clone DL22. C, UPase activity assays. The cell lysates from WT and knockout cell clones were used to check the ability to convert [³H]uridine to [³H]uracil. The activity is expressed in nmol/mg protein/min.

the mRNA transcripts (Fig. 2). To evaluate whether any translation compensation occurs in single knockout cells and exclude the possibility that the truncated and shifted UPase protein was still expressed in the double allele knockout cells, Western blot analysis was performed using an anti-UPase polyclonal antibody generated in our laboratory (11). The data indicate that the expression of the UPase protein was abrogated in the two double knockout clones and halved in the single knockout clone DL362 (Fig. 2). UPase activity was also assayed in these cell extracts indicating that no activity was present in the two double knockout clones, and the uridine conversion was reduced to 50% in single knockout cells compared with the WT control cells (Fig. 2).

To analyze the effects of UPase gene disruption on cellular physiological function, the double knockout clones DL16 and 22, the single knockout clone DL362, and a WT clone were cultured and assayed in regular medium to determine any change in their proliferative rate. We did not observe any obvious difference in growth rate between WT and knockout cells (data not shown). Moreover, the sizes of both pyrimidine and purine ribonucleotide pools did not change, and the Na⁺-dependent active transport of uridine was not affected (data not shown). More interestingly, the intracellular level of R-1-P, a cosubstrate in the phosphorylytic reaction, was not significantly altered in the knockout clones with a concentration of 2 ± 0.15 nmol/mg of proteins in the WT cells and 2.23 ± 0.19 nmol/mg of proteins in the double knockout cells.

UPase Knockout Cells: Effect on Sensitivity to Pyrimidine Analogues. To elucidate the effects of the disruption of UPase activity on cell drug sensitivity, double knockout clones DL16 and 22, the single knockout clone DL362, and the WT ES clone were tested against five pyrimidine analogues and a DNA intercalating antitumor agent, doxorubicin. A 72-h exposure to 5-FU indicated a reduced sensitivity to this pyrimidine antimetabolite with a 10-fold increase in IC₅₀ from 0.2 µM for the WT cells to 2.0 µM for the two UPase double

knockout clones (Fig. 3). The single knockout cells still maintained sensitivity to 5-FU with an IC₅₀ of 0.35 µM. This difference in sensitivity is reflected in the 5-FU incorporation into the nucleic acids of these double knockout cells with a reduction of 2–3-fold compared with the WT cells (Table 1). When the cells were exposed simultaneously to 5-FU in the presence of the specific UPase inhibitor BAU (50 µM), we observed a reduction in 5-FU activity in both WT and UPase single knockout ES cells with IC₅₀s similar to the ones determined for the double knockout clones. The main drawback of the antineoplastic activity of 5-FU is its toxic effect against normal tissues, mostly gastrointestinal mucosa and hematopoietic system. One of the strategies to reduce the toxic side effects of 5-FU has been to administer a nontoxic prodrug that can be selectively activated at tumor level. 5'DFUR represents one of these examples (5, 14). Our results indicated that WT and UPase single knockout cells were much more sensitive to 5'DFUR than the double knockout cells, with IC₅₀ of 0.5, 2.5, and 8.0 µM for the WT, single knockout, and double knockout cells, respectively (Fig. 4), establishing the importance of UPase in the activation of 5'DFUR. The abrogation of UPase had no effects on the cytotoxicity of 2'-deoxy-5-fluorouridine, mainly activated by TK, and 1-β-D-arabinofuranosylcytosine, a deoxycytidine analogue. UPase activity also did not affect the cytotoxic activity of a DNA intercalator and topoisomerase inhibitor such as doxorubicin (data not shown).

Role of UPase Activity on Pyrimidine Salvage Pathway. PALA, a transitional state analogue, intermediate in the condensation of carbamylphosphate with L-aspartic acid, can efficiently inhibit the pyrimidine *de novo* synthesis and deplete the pyrimidine nucleotide

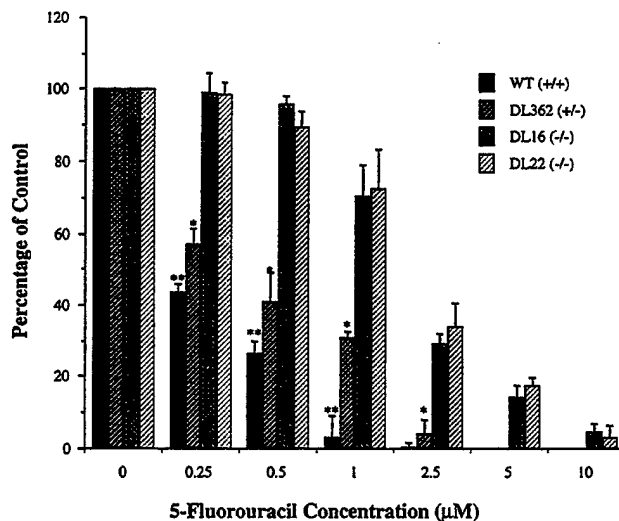


Fig. 3. Antiproliferative activity of 5-FU in WT and knockout ES cells. The WT and knockout ES cells were exposed to different concentration of 5-FU for 72 h, and the amount of viable cells are determined by cell proliferation kit (2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide inner salt). Each column represents the mean of three experiments; bars, ± SD. *, significantly different from the double knockout cells given the same treatment (*P* < 0.001, unpaired *t* test), **, significantly different from both single (*P* < 0.05) and double (*P* < 0.001) knockout cells (unpaired *t* test).

Table 1 Incorporation of radio-labeled 5-FU and uridine

Cell line	[³ H]5-FU (pmol/10 ⁶ cells/24 h)	[³ H]Uridine (nmol/10 ⁶ cells/24 h)
WT (+/+)	56.67 ± 5.69	2.48 ± 0.23
DL362 (+/-)	46.88 ± 8.17	2.73 ± 0.35
DL16 (-/-)	21.18 ± 3.31	3.29 ± 0.39
DL22 (-/-)	16.88 ± 5.67	3.39 ± 0.19

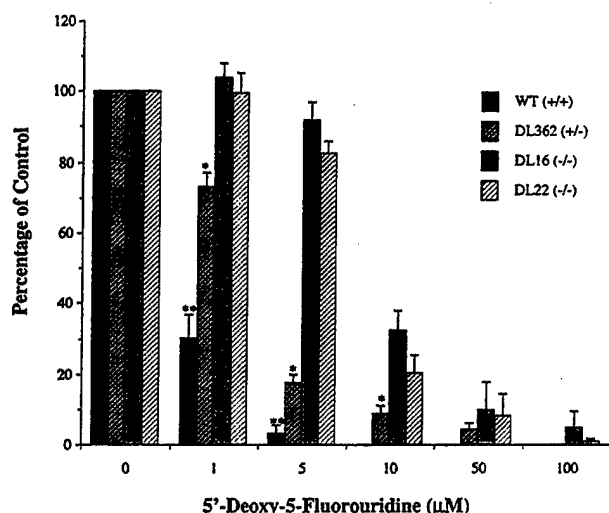


Fig. 4. Role of UPase in the activation of 5'DFUR. WT and knockout ES cells were exposed to different concentration of 5'DFUR for 72 h, and the cell were treated as described in Fig. 4. Each column represents the mean of three separate experiments; bars, \pm SD. *, significantly different from the double knockout cells given the same treatment ($P < 0.01$, unpaired t test); **, significantly different from both single ($P < 0.01$) and double ($P < 0.001$) knockout cells (unpaired t test).

pools via inhibition of aspartate transcarbamylase (15). Our data demonstrate that the disruption of UPase activity causes an increase in the IC_{50} of PALA from 50 μ M in WT ES cells to $>2000 \mu$ M for the double knockout cells (Fig. 5), indicating the diminished role of the *de novo* pyrimidine synthesis in this knockout cell model. This is confirmed by the increased uridine incorporation (Table 1) and uridine kinase activity (Table 2). As expected, uridine rescue (50 μ M) could efficiently protect both WT and single knockout cells from PALA toxicity (data not shown). The elevated uridine kinase activity observed in the UPase double knockout cells is reflected in an increased sensitivity of these murine ES subpopulations to 5-fluorouridine, a direct substrate for uridine kinase. The 0.05 μ M ED_{50} of 5-fluorouridine in WT ES cells was reduced to 0.02 μ M in both UPase double knockout clones. No change in sensitivity to 5-fluorouridine was observed in the UPase single knockout ES cells that did not display any significant alteration in uridine kinase activity. The analysis of TPase, an enzyme that despite a lower efficiency shares substrate specificity with UPase, surprisingly revealed that no detectable activity was present in WT ES cells, and no induction was observed in the UPase knockout ES cells. Similarly, eliminating UPase activity did not cause any alteration in the expression of OPRTase, a key enzyme in the *de novo* biosynthetic pathway and in the activation of 5-FU (Table 2).

DISCUSSION

Using specific gene targeting methodology, we were successfully able to obtain several UPase knockout ES clones. Northern blot and Western blot analyses as well as enzyme activity assays confirmed the absence of the UPase gene products in the double knockout cells and an $\sim 50\%$ reduction in the single knockout cells. These cell clones together with their parental WT cells compose an ideal cell panel, genetically differing only in UPase activity alone, to evaluate the metabolism and the activity of pyrimidine and antipyrimidine metabolites.

Theoretically, the abrogation of UPase activity, the first enzyme of the pyrimidine degradative pathway, should lead to the accumulation of uridine and consequent expansion of pyrimidine nucleotide pools in knockout cells. However, we did not find significant changes in

ribonucleotide pools, in the intracellular level of R-1-P, and in the activity of the enzymes involved in pyrimidine regulation except for uridine kinase that was found elevated in knockout cells.

Uridine homeostasis is apparently maintained by a reduced contribution of the pyrimidine *de novo* synthesis as indicated by a reduced sensitivity of the double knockout cells to PALA and by a greater reliance on the pyrimidine salvage pathway as confirmed by an increased incorporation of preformed pyrimidines (uridine) in the nucleic acids.

Many investigators have reported that UPase is mainly a catabolic enzyme leading to the formation of β -alanine by catalyzing uridine phosphorolysis rather than the ribosylation of uracil (5, 16, 17). Unlike purine bases that can be salvaged by adenine phosphoribosyltransferase and hypoxanthine-guanine phosphoribosyltransferase in a single step reaction, pyrimidine salvage is thought to occur only at the nucleoside level (16). However, recent observations have indicated that UPase may function as an anabolic enzyme using activated ribose to salvage uracil into uridine nucleotides (with uridine as an intermediate) even in the presence of excess inorganic phosphate (6, 18, 19).

Although two main pathways contribute to 5-FU activation, either via the OPRTase pathway in the presence of phosphoribosyl PP, or via UPase-initiated salvage pathway with R-1-P as cosubstrate, it is still controversial as to which pathway plays a predominant role. Our results in UPase knockout ES cells support the hypothesis that UPase substantially contributes to the activation of 5-FU as indicated by a 10-fold increase in IC_{50} for 5-FU, and a reduced 5-FU sensitivity in WT and single knockout cells in the presence of the UPase inhibitor BAU.

We have indicated the role of R-1-P as the rate-limiting factor (5, 20) *in vivo* of the anabolic reactions catalyzed by UPase, and con-

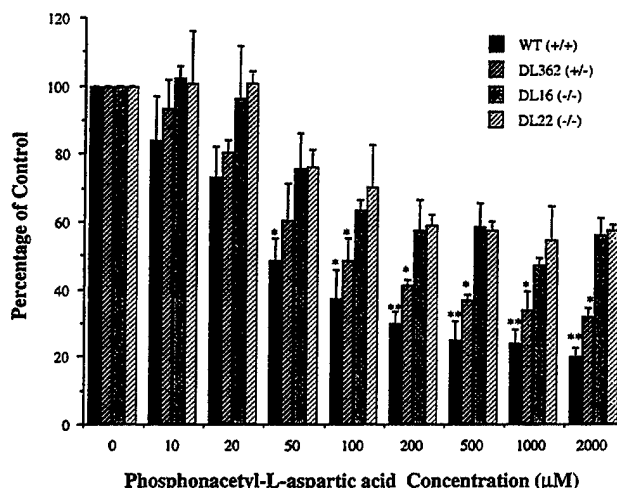


Fig. 5. Disruption of UPase activity results in resistance to PALA. WT and knockout ES cells were exposed to different concentrations of PALA for 72 h, and the cells were treated as described in Fig. 4. Each column represents the mean of three separate experiments; bars, \pm SD. *, significantly different from the double knockout cells given the same treatment ($P < 0.05$, unpaired t test); **, significantly different from both single ($P < 0.01$) and double ($P < 0.005$) knockout cells (unpaired t test).

Table 2 Activities of uridine kinase and OPRTase

Cell line	Uridine kinase (nmol/mg protein/h)	OPRTase (nmol/mg protein/h)
WT (+/+)	185.9 \pm 12.3	103.3 \pm 11.5
DL362 (+/-)	188.2 \pm 15.9	102.5 \pm 10.8
DL16 (-/-)	390.6 \pm 11.8	119.5 \pm 12.5
DL22 (-/-)	322.4 \pm 13.1	107.8 \pm 13.5

templated the possibility that changes in the balance between *de novo* pyrimidine synthesis and salvage pathway could influence the intracellular concentration of R-1-P. As mentioned above, the R-1-P level was not significantly altered in double knockout cells compared with the WT ES population. This finding indirectly reinforces the hypothesis of an interaction between purine and pyrimidine metabolism indicating that inosine and guanosine could actually replace R-1-P as ribose donors and suggesting that in our *in vitro* model R-1-P does not represent a rate-limiting factor in 5-FU activation (6, 17).

Our studies have also confirmed the role of UPase in the activation of 5'DFUR, a nontoxic prodrug of 5-FU designed to be selectively activated in tumor cells. Several investigators have postulated a correlation between growth inhibition by 5'DFUR and the activity of UPase (5, 21, 22). Our data support this hypothesis showing that the sensitivity of the WT cells to 5'DFUR is almost 16-fold higher than UPase double knockout cells, and the single knockout cells had an intermediate sensitivity with an IC₅₀ 3-fold higher than the double knockout cells. These data clarify the activation mechanism and shed new light on the tumor-specific selectivity of capecitabine, an oral fluoropyrimidine prodrug approved recently as a first line agent for the treatment of metastatic colorectal cancer and metastatic breast cancer resistant to paclitaxel and anthracycline-containing chemotherapy. To exert its antineoplastic activity, capecitabine must be activated initially in the liver and subsequently in tumors by a series of enzymatic reactions to generate 5-FU. Capecitabine is first converted by hepatic carboxyl esterase to 5'-deoxy-5-fluorocytidine and then to 5'DFUR by cytidine deaminase. In tumor tissues, 5'DFUR is then selectively metabolized to form 5-FU by phosphorolytic activity (23). Thus far this last metabolic step was totally ascribed to the enzymatic activity of TPase, a protein that has been shown to be overexpressed in some human tumors (24). Our laboratory and others have reported the elevation of UPase activity and its expression in different solid tumors including breast and colorectal compared with adjacent normal tissues (11, 25). Here, we have clearly demonstrated the critical role of UPase in the activation of 5'DFUR, strengthening the rationale of tumor-specific activation of capecitabine, attributable not only to TPase elevation but also and probably mostly because of the presence of increased neoplastic UPase activity.

In conclusion, we have successfully generated a UPase gene knockout cell model by gene targeting technology. This disruption does not affect the basic cell physiology under normal tissue culture conditions but significantly alters the cell sensitivity to 5-FU, 5'DFUR and PALA, thereby indicating that UPase plays a significant role in 5-FU activation and capecitabine tumor selectivity. Our results also question the hypothesis that R-1-P is the rate-limiting factor of UPase-catalyzed anabolic reactions and possibly confirm the role of purines as ribose donors. These findings will have significant importance in the clinical selection of antitumor agents, and in the development of biochemical modulators and new fluoropyrimidine prodrugs.

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Review

Homeostatic control of uridine and the role of uridine phosphorylase: a biological and clinical update

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Abstract

Uridine, a pyrimidine nucleoside essential for the synthesis of RNA and bio-membranes, is a crucial element in the regulation of normal physiological processes as well as pathological states. The biological effects of uridine have been associated with the regulation of the cardio-circulatory system, at the reproduction level, with both peripheral and central nervous system modulation and with the functionality of the respiratory system. Furthermore, uridine plays a role at the clinical level in modulating the cytotoxic effects of fluoropyrimidines in both normal and neoplastic tissues. The concentration of uridine in plasma and tissues is tightly regulated by cellular transport mechanisms and by the activity of uridine phosphorylase (UPase), responsible for the reversible phosphorolysis of uridine to uracil. We have recently completed several studies designed to define the mechanisms regulating UPase expression and better characterize the multiple biological effects of uridine. Immunohistochemical analysis and co-purification studies have revealed the association of UPase with the cytoskeleton and the cellular membrane. The characterization of the promoter region of UPase has indicated a direct regulation of its expression by the tumor suppressor gene p53. The evaluation of human surgical specimens has shown elevated UPase activity in tumor tissue compared to paired normal tissue. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Uridine; Uridine phosphorylase; Pyrimidine; 5-Fluorouracil; p53; Cytoskeleton

1. Introduction: physiological and biological role of uridine

Pyrimidines are synthesized *de novo* in mammalian cells through a multistep process starting from glutamine and carbon dioxide to form, the pyrimidine ring in the second to last intermediate, orotic acid, which is then converted to its nucleotide form in the presence of PRPP. From the degradation of the nucleic acids and nucleotides a large portion of the pyrimidines are salvaged. The relative contribution of *de novo* synthesis and salvage pathway to the maintenance of

the nucleotide pools varies in different cells and tissues [1]. A crucial difference between purine and pyrimidine metabolism is that purines are recycled from their bases while pyrimidines are salvaged from their nucleosides, particularly uridine. In fact, in patients with deficient pyrimidine biosynthesis, only uridine is able to overcome this pathological manifestation but uracil is not [2].

The concentration of circulating plasma uridine of approximately 3–5 μM is tightly regulated throughout different species and individuals [3–5]. The liver appears to have this homeostatic control on uridine degradation and formation [6]. Uridine is essentially cleared in a single pass through the liver and it is replaced in a highly regulated manner by “new uridine” formed by *de novo* synthesis [6]. We have previously reported the cellular basis for the catabolic component of this apparent paradox by the dissociation of the liver into two cell fractions, hepatocytes and a nonparenchymal cell population. Suspensions of the nonparenchymal cells were shown to rapidly cleave uridine to uracil, while in hepatocytes, this activity was barely detectable. Conversely, hepatocytes caused extensive degradation of uracil to β -alanine. These differences correlated with the

Abbreviations: PRPP, 5-Phosphorylribose 1-pyrophosphate; BAU, Benzylacyclouridine; UDPG, Uridine 5'-diphosphoglucose; UPase, Uridine phosphorylase; TNF- α , Tumor necrosis factor- α ; IL-1 α , Interleukin-1 α ; IFN α and γ , Interferon α and γ ; TPase, Thymidine phosphorylase; 5-FU, 5-Fluorouracil; PALA, *N*-(phosphonacetyl)-L-aspartate; MTD, Maximum tolerated dose; TAU, 2',3',5'-tri-*O*-acetyluridine; NBMPR, nitrobenzylthioinosine; PD-ECGF, platelet-derived endothelial cell growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NDPK, nucleoside diphosphate kinase; NTP, nucleotide triphosphates

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uridine phosphorylase (UPase) and dihydrouracil dehydrogenase activity present in each cell type [7].

Besides the critical role of uridine in the synthesis of RNA and bio-membranes, through the formation of pyrimidine-lipid and pyrimidine-sugar conjugates, experimental and clinical evidence suggests a role for uridine in regulating a series of biological functions [8].

Uridine and its nucleosides have shown a complex effect in the regulation of vascular resistance, producing opposing effects in some tissues, either by acting directly on the smooth muscle cells or by stimulating the surrounding endothelial cells [9].

Uridine is present in the seminal fluid at millimolar concentrations, a level that is approximately three orders of magnitude higher compared to other body fluids or tissues [10]. The presence of such a high uridine concentration and its correlation with sperm motility suggests a role of uridine in spermatogenesis [11]. Furthermore, a low level of uridine in prostatic secretions of patients with prostatitis also indicates a possible role of this nucleoside in the etiology of this disease [11].

In the area of the peripheral nervous system, uridine appears to have a modulatory role. It has been shown to hyperpolarize amphibian ganglia and rat superior cervical ganglia at submillimolar concentrations, possibly resulting in an inhibitory activity [12]. Several clinical observations indicate the crucial function of pyrimidine nucleoside salvage in the maintenance of normal CNS activity [13,14]. In the treatment of a form of autism with seizures, oral uridine administration has led to improvement in speech, behavior and decreased frequency in seizures [15]. Deficiencies in the catabolism of pyrimidines due to impaired activity of one of the steps in the catabolic pathway, such as dihydropyrimidine dehydrogenase or β -ureidopropionase, have resulted in autism, convulsions, mental retardation and decreased motor coordination [14].

In animal models, uridine has been shown to potentiate dopaminergic transmission and reduce anxiety [16]. Uridine has been documented as a sleep-inducing factor in rats and shown to increase the activity of barbiturates [17,18]. In mice and rats, high doses of uridine cause a dramatic reduction in body temperature [19]; whereas uridine induces fever in human and rabbits [20,21]. The co-administration of an inhibitor of UPase, benzylacetyluridine (BAU), almost completely prevented the effect on thermoregulation, suggesting that a pyrimidine catabolite, possibly β -alanine, could alter the control of the body temperature [21].

Aside from the 'physiological' effects that we have just briefly outlined, uridine appears to have remarkable functions in tissues under stress or pathological situations and in the clinical setting. In hearts subjected to ischemia, perfusion with uridine rapidly restored myocardial ATP levels, glycogen and UDPG [22]. Similarly, uridine perfusion resulted in the maintenance of brain metabolism during ischemia or severe hypoglycemia [23,24]. Furthermore, uridine induced recovery from neuronal degeneration pro-

duced by diabetic neuropathy [25]. Uridine has been used as a 'rescue' agent in cancer therapy to decrease bone marrow and gastrointestinal toxicity, following 5-fluorouracil-based drug regimens [4,26–31]. In combination with BAU, uridine has shown some activity in reducing neurotoxicity and the effects on the bone marrow of AZT during treatment for HIV infections [32].

1.1. Role of UPase on the pharmacological activity of uridine

Plasma and intracellular concentrations of uridine are regulated by the catabolic activity of UPase and by two transport mechanisms, facilitated diffusion and Na^+ -dependent active transport. UPase catalyzes the reversible phosphorolysis of uridine and to a lesser degree of thymidine. It also cleaves pyrimidine 2'- and 5'-deoxyriboosides at a much lower rate [33–37]. UPase is present in most tissues and in tumors, where its activity is generally elevated [33,35,38]. The mammalian enzyme appears to be a tetrameric protein with subunits of approximately 33,000 molecular weight. Initial velocity and product inhibition studies suggest an ordered bi-bi mechanism where P_i binds first before uridine and ribose-1-phosphate is released after uracil [39]. UPase plays an important role in the homeostatic regulation of uridine concentration in plasma and tissues [22–25] as well as affects activation and catabolism of fluoropyrimidines influencing their therapeutic capacity [40–43]. The expression of UPase has been shown to be induced in different tumor cell lines, such as Colon 26 and HCT-116, when in the presence of cytokines: $\text{TNF-}\alpha$, $\text{IL-1}\alpha$ and $\text{IFN-}\alpha$ and γ , and vitamin D_3 [44–46]. A similar response to cytokines has been observed for thymidine phosphorylase (TPase) [47]. Induction of UPase expression has also been reported in c-H-ras transformed NIH 3T3 cells [48]. In a murine model, hepatic UPase has been found to follow a circadian rhythm which was the inverse of that for plasma uridine concentration, re-emphasizing its role in the regulation of blood uridine level and suggesting its possible involvement in the humoral control of sleep [49].

A misconception surrounds the role of UPase and TPase. Some literature reports UPase as the pyrimidine nucleoside phosphorylase in murine tissues and TPase the main phosphorolytic enzyme in human tissues [50]. UPase, however, has been shown to be present in all human tissues and tumors, whereas TPase activity has been found reduced or absent in many human tumors [4,17,26,27,33,35,40,51–54].

1.2. "Rescue" of 5-fluorouracil toxicity

As previously mentioned, UPase has a critical role in regulating the concentration of uridine in plasma and tissues. A number of clinical studies have demonstrated the ability of uridine to reduce 5-FU toxicity, without affecting its antitumor activity, if properly administered 18–24 h following the cytotoxic agent [26–29].

The combination 'rescue regimens' of 5-FU plus uridine were initially proposed to evaluate the hypothesis that the antitumor effect of 5-FU is primarily due to the inhibition of thymidylate synthase and the host toxicity caused by the incorporation of the fluoropyrimidine into RNA [55]. In vivo studies in a murine model [56] and in vitro data [57] have clearly indicated that the incorporation of 5-FU into RNA appears to be the major cause of gastrointestinal toxicity. Results show that uridine inhibited the incorporation and avoided the cytotoxic effect, whereas thymidine did not prevent 5-FU toxicity. Furthermore, a recent study has indicated that the p53-dependent apoptosis induced by 5-FU in intestinal cells was reduced by uridine administration but not by thymidine [58].

Because of its low oral bioavailability and a rapid half-life, large doses of uridine are necessary to achieve clinically relevant concentrations in plasma causing moderate to severe toxicity including severe diarrhea as dose-limiting toxicity [4,20]. On the other hand, infusion of uridine has resulted in fevers, phlebitis, cellulitis, superior vena cava syndrome, torpor and confusion, and precluded an extensive clinical use [20,59]. Nevertheless, clinical studies of 5-FU in combination with methotrexate and PALA, have shown that patients tolerated combination therapy with delayed uridine (infused over a 72-h period starting 2 h after 5-FU administration, 3 h on and 3 h off) up to a weekly dose of 750 mg/m² of 5-FU, with 25% experiencing moderate mucositis (grade II). In a previous clinical trial without uridine, four out of six patients could not tolerate a 600 mg/m² dose of 5-FU because of mucositis, diarrhea and a decrease in performance status. In another study, 5-FU treatment could be continued with delayed administration of uridine at a weekly dose of 5-FU, which alone caused dose-limiting myelosuppression. In most of the patients who had previously developed leukopenia, the WBC increased markedly despite continued 5-FU administration [59]. In a more recent study of high-dose 5-FU with doxorubicin, high-dose methotrexate and leucovorin, oral uridine administration allowed for dose intensification of 5-FU with a 33% increase in the MTD of 5-FU in the presence of doxorubicin and a 45% increase in 5-FU MTD without doxorubicin [60]. No responses were obtained in patients with gastric cancer (0/11) when uridine was administered 2 h after 5-FU, however 2/3 patients responded with the 24-h uridine delay. This last regimen, with a 24-h interval between the administration of 5-FU and uridine, ensured rescue from 5-FU-induced hematologic toxicity without adverse impact on tumor response [60]. As indicated in pre-clinical studies, properly delayed uridine rescue results in a faster clearance of 5-FU from RNA of bone marrow and tumors and enhancement of the rate of recovery of DNA synthesis only in the bone marrow [37].

Our laboratory has shown that the problem of a rapid disposition of uridine and the administration of large doses of the nucleoside could be overcome by utilizing inhibitors of UPase, such as BAU, to conserve endogenous uridine

with consequent elevation of its concentration in plasma and tissues. This approach has resulted in the reduction in animal models of host toxicity, while maintaining the antineoplastic effect of 5-FU [34]. A phase I clinical trial of oral BAU administered as a single agent has shown the ability of this inhibitor to elevate 2–3-fold the plasma uridine concentration with no significant host toxicity in patients [3].

1.3. *Tri-O-acetyluridine (TAU)*

A new agent 2',3',5'-tri-*O*-acetyluridine (TAU; PN 401) has been tested recently in a clinical trial to rescue 5-FU toxicity. TAU is a uridine pro-drug, the presence of the acetyl groups increases the hydrophobicity, therefore enhancing the gastrointestinal transport and bioavailability, and protecting this agent from the catabolism by UPase. Uridine is then progressively released by plasma esterases resulting in sustained delivery over time without most of the side effects of uridine administration. The sustained elevated level of uridine, up to 50 μM in plasma, has allowed a dose escalation of 5-FU, administered as a rapid intravenous bolus weekly for 6 weeks, from 600 to 1000 mg/m². Still, an oral dose of 6 g of TAU at 6-h intervals was necessary to produce significant uridine levels [61].

2. Regulation of uridine homeostasis: transport mechanisms

Besides catabolism by nucleoside phosphorylase activity, the intracellular concentration of uridine is regulated by its transport through the cell membranes. A facilitated-diffusion mechanism, which equilibrates intracellular and extracellular uridine, has been considered for many years to be responsible for intracellular uridine concentration. This non-energy dependent process displays broad substrate specificity toward synthetic and naturally occurring pyrimidine nucleosides [62–65]. In addition to competition between functional substrates, the facilitated diffusion of nucleosides present in most cell lines can be reversibly inhibited by compounds such as dipyridamole and nitrobenzylthioinosine [66]. Studies by Belt [67,68] have revealed that the facilitated diffusion mechanism in cell lines can be distinguished by the sensitivity to inhibition by NBMPR or dipyridamole. Recently, both transporters, NBMPR-sensitive (es) and NBMPR-insensitive (ei) have been cloned and characterized [69,70].

In addition to the non-concentrative facilitated diffusion mechanism, renal and gut epithelial cells were shown to possess a Na⁺-dependent transporter for nucleosides [71–77]. Our laboratory revealed the potential physiological significance of this Na⁺-dependent transport system by the finding that concentrations of uridine in a variety of freeze-clamped normal murine tissues far exceeded the concentration of uridine in the plasma. We were able to

demonstrate that BAU generated concentrations of uridine in selected tissues that were as much as 50–100 times that in control plasma [34]. The concentrative system in liver, kidney and gut appears to have different substrate specificity than in lymphoid cells (spleen or thymus). In addition, we have demonstrated the generality of the expression of a concentrative, active transport mechanism for uridine in a variety of normal murine tissues [53]. These observations changed the perception of the Na^+ -dependent uridine transport process from a rate phenomenon observed in isolated cells to a major physiological effect that afforded therapeutic opportunities considering that the intracellular concentrations of uridine in a wide variety of neoplastic cell lines did not exceed those in the media. It has been shown that some lines express the concentrative mechanism to a very limited degree [78] but that it is overwhelmed by the equilibration of nucleoside achieved by an active facilitated diffusion mechanism. At this moment, five major Na^+ -dependent active transport systems have been isolated: (1) the purine selective N1 system, or cif, shown to be present in rat intestinal epithelium, mouse enterocytes and lately in human kidney [79–82]; (2) the pyrimidine selective N2 system or cit described by Jarvis and Griffith [83], present in rabbit small intestine with selectivity for pyrimidine nucleosides and adenosine; (3) the N3 transport system (cib) isolated in rat jejunum sensitive to inhibition by both purine and pyrimidines [84]; (4) the N4 transport system, expressed in human kidneys, which is identical to the N2 system in substrate specificity [85]; and the N5, a cs transport system, found in freshly isolated human leukemia cells [86].

3. UPase: clinical, biological and regulation update

Over the past few years, we have focused on the role of UPase in regulating uridine metabolism and its intracellular levels in normal and neoplastic tissues and on the mechanisms controlling the expression and localization of UPase. We have determined that the activity of UPase is elevated in tumors as compared to their normal tissue counterpart and discovered variant uridine phosphorolytic activity in selected human tumors [54]. We have also defined the genomic structure of the UPase gene, characterized its promoter region, the p53-dependent control of its expression [87,88] and established its intracellular localization and association with cytoskeletal elements [89].

3.1. Expression and detection of UPase and identification of variant phosphorolytic activity in human tumors

TPase catalyzes the reversible phosphorolysis of thymidine to thymine and with less efficiency also contributes to the initial degradation of uridine. Since TPase has been shown to be identical to platelet-derived endothelial cell growth factor (PD-ECGF) and its angiogenic activity demonstrated [90,91], several studies have investigated the role

of this protein in tumor progression and clinical outcome. Increased expression has been found in some solid tumors compared to the adjacent normal tissues [92] and TPase has been shown to be a negative prognostic indicator in bladder [93], colo-rectal [94,95], ovarian [92], pancreatic [96] and renal [97] cancers but its role is still controversial in breast carcinoma [98,99]. PD-ECGF/TPase has been shown to promote angiogenesis only when enzymatically active and in the presence of thymidine [100].

The intense investigation of the role of TPase on cancer invasiveness and malignancy has not translated to a similar interest for a potential involvement of UPase, despite its complementary phosphorolytic activity.

We have evaluated the activity of UPase in fresh tumor specimens and adjacent normal tissues of patients undergoing surgical resection of their malignancy. The enzymatic activity was variable among the different tissue specimens, but overall it was 2–3-fold higher in tumors compared with the paired normal tissue (Fig. 1). In the tissues that we were able to collect the most clinical specimens, breast ($n=28$) and colon ($n=9$) carcinomas, the difference in activity between tumor and normal tissues was statistically significant with P values of 0.012 and 0.021, respectively [54]. These results have been confirmed in a recent study in 35 human colo-rectal carcinomas also indicating higher UPase mRNA gene expression in tumor compared to paired normal tissue [101]. The same study concluded that higher UPase gene expression was a negative prognostic factor for the patient [101]. In all normal tissues and most tumor specimens evaluated in our investigation [54], UPase activity was completely inhibited by the UPase inhibitor BAU at 10 μM concentration. However, breast, head-neck, and ovarian tumors showed partial sensitivity to the inhibitor with ~ 40% of residual phosphorolytic activity still present after the addition of 100 μM BAU. Using the TPase inhibitor 5-bromo-6-aminouracil [102], we were able to establish that TPase does not significantly contribute to the BAU insensitive phosphorolytic activity present in breast tumor tissue.

The evaluation of the clinical specimens has clearly indicated that normal tissues like gastrointestinal tract and bone marrow, that are the most sensitive to fluoropyrimidine toxicity, possess UPase activity completely inhibitable by BAU. However, human breast tumors possess distinct phosphorolytic activity that is partially insensitive to the classical UPase inhibitors, therefore resulting in a more rapid degradation of uridine. This differential catabolism of the pyrimidine nucleoside could be exploited to create, in the presence of BAU, a selective rescue effect for normal tissues without affecting the antineoplastic activity in breast neoplastic tissues therefore enhancing the therapeutic index of the fluoropyrimidine.

Our study, on human clinical specimens revealing the presence of higher UPase enzymatic activity in tumor tissues as compared to paired normal tissue is in contrast with a previous report from Maehara et al. [48] indicating no difference in UPase activity between tumor and normal

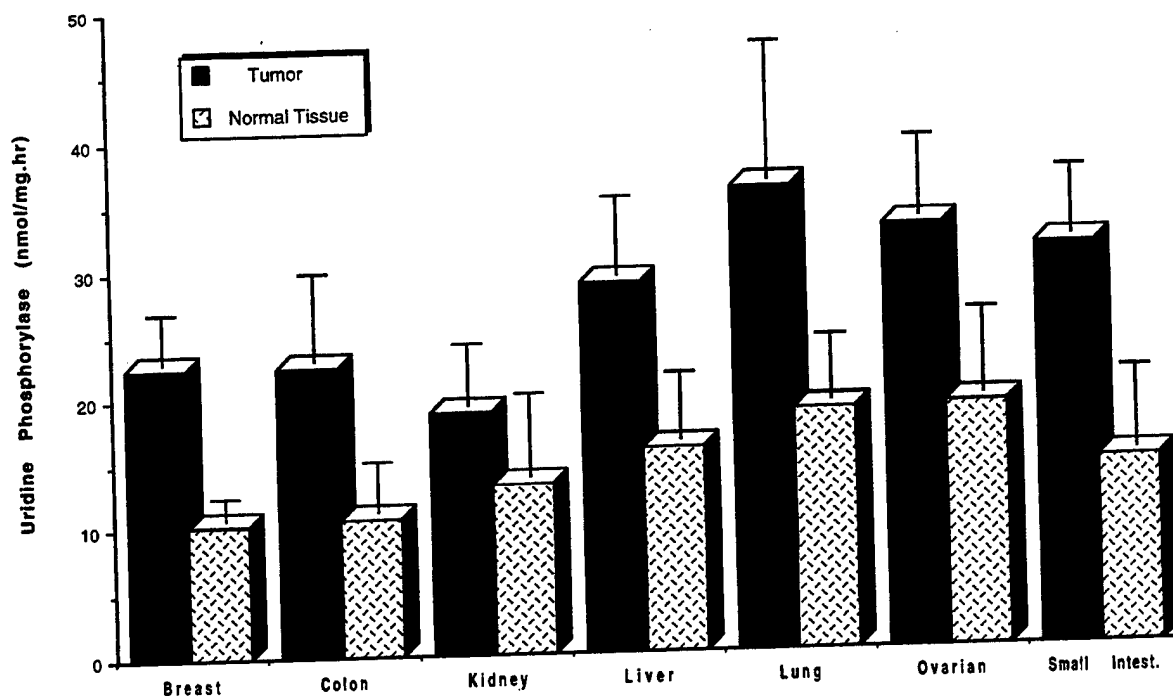


Fig. 1. UPase activity in matched pairs of human breast tumor and normal tissues following surgical excision of the malignancy.

tissues. We also disagree with another finding from the same group indicating that the main pyrimidine nucleoside phosphorylase in human is TPase with activity 20-fold higher than UPase [48]. In our evaluation, we have observed a degree of variation in the ratio of TPase to UPase activity in human tissues with an overall ratio of 2. However, in many tissues including breast tumors, the ratio was actually in favor of UPase. Our investigation has also indicated that both bone marrow and gut mucosa specimens possess low phosphorylase activity compared to tumor tissues, suggesting that uridine rescue could specifically benefit these tissues representing the primary targets of 5-fluorouracil toxicity, as reported by Pritchard et al. [58] in a study on its effect on the intestinal mucosa.

A recent article from Kanzaki et al. [103] has examined the mRNA expression of UPase and TPase in surgical specimens of 43 patients with breast carcinoma and examined the correlation with clinical pathological factors. The investigators have found a large variation in the expression level of both genes with the highest level measured as more than 1000-fold higher than that in samples expressing the lowest level. There was a significant correlation between TPase expression and microvessel density but no correlation with UPase expression suggesting that UPase does not have any angiogenic activity in human breast carcinoma. In addition, no correlation was found between UPase gene expression and TPase expression level in those breast tumor samples. However, UPase gene expression was higher in patients who relapsed than in patients that did not and patients with high UPase mRNA levels had a significantly poorer overall survival than patients with lower levels.

TPase gene expression did not correlate with either relapse or overall survival in these breast cancer patients [103]. This critical study confirms a previous study from the same group indicating a lack of correlation between clinical outcome and TPase mRNA expression in breast cancer [98]. However, it suggests that UPase could be an independent prognostic factor in breast cancer patients [103].

3.2. UPase genomic structure, characterization of its promoter region and p53-dependent control of its expression

We have recently isolated from a murine BAC library a genomic DNA fragment which included the entire murine UPase gene, whose full length approximates 18.0 kb. The UPase gene has been mapped by FISH to the murine chromosome 11A1–2. A series of oligonucleotide primers based on the cDNA sequence of murine UPase have been utilized to elucidate the intron–exon boundaries [87]. Our results indicate that the murine UPase gene consists of nine exons, ranging in length from 66 to 210 bp, and eight introns varying in size from 240 to 6.0 kb, with typical donor and acceptor sites (GT-AG rule). Exon 1, 2 and the 5' end part of exon 3 do not encode amino acids, the first in-frame ATG codon is located in exon 3. Exon 8 encodes the C-terminus of murine UPase protein and contains a translation stop codon TGA. It also contains the first 70 bp of the 3'-untranslated region. A polyadenylation signal, AATAAA, is present at 45 bp downstream of the TGA codon [87]. We have now also concluded the characterization of the human UPase gene that presents the same basic structure with nine

exons and eight introns. Chromosomal mapping of human UPase identified its location at 7p12, a position where frequent LOH has been found in human breast cancers [104].

The sequence of the 3'-untranslated flanking region of the murine UPase gene shows a GT-rich region present 22 bp downstream of the AATAAA polyadenylation signal. A TGGGG tandem repeat, TGGGGG(TGGGG)₄, is present at 154 bp downstream of AATAAA polyadenylation signal, which represents a putative recombination consensus sequence found in the immunoglobulin switch region (S region), in the α -globin gene cluster, in the putative arrest sites for polymerase α , and in the deletion hot spot (exon 8) of the survival motor-neuron (SMN) gene [105–107].

The 5' flanking region of the murine UPase gene, the immediate full-length sequence (1703 bp) that has shown promoter activity in our studies, doesn't contain canonical CAAT box although a TATA-like sequence, CAATAAAA, is present from -41 to -49 bp upstream of the transcription start point at +1 bp. The lack of both canonical TATA and CAAT consensus sequences is a feature present in a group of genes, many of which have a housekeeping function, such as *N-ras* and transforming growth factor α [108]. At the 5' end of UPase promoter (from -1619 to -1110) we identified a series of microsatellite and minisatellite repeat bases. In addition, an abundance of promoter regulatory elements are seen in the murine UPase promoter region including the presence of the consensus motifs for GATA-1 and two transcription factors. These factors mainly function as regulatory elements in the control of cellular differentiation of hematopoietic cells [109,110]. An IRF-1-like consensus element present just upstream (from -21 to -33) of the putative transcription start site of the UPase gene represents an important transcription factor in the regulation of the interferon response system for infection, cell growth and apoptosis [111,112]. Finally, two potential proto-oncogene binding sites for C-Myb and V-Myb [113–115], and a tumor suppressor gene, p53 putative regulatory element [116–118], located in the sequence -303 bp to -294 bp, have been found in the UPase promoter region.

To explore the possible effect of p53 on UPase expression, we have analyzed the effects of p53 on the murine UPase promoter activity [88]. We found that the deletion from -1619 to -445 of the UPase promoter had no effect on the ability of p53 to inhibit gene expression, however, the inhibitory activity was altered when the promoter region between -445 and -274 bp was deleted. Using transient-expression assays in EMT6 and NIH 3T3 cells, co-transfection with the wild-type p53 construct resulted in significantly less luciferase activity in the constructs from -1619 to -445 bp, whereas down to -274 bp and more, the promoter activity was not affected. These data indicate that the region between -445 and -274 bp is susceptible to regulation by p53 in the UPase promoter. This phenomenon was further confirmed in p53 nullified cells [88]. Sequencing analysis of this region found a putative p53-binding

motif AGcCTTGTC located at -303 to -294. This binding motif differs in one base (small case base) from the consensus binding element of p53 [119]. The gel mobility shift assay and DNase I footprinting have indicated that this putative regulatory motif exhibited specific binding with the p53 protein [88].

p53 has been shown, by Linke et al. [120], to be activated by ribonucleotide depletion caused by antimetabolite drugs such as PALA even in the absence of DNA damage. As previously mentioned, the phosphorolytic activity of UPase regulating intracellular uridine levels reveals the critical role of this enzyme in modulating the pyrimidine salvage pathway. The suppressive regulation of p53 on UPase gene indicates the presence of a negative control of the pyrimidine salvage pathway by p53 through UPase, probably as a cellular self-protection mechanism in case of ribonucleotide depletion. p53 has previously been shown to: (a) activate genes that initiate apoptosis to eliminate damaged cells and protect an organism from more severe damage and (b) cause cell-cycle arrest following DNA damage to prevent the replication of altered DNA. However, so far, any indication of the contribution of p53 to damage repair is quite limited. A recent report by Tanaka et al. [121] has described a p53-induced gene, p53R2 that encodes for a protein similar to one of the two subunits of ribonucleotide reductase, the rate-limiting step in the conversion of ribonucleotides to deoxyribonucleotides. The p53 regulated R2 subunit is found in the nucleus and its expression is induced by cellular damage (γ -radiation and doxorubicin treatment) suggesting that when repair is needed, the nuclear precursors have to be concentrated near the site of damage.

Somehow, the p53-regulated suppression of UPase expression exerts similar functions to the control that p53 has on p53R2. A cellular damage causing loss or imbalance in the ribonucleotide pools could cause activation of p53 leading to suppression of UPase expression and activation of the pyrimidine salvage pathway to replenish the affected pyrimidine nucleotide pools (Fig. 2). These two p53-regu-

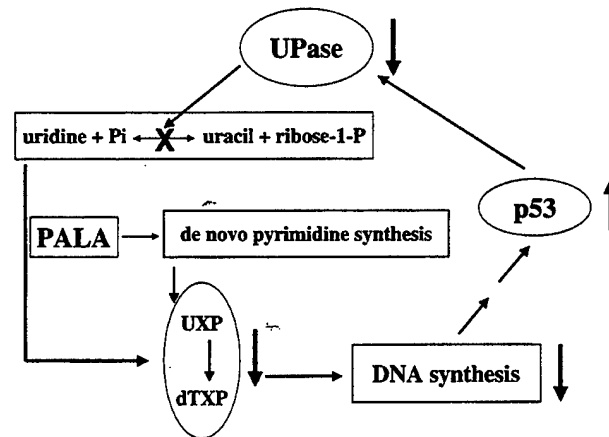


Fig. 2. p53-dependent control of UPase expression and regulation of pyrimidine salvage pathway following PALA inhibition of de novo pyrimidine biosynthesis.

lated mechanisms provide a new level of control on ribo- and deoxy-ribonucleotide pools. Under normal replication conditions, the regulating mechanisms that control the appropriate balance of nucleotides are mostly based on the direct feedback regulation of the biosynthetic enzymes by some of the precursors or final products. However, the p53R2 study and our data on UPase possibly indicate that in case of cellular damage with depletion of nucleotide pools a more sophisticated level of regulation is triggered to more rapidly provide precursors for nuclear repair [88,121].

The elucidation of the negative control regulation of p53 on the UPase gene promoter and UPase expression could also have considerable implication at the clinical level since the human UPase DNA promoter presents, as we have shown for the murine gene, a p53 regulatory element [88]. It is conceivable that mutations and loss of functionality of the p53 gene product, which is a common event in many forms of cancer [122], could alter the suppressive regulatory control on UPase resulting in higher UPase mRNA expression and elevated UPase activity seen in many tumors as compared to paired normal tissues [54].

3.3. Intracellular distribution, localization and association with vimentin

We have established that UPase is associated with the intermediate filament vimentin in NIH 3T3 fibroblasts and Colon 26 cells through co-purification studies using a 5'-amino benzylacetyloumidine affinity matrix. The separation of cytosolic proteins using gel filtration chromatography yields a high molecular weight complex containing UPase and vimentin in a 1:1 stoichiometry. Immunofluorescent techniques have confirmed that UPase is associated with vimentin and that the depolymerization of the microtubule system using nocodazole results in UPase remaining associated with the collapsed intermediate filament, vimentin [89].

UPase is associated with both the soluble pool of vimentin and also with its insoluble pool, with approximately 50–70% of the total UPase present in the cytosol as a soluble protein. However, sequential cell extraction liberates an additional 15–25% UPase activity associated with a Triton-X-100 soluble fraction and a remaining 10–20% UPase activity associated with a Triton-X-100 insoluble pool [89].

The role of UPase in the salvage pathway of pyrimidine nucleoside biosynthesis does not readily translate into a role for this enzyme in association with the cytoskeleton and more specifically with the intermediate filament vimentin. While a number of theories have been proposed for the function of this network, the data are not yet conclusive. Cellular processes as diverse as differentiation, motility, signal transduction, cell division, cytoskeletal stability and vesicular trafficking have been associated with alterations in the dynamics of the intermediate filaments [123–127].

A number of proteins have been shown to be associated with the vimentin intermediate filament scaffold including

p53 [128], protein kinase C [129], Yes and cGMP kinase [130,131], glycolytic enzymes creatine phosphokinase and GAPDH [132–134] and nucleoside diphosphate kinase [132,135] as well as the cross-linking proteins plectin, IFAP-300 and filamin that link intermediate filaments to other cytoskeletal elements and membranes [136–139]. It is particularly interesting to note the number of proteins involved in signal transduction and energy metabolism that have been associated with vimentin. The proposed role for NDPK in nucleotide channeling [135], its co-purification with vimentin and enzymes involved in ATP formation/regeneration [132] together with our observation of UPase co-localization with this same cellular machinery, is making it more likely that such observations are biologically relevant. UPase, a nucleoside phosphorylase and NDPK, an enzyme that provides the majority of cellular non-ATP nucleoside triphosphates have both been co-localized to the intermediate filament vimentin. Since a number of biological responses have been associated with UTP and UDP [140] through the activation of pyrimidine receptors, it is possible that vimentin may play a role in the coordination of these signaling events.

In vitro enzymatic analyses of the detergent-resistant pool of UPase demonstrated that this source of enzyme retains enzymatic activity. The UPase found in association with the polymeric vimentin network may represent a mobilizable pool of enzyme that is only active when liberated from its three-dimensional network. It is also possible that UPase, in association with the insoluble vimentin network, represents a way of localizing enzymatic activity to a particular area within the cell. Vimentin has been proposed as a network that might target mRNA to areas of active protein synthesis [124]. This function for vimentin might explain the necessity of having machinery for pyrimidine synthesis/degradation in close proximity to areas of mRNA translation.

The interdependence of the dynein and kinesin motor proteins, microtubule and intermediate filament systems and the need to furnish this cellular machinery with high quantities of nucleotide triphosphates provides a basis for investigating the mechanisms responsible for the local delivery of high quantities of NTPs to the areas of active energy utilization. The question of what role UPase may play in close proximity to such machinery is at this moment cause for speculation.

4. Conclusions

Many factors have contributed to the limited attention uridine and UPase have received despite their physiologic and pharmacological role in comparison to the interest reserved over the years to adenosine and TPase.

Adenosine has been shown to have a general inhibitory effect on neuronal activity including regulation of sleep, neuroprotection and seizure control [141]. Furthermore, this

purine nucleoside appears to have cardioprotective and immunomodulatory functions [142]. These regulatory and modulatory activities of adenosine are mediated by four subtypes of G-protein-coupled receptors [143]. As we have indicated in our introduction, uridine exerts very similar modulatory and regulatory functions to adenosine, however, no clear mechanism has been identified modulating these physiological activities. Receptors for uridine nucleotides have been mentioned and also the possible existence of a specific receptor for uridine itself has been postulated. A very recent report suggests the presence of a new receptor identified as "uridine receptor" regulating the hypnotic activity of uridine derivatives in rat brain [144]. More studies are needed to confirm this finding, to elucidate the biological and structural characteristic of the receptor, and the interactions with other receptors and substrates. It is critical to extend these studies to other organs to determine the presence of "uridine receptor(s)" not only in the central nervous system but also in the cardio-circulatory system and at the reproduction level. These studies will lead to the discovery of a new interacting molecules and the development of new class of therapeutic agents for various human diseases.

Similarly, the attention dedicated to TPase is associated to its angiogenic properties [144] and more recently, to its link to a human genetic disease, MNGIE [145], and it could be matched by UPase only after we have better defined its physiological function and its possible role in human diseases. Our discoveries of an elevated expression of UPase in human tumors [54], its altered pattern of inhibition in breast cancers [54] and the intracellular association with the cytoskeleton [89] only represent starting points to gain new insight in its biological functions and define its clinical-pathological role.

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p53-dependent Suppression of Uridine Phosphorylase Gene Expression through Direct Promoter Interaction¹

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ABSTRACT

Uridine phosphorylase (UPase) is a key enzyme in the pyrimidine salvage pathway. It reversibly catalyzes the catabolism of uridine to uracil; controls the homeostatic regulation of uridine concentration in plasma and tissues; and plays a role in the intracellular activation of 5-fluorouracil. We cloned the murine UPase gene promoter, a 1703-bp fragment, and determined the transcription initiation sites located at +1 and +92 bp of the cDNA sequence. Through transient expression analysis of the 5'-flanking region of UPase gene, we have evaluated the promoter activity for a series of fragments with 5'- to 3'-deletion in murine breast cancer EMT-6 cells and immortalized murine fibroblast NIH 3T3 cells. Cotransfection of the UPase promoter constructs (from -1619 to -445) containing p53 binding motif with the wild-type p53 construct resulted in a significant reduction of luciferase activity; however, this effect disappeared with the additional deletion of the -445 to -274 sequence to suggest the existence in this promoter region of a putative p53 recognition element. Similar cotransfection in murine embryo fibroblasts p53-/- confirmed the inhibitory role of p53 on the UPase promoter activity. The specificity of the interaction is demonstrated by nuclear protein-specific binding to the putative p53 recognition sequence using gel mobility shift assay and DNase I footprinting analysis. These data indicate the UPase gene is a novel target of p53, and its expression is down-regulated by p53 at the promoter level.

INTRODUCTION

Uridine, a pyrimidine nucleoside essential for the synthesis of RNA and biomembranes, has also been shown to be a crucial element in the regulation of normal physiological processes as well as pathological states. The biological effects of uridine have been associated with the regulation of the cardiocirculatory system, at the reproduction level, with both peripheral and central nervous system modulation, and with the functionality of the respiratory system (1). The concentration of uridine in plasma and tissues is tightly regulated, and the liver has been shown to maintain uridine homeostasis by degrading "old" uridine and resynthesizing new uridine in a single pass (2, 3). Pharmacologically, uridine has been used to protect normal tissues from the toxic side effects of pyrimidine-based anticancer chemotherapy, mostly as a "rescue" therapy for myeloid and gastrointestinal toxicity produced by FU (4, 5). Uridine in combination with 5-benzylacyclouridine (an inhibitor of UPase)³ has also been shown to protect mice against the neurotoxic side effects of FU-containing drug regimens (6-8). UPase is the key enzyme responsible for the reversible phosphorylation of uridine to uracil and plays a critical role in the homeostatic regulation of uridine concentration in plasma and tissues.

We have shown recently that UPase is elevated in many solid

tumors (9), and specific mutations have been found in human breast cancer specimens but not in paired normal tissues (10, 11). Expression of UPase has been shown to be induced in different tumor cell lines, such as colon 26 and HCT-116, when in the presence of the following cytokines: tumor necrosis factor- α , interleukin 1 α , IFN- α and - γ , and vitamin D₃ (12). In the treatment of advanced colorectal carcinoma, IFN- α in combination with FU has resulted in a significant increase in response rate and patient survival when compared with FU alone (13). In colon 26 tumor cells, a mixture of tumor necrosis factor- α , interleukin 1 α , and IFN- γ effectively enhanced FU and 5-fluoro-2'-deoxyuridine (5-dFUrd) cytotoxicity 2.7- and 12.4-fold respectively, because of induction of UPase expression (14). Induction of UPase expression has also been reported in c-H-ras-transformed NIH 3T3 cells resulting in an increased sensitivity to 5'-dFUrd (15). We have reported recently that the murine UPase gene contains 9 exons and 8 introns, spanning a total of ~18 kb (16). We have also cloned and partially characterized the UPase promoter region that appears to contain putative regulatory elements for several oncogenic factors and tumor suppressor genes including p53 (16). Thus, understanding the regulation of the UPase gene affecting both catalytic activity and expression has become critical to elucidate its potential role in the tumorigenesis and to modulate the selectivity of cancer treatment.

The p53 tumor suppressor gene plays a crucial role in cell growth control, DNA damage repair, and apoptosis (17). p53 functions as a transcription factor regulating a number of target genes at the transcriptional level. Despite the progress achieved toward understanding p53 functions, the mechanisms by which p53 acts as a key regulator of cell growth and tumorigenesis have not been completely elucidated.

The isolation and functional characterization of transcriptional regulatory elements are prerequisites for understanding gene expression. In this study, we report a more in-depth characterization of the UPase promoter region, the mapping of the transcription initiation sites, and conduct the functional analysis of the murine UPase promoter in different murine cell lines. Our analysis demonstrates that wild-type p53 can regulate and repress the activity of UPase at the gene promoter level, possibly regulating the pyrimidine salvage pathway after perturbation of the ribonucleotide pools.

MATERIALS AND METHODS

Cell Culture. NIH 3T3 fibroblast cells used in this study were originally obtained from the American Type Culture Collection. The murine breast cancer cell line EMT6 was kindly made available by Dr. Sarah Rockwell (Yale University, New Haven, CT). Early passages p53 -/- and +/- MEF cells were generously provided by Dr. Larry Donchower (Baylor College of Medicine, Houston, TX; Ref. 18). Colon 26 cell cultures were established in our laboratory from *in vivo* growing tumors. All of the cell lines were maintained in DMEM supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μ g/ml) at 37°C in a 5% CO₂ incubator.

Cloning and Sequencing of the 5'-Flanking Region of UPase Gene. A genomic DNA clone that contains the immediate full-length 5'-flanking UPase sequence was obtained by screening a ES-129/SvJ bacterial artificial chromosome library with a murine UPase cDNA probe. The 1703-bp *Xba*I/*Xba*I fragment immediately upstream of the murine UPase gene containing 84 bp of the 5'-untranslated region of cDNA was subcloned into a pBluescript KS II cloning vector (Stratagene). The complete sequence was determined with

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³ The abbreviations used are: UPase, uridine phosphorylase; MEF, murine embryo fibroblast; FU, 5-fluorouracil; EMSA, electrophoretic mobility shift analysis; RT-PCR, reverse transcription-PCR.

autosequencing by the Protein and Nucleic Acid Chemistry Facility of the Yale Cancer Center (Yale University).

Primer Extension Analysis. A 33-mer antisense primer corresponding to bases +133 to +101 of the murine UPase cDNA sequence was end-labeled with T4 polynucleotide kinase using [γ - 32 P]ATP. Total cellular RNA (15 μ g) from colon 26 tumor, which presents high UPase expression, was hybridized with 10^5 cpm of the 32 P-labeled oligonucleotide by heating at 90°C for 5 min in 20 μ l of hybridization buffer [50 mM Tris-Cl (pH 8.3), 150 mM KCl, and 1 mM EDTA] followed by incubation at 42°C overnight. The DNA-RNA hybrid was then collected by ethanol precipitation and dissolved in 20 μ l of reverse transcription buffer [50 mM Tris-Cl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, and 0.5 mM deoxynucleotide triphosphate]. The primer was extended by SuperScript II RNaseH⁻ Reverse Transcriptase (Life Technologies, Inc.) at 42°C for 1 h. After completion of the reaction, the samples were extracted with phenol-chloroform, precipitated with ethanol, and analyzed on 6% denaturing polyacrylamide gel. The same primer was used in sequencing reactions with the Thermo Sequence radiolabeled cycle sequencing terminator kit (Amersham Pharmacia Biotech).

Plasmid Constructions. To create the p-1619/+84 plasmid, the genomic clone including the 5'-flanking region of UPase gene was digested with *Xba*I (position -1619 and +84). The promoterless pGL3 luciferase reporter gene vector (Promega) was digested by *Hind*III. The single-strand ends of the released fragment and linear vector were made double-stranded using the Klenow fragment of DNA polymerase I. The fragment was then blunt-end ligated into the *Hind*III site of the pGL3 vector. A series of luciferase expression constructs, based on the p-1619 plasmid, that contained various lengths of the 5'-upstream sequence of the UPase gene were prepared using different restriction enzymes but maintaining the same 3' end digested by *Sma*I. These include p-1470 (*Acc*I), p-1081 (*Bst*EII), p-570 (*Nhe*I), p-445 (*Bgl*II), p-274 (*Bst*XI), p-212 (*Pvu*II), and p-84 (*Eco*RV). All of the restriction enzymes used for the plasmid construction, except for *Acc*I, present only a single cutting point on the UPase promoter. For the fragment obtained at the *Acc*I cutting site, we conducted a partial digestion for the plasmid construct.

Wild-type p53 plasmid construct was kindly provided by Dr. Albert B. Deisseroth (Yale University; Ref. 19).

Transfection and Luciferase Assays. All of the transfections were done in triplicate in 6-well plates. Approximately 10^5 cells/well were seeded 24 h before transfection. Plasmids were transfected into cells using LipofectAMINE reagent (Life Technologies, Inc.). The cells were incubated in transfection buffer (serum-free DMEM) for 3 h and then harvested after 45 h in culture. Luciferase assays were performed using the Dual Luciferase Assay System (Promega) that already contains an internal control detectable simultaneously with the luciferase reporter gene. Each experiment was conducted at least in triplicate.

Nuclear Extract Preparation and EMSA. Nuclear extracts were prepared according to the method of Lassar *et al.* (20). To obtain the nuclear extract, cell-containing plates were washed three times with Tris-buffered saline solution and 2.5 ml of lysis buffer [20 mM HEPES (pH 7.6), 20% glycerol, 10 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.1% Triton X-100, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml leupeptin, 10 mg/ml pepstatin, and 100 mg/ml aprotinin] was added to each 15-cm diameter dish. Cell were removed by centrifugation for 5 min at 2,000 rpm at 4°C. Nuclei were resuspended at 2.5×10^7 nuclei/ml in nuclear extraction buffer (identical to the lysis buffer with the addition of 500 mM NaCl). Nuclei were gently shaken for 1 h at 4°C, centrifuged at 10,000 rpm for 10 min, frozen quickly in liquid nitrogen, and stored at -80°C.

The double-stranded DNA probe used in the gel mobility shift assays was the following: 5'-CACCCCATTCCTCCCAAGCCTTGCTCTTCGCCAGA-3' from the position -317 bp to -283 bp (coordinated relative to the primary transcription start site) of the murine UPase promoter, synthesized and purified (using oligonucleotide purification cartridge from Perkin-Elmer) by the Protein and Nucleic Acid Chemistry Facility of the Yale Cancer Center (Yale University). This probe included the p53-binding motif. EMSA was performed according to the manufacturer's instructions for Gel Shift Assay Systems (Promega). Briefly, 2 μ g of nuclear extract were mixed with 1 ng of each labeled probe in binding buffer containing 0.5 mM EDTA, 0.5 mM DTT, 4% glycerol, 1 mM MgCl₂, 50 mM NaCl, 10 mM Tris-HCl (pH 7.5), and 0.05 mg/ml poly(dI-dC)poly(dI-dC) and incubated for 20 min at room temperature. To demonstrate the sequence-specific binding, a 100-fold excess of the same

unlabeled probe and other unlabeled probes as specific and nonspecific competitors were included in a separate reaction. The reaction mixtures were then separated on a 6% nondenaturing polyacrylamide gel at room temperature in $0.5 \times$ TBE buffer at 100 V for ~3 h. The gel was transferred to Whatman No. 3MM paper, dried, and exposed to X-ray film overnight at -70°C with an intensifying screen.

DNase I Footprinting. Single end-labeled probe (1×10^{-4} cpm) from -445 to -274 bp of murine UPase promoter was incubated with 30 μ g of nuclear extract from EMT6 cells in binding buffer containing 25 mM Tris (pH 8.0), 50 mM KCl, 6.25 mM MgCl₂, 0.5 mM EDTA, 10% glycerol, 0.5 mM DTT, and 2 μ g of poly(dI-dC)poly(dI-dC) on ice for 15 min in a total volume of 50 μ l. Then, 50 μ l of a solution containing 5 mM CaCl₂ and 10 mM MgCl₂ was added to the mixture. After incubating the samples at room temperature for 1 min, 0.8 units of DNase I were added to each tube, and the incubation was continued at room temperature for an additional 1 min. The reaction was stopped by adding 90 μ l of a solution containing 200 mM NaCl, 30 mM EDTA, 1% SDS, and 100 μ g/ml yeast tRNA. The DNA samples were purified by a phenol-chloroform extraction and ethanol precipitation and resuspended in loading buffer (0.1 M NaOH-formamide, 0.1% xylene cyanol, and 0.1% bromophenol blue). After denaturation at 75°C, the samples were separated by electrophoresis on a 6% sequencing gel.

Quantitative RT-PCR. Total RNA was extracted from MEF p53 +/+ and p53 -/- cells using TriZol (Life Technologies, Inc.). For RT-PCR analysis, DNase I-treated total RNA was reverse transcribed using oligo(dT) and SuperScript II (Life Technologies, Inc.). The cDNAs were amplified using mUPase primers P190 (5'-GAC GAA GTG ATT GAC TGG TGG TC-3') and P720a (5'-CGC TGC AAG TGC CAA TGC G-3') together with the internal control mS16 primers (5'-AGG AGC GAT TTG CTG GTG TGG A-3' and 5'-GCT ACC AGG CCT TTG AGA TGG A-3'). PCR products were separated on a 1.0% agarose gel and stained with ethidium bromide.

Western Blot and Enzymatic Activity Assay. Primary antibodies against UPase were prepared at Yale University (rabbit anti-UPase polyclonal antibody to human recombinant UPase). The Western blot method is reported in detail (9). UPase enzymatic activity was measured by uridine conversion to uracil, using TLC chromatographic separation as described (21). Briefly, cell lysates were prepared using 50 mM Tris-HCl, and the supernatant after the 30,000 \times g centrifugation was analyzed for both enzymatic activity and Western blot. Enzymatic activity was measured as the percentage of conversion of [3 H]uridine to [3 H]uracil after separation on silica TLC plates (Kieselgel 60; Merck), using an 85:15:5 mixture of chloroform, methanol, and acetic acid, respectively.

RESULTS

Cloning and Sequencing of the Murine UPase Gene 5'-Flanking Fragment. We have cloned previously a 1.2-kb fragment of the UPase gene promoter (16). Now, to characterize the promoter region of the murine UPase gene and study the possible regulatory elements that control UPase transcriptional activity, we isolated a genomic DNA clone that contains the immediate full-length 5'-flanking UPase sequence (1703 bp). A murine ES-129/SvJ bacterial artificial chromosome library was screened using the murine UPase cDNA probe. The 1703-bp *Xba*I/*Xba*I fragment immediately upstream of the murine UPase gene containing 84 bp of the 5'-untranslated region of cDNA was subcloned into a pBluescript KS II cloning vector (p-1619/+84), and the orientation was verified by sequencing both DNA strands. Analysis of the nucleotide sequence of the 5'-flanking region of the murine UPase revealed the absence of a canonical TATA box. At the 5' end of UPase promoter (from -1619 to -1110) are a series of microsatellite and minisatellite repeat bases (Fig. 1). A potential p53 binding motif, AGcCTTGTCc, is located in the sequence -303 bp to -294 bp presenting one base difference (the small-case c) from the consensus p53 binding sequence, 5'-PuPuPuC(A/T; A/T)GPpPyPy-3' (22).

Mapping of UPase Transcription Start Sites. The transcription initiation sites were determined to facilitate the preparation of the UPase gene promoter constructs. Mapping of the transcription start

A



B

-1617 GTTCTAGAAA GCTGCATGGA TACTGGTATC ATCAGAGTTT CTAGTTCCT
 -1567 TTATGCTGCT TTAGTGATAA ACACGGAGAC CGAAGTTTGT AAAAAATTA
 -1517 TATATATAAT AATAATAAAA ATTAATAAAA ATAAGGGAAA TGTTTTTGT
 -1467 TACATTGAA GCAGAGCTTT TTTTTCCTTC CAGTTTGGCT TCAGACCCCTG
 -1417 ACCTCTAGGT TCTCCAGCG TTCCCTCAGTC TCCCTCCTTC ACACCTTATC
 -1367 GAAAGGCTTT TCCCCCATGT AATCCAGACA TTTTGGGTT TTCCCTCTTT
 -1317 CCTTCCTTCC TCCCTCCTTT CCTTCCCTCC TTCCCTCCTT CCTTCCCTTC
 -1267 TTCTCCCTCC CTTCCCTCCC TCCTCCCTCC CTTCCCTCCC TCCTCCCTTC
 -1217 CCTCCCTCCG TCCTCCCTCC TCTCTCTCTC TCTCTCTCTT TCTTCTTTTC
 -1167 TTTCTTCTTT TCTTCTTTTC TTCTTCTTCT TCTTCTTTTC TTTTCTCTCC
 -1117 CCTCCCCACA TGGTGAATCT CCTGGCTCTC GTCCCTGGGT GACCTTAGCC
 -1067 GAGAGTGAGA GCAGCTTCCC GCAATAAACC AGCATTGAAT GTTATCTAAC
 -1017 CTGTGCTGAG TTGGCTCAAA ATAACTCATC ACTTCTTATT TTGCTGGCAC
 -967 TAGAATAACA ATGAACAGAA TGGGTTCCCTG GTGAGATTGG ATAAACAAGG
 -917 GCTGGTACTC GTCTCACACC AACTTTACAA TCATGGGAGG GGCAGCTTCT
 -867 ACTTGCACTT GCCTCAGGAA CAGGGAAGA GCAGCAGTTA GGGAAAGAGG
 -817 AGCCAGTTAT TCTCTAANA ACATATTGCT TTAGTGGCT ATGACATAGT
 -767 TGGGGTGTG TCCCGCTTT TTGTTACTTA ACAGTAATGG GGCCTTATTC
 -717 TCTGGTCTC TTGCCTGGA TAGCCCTCC CTCTTGTTA TGCTAAAGTT
 -667 TAATGGAGAT CCACCAAGC CTTGGGATGC TGGCTCACAA CCTTGGCCCT
 -617 TGTTTTTTTT TCAGCCAGAC GCAGAGCAGC TGGAGGAGG CTGTGTGTC
 -567 TAGCTTTACT CTCTGCACA GACTTTGATG ATGGTTTTCAG GAGTGGAGGA
 -517 GAAATTCGCT GACACGATTT TGTGCTCAT CTGAGTCTGG TTTCACTTGT
 -467 AGATTCTGAT ACATGAATGG GAGAGATCTT CATCTCTCA GAACAGGAT
 -417 CATAGGCAGT TCTGCTACGG CTGATGCTCA GGAGCAGAG TCCTGCTGG
 -367 AGGCAACAG TGATTGTTGC CCGCTCTACT CTGCTCTCC CCTTCCCTCC
 -317 CACCCCATC CCAAGCCTT GTCCTTTGCG CAGAGTGCCA GCTCGCTGGG
 p53
 -267 GAAGCCAAGC TAGGAATGT GTGAGAATTA GAGGCCACTG GCAGGCCCGG
 -217 ATGGCAGCTG GGGCGGGGA GTACAGTGC AGGGCAGGGC AGAGTGCCT
 -167 TCTGCAGAT AGCTGTGACT GAATGAATGA GTCAATTCCC CTACTGGGCA
 -117 AGGCTCCCC TGGCGTCCC ACAACTACGG AGGATATCCC TGGATCAGGA
 -67 AGGACCTGA GGGGAAGACA ATAAATATCA AAACAAAACA GAACTGGCA
 -17 CGGGTACTG AARGGACAC GTTTACACA GCCTAGCTCT GCATTCTGA
 +1
 CCAGTCCAC AOGGCCATC ATCTCTGGTA CATTCTTCA TCATTCTAG
 AAAAATGG ACTTGGACCC TGGCACTAGA GGTGACCT
 +92

Fig. 1. Restriction map and sequence of the 5'-flanking region of the murine UPase gene. A, schematic restriction map for the subcloned UPase gene promoter region. B, nucleotide sequence of the cloned UPase gene promoter and part of exon 1. The potential p53-binding motif is underlined; bold and * base represents the only difference from the consensus-binding sequence. Larger arrow, major transcription initiation site; smaller arrow, minor transcription initiation site.

site of the murine UPase gene was accomplished by primer extension analysis. For the primer extension assay, we used the primer that is located +133 to +101 bp downstream of cDNA sequence and complementary to the minus DNA strand of the murine UPase cDNA. As shown in Fig. 2, Lane 1, the primer extension reaction yielded 133-bp and 225-bp products using RNA extracted from murine colon 26, a tumor cell line with high UPase expression. Sequencing reactions were performed with the same primer on the noncoding DNA strand from murine colon 26 cells to serve as the sequencing ladder and determine the size and nucleotide position of the start sites (Fig. 2). These experiments determined that two transcriptional start sites are located +1 and +92 bp of the most 5' end of the reported cDNA sequence.

Functional Characterization of the Murine UPase Promoter. Several 5' deletions of UPase gene promoter transgene constructs were generated to define the DNA regulatory elements. Two distinct cell lines, EMT6 and NIH 3T3, were cotransfected with the UPase plasmid DNA constructs and the pRL-TK vector as an internal control for transfection efficiency. The full-length promoter construct (-1619/+84) was consistently expressed in EMT6 and NIH 3T3 cells. Progressive 5'-deletion mutations of the full-length promoter revealed a pattern of functional activity in the transfected cells (Fig. 3). The plasmids containing 5' deletions of various lengths from

-1081 to -445 bp produced a very modest decrease in promoter activity. Additional deletions from -445 to -274 bp led to an increase in promoter activity higher than the expression obtained with the full-length promoter. Deletion to -84 bp resulted in an extreme reduction of the activity in EMT6 cells with a more modest effect in NIH 3T3 cells (Fig. 3).

Suppression of the UPase Gene Promoter Activity by Wild-type p53. Using transient-expression assays in EMT6 and NIH 3T3 cells, cotransfection of UPase promoter construct p-1619/+84 with the wild-type p53 construct resulted in significantly less luciferase activity compared with a cotransfection experiment using an empty vector, an average of 4- and 3.5-fold reduction in relative luciferase activity in EMT6 and NIH 3T3 cells, respectively. To locate the DNA element(s) in the UPase promoter that mediate the transcriptional regulation by wild-type p53, we tested a series of 5'-deletion mutants of the UPase promoter for p53 sensitivity in cotransfection assays (Fig. 4). The transcription of 5' deletion constructs between -1619 and -445 bp was suppressed by wild-type p53, whereas the constructs missing the putative p53-binding element were not affected. These data indicate that the region between -445 and -274 bp in the UPase promoter is susceptible to regulation by p53. The nucleotide sequence analysis of the murine UPase gene 5'-flanking region had identified a potential p53 binding site at -303 to -294 bp, AGcCTTGTCC. This binding motif differs in one base (small-case base) from the consensus element of p53 binding. In p53 null MEF cells, unlike EMT6, NIH 3T3, and MEF p53 +/+ cells, the luciferase activity was not altered when the constructs deleted from -445 to -274 bp were cotransfected. However, UPase gene promoter activity was repressed significantly by cotransfection of wild-type p53 in the p53 knockout MEF cells with the p-1619/+84 and p-445/+84 constructs. As expected, no change in luciferase activity was observed for the construct p-274/+84 that does not contain the p53 binding sequence (Fig. 5).

p53 Suppresses UPase mRNA and Protein Expression. To additionally confirm our observation of p53 down-regulation of UPase gene expression, we evaluated the UPase mRNA and protein expression in MEF p53 -/- and p53 +/+ cells using quantitative RT-PCR, Western blot, and enzyme activity. The data reported in Fig. 6 indicated that the level of UPase mRNA expression in MEF p53 -/- cells was elevated compared with the MEF p53 +/+ control cells. The Western blot showed that UPase protein expression was repressed



Fig. 2. Determination of the transcription start site of the murine UPase gene. The transcriptional start site was mapped by primer extension analysis. For the primer extension reaction, an oligonucleotide primer corresponding to complementary to +133 to +101 nucleotides of the UPase cDNA was end-labeled with [γ - 32 P]ATP and hybridized with 15 μ g of total RNA from colon 26 tumor cells. Lane 1, primer extension with murine colon 26 cell RNA. Lanes 2-5 correspond to A, G, T, and C nucleotide sequencing reaction using the same primer. Arrows designate the primer extension products. Two transcriptional start sites are located +1 and +92 bp, respectively, of the most 5' end of the reported cDNA sequence.

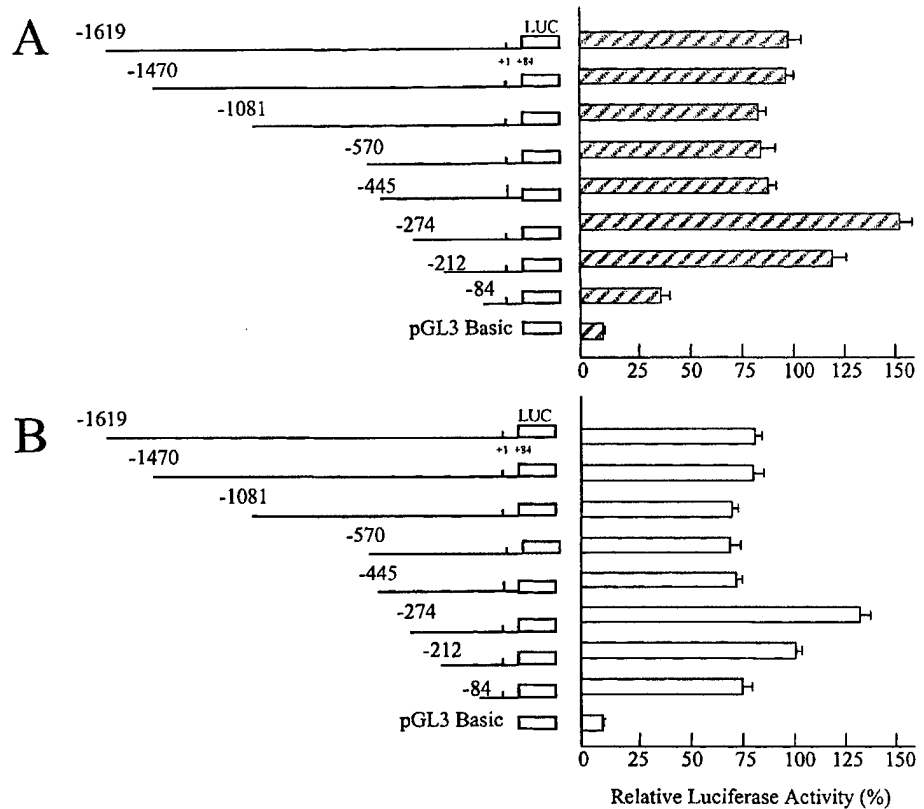


Fig. 3. Deletion analysis of the murine UPase promoter region in (A) EMT6 and (B) NIH 3T3 cells. *Left*, schematic representation of the 5'-flanking region of the UPase/reporter gene constructs used in the transient transfection analysis of promoter activity. The restriction enzyme sites used in the preparation of the constructs are indicated in Fig. 1. The UPase luciferase constructs were cotransfected with a control plasmid pRL-TK and assayed 48 h after transfection. The luciferase activity elicited by each deletion mutant is expressed as percentage of the activity obtained by the full-length (1703 bp) promoter activity in EMT6 cells. *Bars*, SE from three samples in three independent experiments.

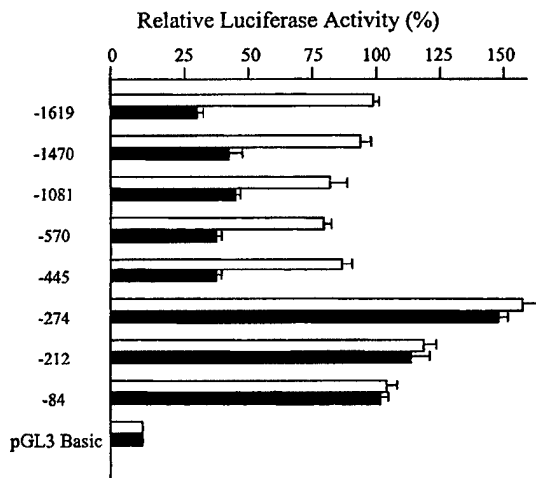


Fig. 4. Suppression of UPase promoter activity by wild-type p53 in NIH/3T3 cells. Cotransfection of murine UPase promoter-reporter gene constructs with wild-type p53 in NIH 3T3 cells. ■, luciferase activity of the UPase constructs cotransfected in the presence of wild-type p53 constructs; □, luciferase activity of the constructs cotransfected with empty vectors; *bars*, \pm SE.

by p53 in MEF p53 $+/+$ cells compared with MEF p53 $-/-$ cells. The enzyme activity of UPase in MEF p53 $-/-$ was \sim 30-fold higher than in the MEF p53 $+/+$ cells (Fig. 7).

p53-specific Binding to the UPase Promoter. To complete the elucidation of the p53 regulation of the UPase gene, a gel mobility shift assay (EMSA) was performed. A synthesized 34-bp DNA fragment (-317 to -284 bp) containing only the p53-binding element as a probe and nuclear proteins including the full-length wild type p53 protein (393 amino acids) were used in the EMSA. The mobility of the

labeled DNA probe was altered in the presence of the p53 protein (Fig. 8) because of the formation of a binding complex. The amount of the shifted complex was diminished by increasing the concentration of self-competitor (nonradiolabeled p53 probe) but not by the addition of nonspecific competitor (SP1 and AP2). The SP1 and AP2 probes used as nonspecific competitors were supplied by Gel Shift Assay Systems (Promega). DNase I footprinting indicated that the binding site was located from -294 to -303 bp of UPase promoter, the position of the p53 binding motif (Fig. 9). This p53-binding motif is 90% homologous to the reported consensus-binding element of p53.

DISCUSSION

To study the UPase gene regulation and to understand the alterations in its expression observed in human tumors, we have isolated

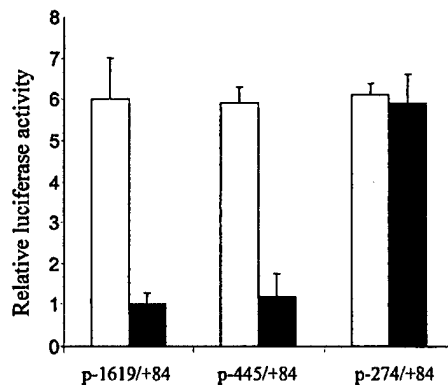


Fig. 5. Suppression of UPase promoter activity by wild-type p53 in MEF p53 $-/-$ cells. Cotransfection of UPase promoter constructs p-1619/+84, p-445/+84, and p-274/+84 with the wild-type p53 construct (■) and a control vector (□); *bars*, \pm SE.

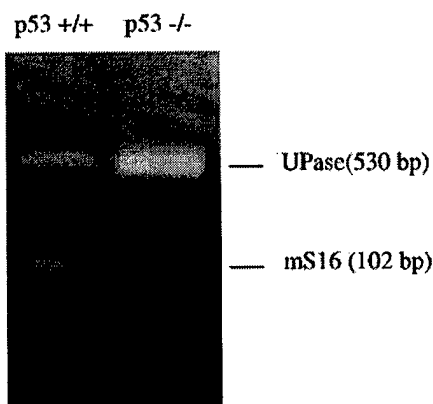


Fig. 6. Suppression of UPase gene mRNA expression by wild-type p53. UPase mRNA expression in MEF p53 +/+ and p53 -/- cells was detected by quantitative RT-PCR. The mS16 primers were used as internal controls in the same PCR reaction.

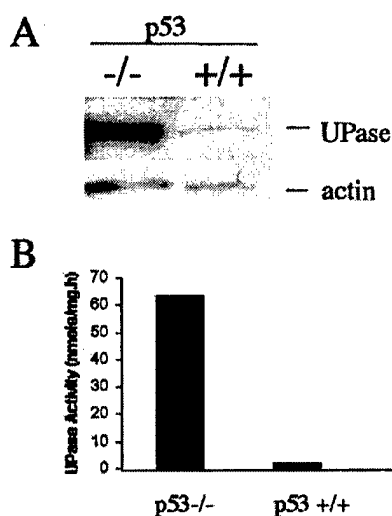


Fig. 7. Western Blot analysis and enzymatic activity of UPase in p53 -/- and p53 +/+ cells. The cell extracts were analyzed by Western blot after SDS-PAGE separation and the enzymatic activity determined as described in "Materials and Methods."

and sequenced a 1703-bp fragment corresponding to the promoter region of murine UPase and characterized its functional activity in EMT6 and NIH 3T3 cells. DNA sequencing of UPase 5'-flanking region has revealed that the UPase gene lacks basal elements like the TATA box or an initiator sequence. The absence of such sites and the presence of SP1 sites, including one in the basal promoter of UPase (16), might have been expected to result in the initiation of transcription at several locations. Two transcription start sites were mapped at +1 and +92 bp of the most 5' end of the reported murine UPase cDNA sequence. Promoter constructs containing the whole 1703 bp of 5'-flanking sequence showed comparable luciferase activity in both EMT6 and NIH 3T3 cells. The promoter activity was altered in EMT6 and NIH 3T3 cells after progressive 5'-deletion mutations of the promoter sequence. We found that deletions from -1619 to -445 bp produced a slight decrease in promoter activity. However, additional deletion to -274 bp resulted in the elevation of the activity to levels higher than the full-length promoter. Cotransfection of the UPase promoter constructs with the wild-type p53 construct resulted in significantly less luciferase activity compared with a cotransfection experiment using an empty vector; however, this phenomenon disappeared with the deletion from -445 to -274 bp. This indicates the existence of a p53-inhibitory element in this promoter sequence. A

significant promoter activity was also detected between -274 and -84 bp, suggesting that this region contains information necessary for an active transcription of UPase in these cells. In contrast, an additional deletion to -84 resulted in an extreme reduction of activity in EMT6 cell but not in 3T3 cells. A putative IRF-1 binding site is located in this region and could possibly play a different regulatory role in these two cell lines. Additional elucidation of this regulatory element and its function in UPase expression is currently under investigation to clarify the role of cytokines in FU activation and ultimately in pyrimidine-based cancer therapy.

p53 functions as a transcription factor and regulates a number of target genes at the transcriptional level. The central region of the p53 protein interacts with the promoter of the target gene in a sequence-specific manner, binding to two copies of a consensus element {5'-PuPuPuC(A/T; A/T)GPyPyPy-3'; Ref. 22}. Wild-type p53 efficiently binds to this sequence and transactivates expression of the target

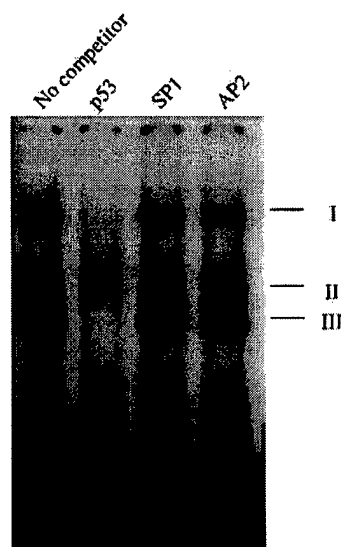


Fig. 8. Electrophoretic mobility shift assay. A radiolabeled double-stranded DNA probe (34-bp long) containing the p53 promoter-binding region was incubated with NIH 3T3 cell extract and separated on a 6% polyacrylamide gel. To determine binding specificity, cold p53 and other control probes were added as specific and nonspecific competitors, as indicated above the corresponding lanes.

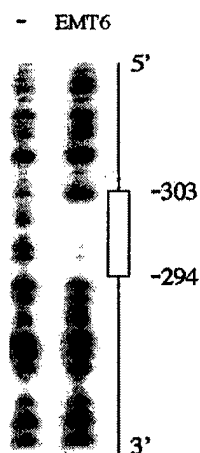


Fig. 9. DNase I footprinting analysis of the murine UPase promoter. 5'-End-labeled fragment, including the sequence from -445 to -274 bp was incubated without extract or with 2 μ g of EMT6 nuclear extract proteins and partially digested with DNase I. Right, the putative p53 binding site sequence is indicated by \square .

genes (22–26). p53 can also repress a wide variety of cellular and viral promoters (27). There are several possible mechanisms to account for the inhibitory activity of p53 on promoter activity. First, p53 inhibition might directly or indirectly inactivate a critical component of the transcription machinery, leading to general inhibition of transcription. However, we found that the promoter of UPase at p-274 still had a high promoter activity even in the presence of cotransfected p53, indicating that the transcriptional machinery is still active under our experimental conditions.

A second possibility is that p53 may inhibit the activity of the promoter by "squenching" or sequestering general transcription factors (28). Squenching would be expected to inhibit the activity of promoters lacking p53; in some promoters the p53 regulation occurs through binding to the TATA-binding protein causing suppression of the promoter activity (29, 30).

A third case is that p53 might inhibit the promoter by directly or indirectly blocking the activity of other factors important for the promoter activity, like SP1 (31), CCAAT-binding factor (32), cyclin AMP response element-binding protein (33), and glucocorticoid receptors (34).

A fourth alternative, which appears to be the most likely in the case of the UPase gene, is that the inhibition is attributable to the presence of a specific p53-negative response element that is distinct from the core promoter region, as observed previously for the Rb (35), bcl-2 (36), and topoisomerase IIa (37) promoters. To explore this possibility, we first analyzed the effects of p53 on UPase promoter activity. We found that the deletion from -1619 to -445 of the UPase promoter had no effect on the ability of p53 to inhibit gene expression; however, the inhibitory activity was altered when the promoter region between -445 and -274 bp was deleted. Using transient-expression assays in EMT6 and NIH 3T3 cells, cotransfection with the wild-type p53 construct resulted in significantly less luciferase activity in the constructs from -1619 to -445, whereas further deletions of the promoter did not affect the activity. These data indicate that the region between -445 and -274 bp is susceptible to regulation by p53 in the UPase promoter. This phenomenon was additionally confirmed in p53-nullified cells. Sequencing analysis of this region found a putative p53-binding motif AGcCTTGTC located at -303 to -294. This binding motif differs in one base (small-case base) from the consensus-binding element of p53. The gel mobility shift assay and DNase I footprinting have indicated that this putative regulatory motif exhibited specific binding with the p53 protein.

p53 has been shown to be activated by ribonucleotide depletion caused by antimetabolite drugs such as PALA (*N*-(phosphonacetyl)-L-aspartic acid even in the absence of DNA damage (38). As mentioned previously, the phosphorolytic activity of Upase-regulating intracellular uridine levels reveals the critical role of this enzyme in modulating the pyrimidine salvage pathway. The suppressive regulation of p53 on UPase gene indicates the presence of a negative control of the pyrimidine salvage pathway by p53 through UPase, probably as a cellular self-protection mechanism in case of ribonucleotide depletion. p53 has been shown previously to: (a) activate genes that initiate apoptosis to eliminate damaged cells and protect an organism from more severe damage; and (b) cause cell-cycle arrest after DNA damage to prevent the replication of altered DNA. However, thus far, indication of the contribution of p53 to damage repair is quite limited. A recent report (39) has described a p53-induced gene, *p53R2*, that encodes for a protein similar to one of the two subunits of ribonucleotide reductase, the rate-limiting step in the conversion of ribonucleotides to deoxyribonucleotides. The p53-regulated R2 subunit is found in the nucleus, and its expression is induced by cellular damage (γ -radiation and Adriamycin treatment), suggesting that when repair

is needed the nuclear precursors have to be concentrated near the site of damage.

Somehow the p53-regulated suppression of UPase expression exerts similar functions to the control that p53 has on *p53R2*. A cellular damage causing loss or imbalance in the ribonucleotide pools could cause activation of p53 leading to suppression of UPase expression and activation of the pyrimidine salvage pathway to replenish the affected pyrimidine nucleotide pools. These two p53-regulated mechanisms provide a new level of control on ribo- and deoxyribonucleotide pools. Under normal replication conditions, the regulating mechanisms that control the appropriate balance of nucleotides are based mostly on the direct feedback regulation of the biosynthetic enzymes by some of the precursors or final products. For example, in the case of the pyrimidine nucleotide biosynthesis CTP or UTP (depending on the organism) inhibit the activity of aspartate transcarbamoylase that catalyzes the first reaction of the *de novo* synthesis. Similarly in the deoxyribonucleotide biosynthesis, dGTP and dTTP stimulate the reduction of ADP and GDP to the corresponding deoxyribonucleotide forms. The report on *p53R2* and our data on UPase possibly indicate that in case of cellular damage a more sophisticated level of regulation is triggered to more rapidly provide precursors for nuclear repair.

The elucidation of the negative control regulation of p53 on the UPase gene promoter and UPase expression could also have considerable implication at the clinical level on the therapeutic outcome in the presence of tumors with specific p53 mutations when undergoing antimetabolite-based cancer therapy.

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Uridine Phosphorylase Association with Vimentin

INTRACELLULAR DISTRIBUTION AND LOCALIZATION*

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Uridine phosphorylase (UPase), a key enzyme in the pyrimidine salvage pathway, is associated with the intermediate filament protein vimentin, in NIH 3T3 fibroblasts and colon 26 cells. Affinity chromatography was utilized to purify UPase from colon 26 and NIH 3T3 cells using the uridine phosphorylase inhibitor 5'-amino benzylacetylouridine linked to an agarose matrix. Vimentin copurification with UPase was confirmed using both Western blot analysis and MALDI-MS methods. Separation of cytosolic proteins using gel filtration chromatography yields a high molecular weight complex containing UPase and vimentin. Purified recombinant UPase and recombinant vimentin were shown to bind *in vitro* with an affinity of 120 pM and a stoichiometry of 1:2. Immunofluorescence techniques confirm that UPase is associated with vimentin in both NIH 3T3 and colon 26 cells and that depolymerization of the microtubule system using nocodazole results in UPase remaining associated with the collapsed intermediate filament, vimentin. Our data demonstrate that UPase is associated with both the soluble and insoluble pools of vimentin. Approximately 60–70% of the total UPase exists in the cytosol as a soluble protein. Sequential extraction of NIH 3T3 or colon 26 cells liberates an additional 30–40% UPase activity associated with a detergent extractable fraction. All pools of UPase have been shown to possess enzymatic activity. We demonstrate for the first time that UPase is associated with vimentin and the existence of an enzymatically active cytoskeleton-associated UPase.

The ability of cells to maintain a constant supply of pyrimidine and purine nucleotides is dependent on both *de novo* synthetic and salvage pathways. The relative importance of either the *de novo* or the salvage pathway in the maintenance of nucleotide pools is variable and dependent on the cell or tissue type (reviewed in Refs. 1, 2). Uridine phosphorylase (UPase)¹ is an important enzyme in the pyrimidine salvage pathway and catalyzes the reversible phosphorolysis of uridine

to uracil (3–5). This enzyme is present in most human cells and tissues analyzed, and it is frequently elevated in tumors (4, 5). Enzymatic activity may also be induced in different cell lines by cytokines such as tumor necrosis factor- α , interleukin-1 α , and interferon- α and - γ as well as vitamin D₃ (6–8). UPase has also been shown to be important in the activation and catabolism of fluoropyrimidines (9, 10), and the modulation of its enzymatic activity may affect the therapeutic efficacy of these chemotherapeutic agents (11, 14).

UPase also plays an important role in the homeostatic regulation of both intracellular and plasma uridine concentrations (11–14). Uridine plasma concentration is under very stringent regulation (15, 16) mostly as a function of liver metabolic control (17), intracellular UPase enzymatic activity (11–14), and cellular transport by both facilitated diffusion and Na⁺-dependent active transport mechanisms (18–25). Uridine is critical in the synthesis of RNA and biological membranes through the formation of pyrimidine-lipid and pyrimidine-sugar conjugates (reviewed in Ref. 1), and it has been associated with the regulation of a number of biological processes (1).

Whereas there is evidence that uridine and its nucleotides are associated with different biological processes (reviewed in Refs. 1 and 2) the precise mechanisms that allow uridine to modulate these processes are not well defined. Uridine has been shown to cause increased vascular resistance (25), hyperpolarize amphibian and rat ganglia (26, 27), potentiate dopaminergic transmission, and reduce anxiety in animal models (28, 29), as well as potentiate barbiturate effects and induce sleep in rats (30, 31). Uridine perfusion has been shown to maintain brain metabolism during ischemia (32, 33) and to rapidly restore myocardial ATP and UDPG (34) following myocardial ischemia.

Characterization of UPase intracellular localization and association with other proteins may provide some insight into the mechanisms that control uridine metabolism in cells. In this study, we characterize the cellular distribution and the associated enzymatic activity of UPase.

EXPERIMENTAL PROCEDURES

Materials—Primary antibodies against UPase were prepared at Yale (rabbit anti-UPase polyclonal antibody to human recombinant UPase) (35), or purchased from Sigma Chemical Co. (St. Louis, MO) (mouse anti-Vimentin Clone V9). Secondary antibodies, horseradish peroxidase-conjugated donkey anti-mouse or anti-rabbit (fluorescein isothiocyanate-conjugated and Texas red-conjugated donkey anti-rabbit) or (fluorescein isothiocyanate- or Texas red-conjugated sheep anti-mouse) were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Antibodies for vimentin (V9 or monoclonal antibody clone 13.2) or polyclonal goat anti-vimentin were purchased from Sigma (St. Louis, MO). Immunoaffinity support for antibody immobilization and antibody purification was purchased from Pierce. Affi-Gel-10-activated agarose and protein assay reagent were purchased from Bio-Rad (Hercules, CA). 5'-Amino benzylacetylouridine and benzylacetylouridine (BAU) were

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¹ The abbreviations used are: UPase, uridine phosphorylase; MALDI-MS, matrix-assisted laser desorption ionization-mass spectrometry; BAU, benzylacetylouridine; NDPK, nucleoside diphosphate kinase; RIPA buffer, radioimmune precipitation buffer; PBS, phosphate-buffered saline; BSA, bovine serum albumin.

a generous gift from Dr. S. Chu at Brown University (Providence, RI).

Tissue Culture—Cells (NIH 3T3 and colon 26) were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Sigma) and were maintained in a humidified atmosphere containing 5% CO₂ in air. NIH 3T3 cells were purchased from ATCC, and colon 26 cells were derived from the solid tumor (grown *in vivo*) using standard procedures. Colon 26 cells were originally obtained from Southern Research Institute (Birmingham, AL) and grown subcutaneously in BALB/C mice.

Affinity Chromatography—The BAU affinity column was prepared by coupling amino-BAU to an Affi-Gel-10-agarose matrix as previously described (35). Antibody affinity columns were prepared using a cyanoborohydride coupling procedure (Pierce) following manufacturer guidelines. Vimentin antibody was purchased from Sigma (goat anti-vimentin and clone V9 mouse anti-vimentin were both used to prepare affinity columns).

In all affinity chromatographic procedures, cells (colon 26 or NIH 3T3) or solid tumors (colon 26) were lysed in 50 mM Tris-HCl, pH 7.4 (containing 2 mM dithiothreitol), using a Dounce homogenization apparatus or tissue homogenizer, respectively. Cell lysates were prepared at 4 °C, and the supernatant from the 30,000 × *g* centrifugation was applied to the column. Following sample application, the column was washed with ~10 volumes of buffer or until the column effluent contained no detectable protein (Bio-Rad Coomassie Brilliant Blue G-250 dye reagent). Sample elution was accomplished using 4–8 column volumes of 0.1 M glycine (antibody affinity column) or 20 mM uridine (BAU affinity column). The eluted proteins were concentrated using Ultra-free-4 centrifugal filters (Millipore) and analyzed by SDS-polyacrylamide gel electrophoresis and Western blot techniques.

Gel Filtration Chromatography—Cell lysates from colon 26 cells grown as a monolayer in 150-cm² dishes were prepared in 20 mM Tris-HCl, 137 mM NaCl pH 7.4 (TBS). Cytosolic fractions were centrifuged at 100,000 × *g* for 1 h and applied to a Sephacryl-s300 column (Amersham Pharmacia Biotech) that had been calibrated using known molecular weight markers (Amersham Pharmacia Biotech). The mobile phase was 20 mM Tris-HCl, 137 mM NaCl with a flow rate of 1 ml/min. Protein elution of standards was monitored in fractions using the Bio-Rad Coomassie Blue G-250 dye reagent.

In Vitro Protein Binding Assay—The ability of UPase and vimentin to form a stoichiometric complex was evaluated using purified recombinant vimentin (cytoskeleton) directly applied to nitrocellulose membranes. Known quantities of recombinant vimentin (431–3.4 μM) or similar protein concentrations of BSA as a negative control was applied to nitrocellulose membranes and allowed to air dry. Membranes were blocked for 1 h at room temperature in 5% nonfat milk. Purified UPase was directly coupled to horseradish peroxidase (Pierce) and used to probe the nitrocellulose membranes containing BSA and vimentin. The concentration of UPase bound to vimentin was calculated from a standard curve containing 5–0.15 μg of horseradish peroxidase-UPase directly applied to nitrocellulose and exposed to ECL (Amersham Pharmacia Biotech) at the same time as the vimentin membrane.

Construction of Prokaryotic Expression Vector and Preparation of Recombinant UPase Protein—Large quantities of human UPase recombinant protein were prepared using the pQE expression system (Qiagen, Santa Clarita, CA). Briefly, human UPase cDNA was released from pMal/Hup with *Eco*RI and *Hind*II (35) and inserted into pBluescript KS II vector (Stratagene, La Jolla, CA) generating a pBlue/Hup construct. Then, human UPase cDNA was released by *Bam*HI and *Hind*III and inserted into a pQE 30 vector, generating a prokaryotic expression vector, pQE/Hup, that produces the full-length human UPase recombinant protein. After the construct was confirmed by restriction enzyme digestions, a single M15 transformant was incubated overnight in LB broth with 100 μg/ml ampicillin and 50 μg/ml kanamycin. The overnight culture was diluted 1:100 in 1 liter of LB broth and grown until A₆₀₀ reached 0.5. The growth was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h at 37 °C. Bacteria were pelleted by centrifugation for 15 min, 7000 × *g* at 4 °C, and resuspended in 30 ml lysis buffer (50 mM NaH₂PO₄, 500 mM NaCl, 10 mM β-mercaptoethanol, 0.5% Triton X-100, 10% glycerol, and 20 mM imidazole, pH 8.0). The bacterial suspensions were incubated on ice for 30 min with 1 mg/ml lysozyme, followed by sonication at 300 watts for 6 × 10 s with 10-s intervals, and the lysate was clarified by centrifuging at 10,000 × *g* for 20 min at 4 °C (Sorvall, Newton, CT). Protein binding was performed at 4 °C for 2 h by addition of 2 ml of nickel-nitrilotriacetic acid resin into the supernatant. The resin-protein mix was then loaded onto the column and washed with wash buffer (same as lysis buffer except for imidazole increased to 40 mM). UPase protein tagged with 6× histidine remained on the column and was eluted by imidazole step-gradient buffer (50 mM

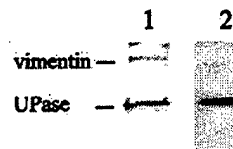


FIG. 1. UPase was purified from colon 26 cells using BAU affinity column as described under "Experimental Procedures." Lane 1 is the Coomassie Blue-stained polyvinylidene difluoride membrane; lane 2 is the Western blot of the same polyvinylidene difluoride membrane using the UPase antibody.

NaH₂PO₄, 500 mM NaCl, 10% glycerol, plus imidazole at 120, 180, 240, and 300 mM). The presence of UPase in the fractions was examined by SDS-polyacrylamide gel electrophoresis/Coomassie Blue staining and confirmed by enzyme activity assay.

Enzyme Assay—UPase enzyme activity was measured by uridine conversion to uracil, using TLC chromatographic separation as described previously (35). Briefly, cell lysates were prepared using 50 mM Tris-HCl. The cell pellet remaining following disruption by Dounce homogenization was sequentially extracted using 1% Triton X-100 in 50 mM Tris-HCl, and the supernatant following the 30,000 × *g* centrifugation was analyzed for enzyme activity. Finally, the pellet remaining after Triton X-100 solubilization was extracted using radioimmune precipitation (RIPA) buffer (1% Triton X-100, 0.5% deoxycholic acid, and 0.1% SDS), and the supernatant was analyzed for enzyme activity. Enzyme activity was measured as the percent conversion of [³H]uridine to [³H]uracil (scintillation counting) following separation on silica TLC plates (Kieselgel 60, Merck), using an 85:15:5 mixture of chloroform and methanol to acetic acid, respectively. The effect of detergents on UPase enzymatic activity was evaluated using purified recombinant UPase to which the detergents were added to the final concentrations used in the extraction methods. No significant alteration in activity was noted in the presence of the detergents used.

Immunofluorescence Techniques—For whole cell immunofluorescence analyses, cells were grown to 50–70% confluence on glass cell culture slides. After a brief wash with PBS, cell monolayers were fixed with 3.8% paraformaldehyde in PBS for 10 min at 4 °C. Fixed cells were washed briefly in PBS (5 min) and permeabilized using 0.1% Triton X-100 in PBS (10 min), and nonspecific binding was blocked using 3% BSA or serum of the same specificity as the secondary antibody when available (10 min). Incubation with the primary antibody was performed for 1 h at room temperature. After washing the excess unbound antibody (two times 5 min with PBS), sample was exposed for 1 h to secondary antibody at room temperature. The excess secondary antibody was washed with PBS, and the slides were mounted in fluorescent antibody-compatible medium (Molecular Probes, Eugene, OR). Photographs were taken using a Zeiss Axiophot microscope and camera apparatus. All experiments included a negative nonspecific serum (pre-immune serum) control to ensure specificity of the observed fluorescence. In the case of dual antibody detection, reagent compatibility was determined using normal or preimmune serum and secondary antibodies as negative controls singly and in combination.

Cytoskeleton immunofluorescence was performed as described previously (36). Cells grown to 70% confluence on glass slide covers were rinsed briefly in PBS, and then soluble proteins were extracted for 3–5 min at room temperature using cytoskeleton buffer (100 mM PIPES, pH 6.9, 1 mM MgCl₂, 1 mM EGTA containing 4% polyethylene glycol and 1% Triton X-100). Following extraction, cell cytoskeletons were rinsed three times with cytoskeleton stabilizing buffer without detergent. The immunofluorescence analysis was performed as described above.

MALDI-MS—The unknown protein band was excised from a Coomassie Blue-stained gel and digested overnight using trypsin as described (37). The peptides were subsequently analyzed using MALDI-MS and the Profound peptide data base at the Howard Hughes Medical Institute Biopolymer/W. M. Keck Foundation Biotechnology Resource Laboratory at Yale University (38, 39).

RESULTS

Uridine phosphorylase was purified from NIH 3T3 and colon 26 cells using an affinity column coupled to the high affinity uridine phosphorylase inhibitor, 5'-amino-benzylacetyluridine. For colon 26 cells, solid tumor homogenate was affixed to the BAU column. The Coomassie Blue-stained blot of UPase eluted using 20 mM uridine is shown in Fig. 1 (lane 1). The one-step purification procedure results in the isolation of UPase and the

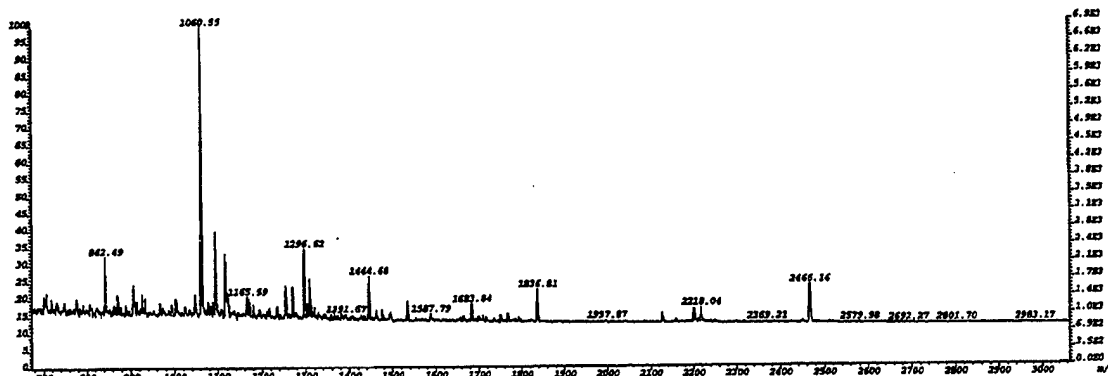


FIG. 2. The tryptic digest of the *upper band* shown in Fig. 1 was identified as vimentin using MALDI-MS. The internal standards were 100 fmol of bradykinin, which has a protonated, monoisotopic mass of 1060.57, and adrenocorticotrophic hormone clip, which has a protonated, monoisotopic mass of 2465.2.

copurification of a 58-kDa species. The identification of the *lower band* as UPase using Western blot analysis is shown in Fig. 1 (*lane 2*). No cross reactivity of the UPase antibody with the *upper band* was noted. The *lower band* at 34 kDa in Fig. 1, *lane 1*, represents purified UPase, and the *upper band* (~58 kDa) was identified as vimentin using matrix-assisted laser desorption ionization-mass spectrometry (MALDI-MS) of the tryptic digest (Fig. 2). The tryptic digest of the Coomassie Blue-stained 58-kDa band excised from acrylamide gel covered 57% of the protein using a mass tolerance of ± 0.2 atomic mass unit for monoisotopic and ± 0.5 atomic mass unit for observed average masses.

We analyzed NIH 3T3 cells to evaluate whether the copurification of UPase with vimentin was a phenomenon specific to colon 26 tumor cells, which contain highly elevated levels of UPase. UPase was purified from NIH 3T3 and colon 26 cell lines using the BAU column as described, and the results of the Western blots are shown in Fig. 3 (A and B), respectively. In both cases, vimentin was identified in the BAU eluate using Clone V9 monoclonal antibody (Sigma) as shown in Figs. 3A, *lane 2*, and 3B, *lane 2*.

We, and others (35, 40) have previously shown that the majority of the UPase in cells exists in a soluble form that is readily extractable using near-physiologic buffers (40). This is in contrast to what is known about the intermediate filament vimentin, which is one of the most insoluble proteins known. In fact, it has been shown that less than 1% of the total vimentin exists in cells as soluble tetramers (41). The fact that UPase and vimentin are copurified in cell/tumor extracts using physiologic buffers suggests that a fraction of UPase exists in combination with this soluble pool of vimentin. To confirm the association of UPase with vimentin in the cytosol, we performed gel filtration chromatography using a Sephacryl-s300 column to separate the UPase monomer from that which is associated with vimentin. As shown in Fig. 4A, UPase elutes from the column as two distinct peaks. The high molecular mass species elutes at ~400–500 kDa, and the low molecular mass peak elutes as a broad peak with a mean value of about 34 kDa, suggesting that it exists predominantly as a monomer in the cytoplasm. The shoulder on the low molecular mass peak may indicate that UPase exists in both monomeric and dimeric forms or is associated with another protein, under these experimental conditions. Vimentin coelutes from the Sephacryl-s300 column in the same fractions containing the high molecular mass UPase, again suggesting they exist in the cytosolic pool as a complex. The Western blot shown in Fig. 4B shows the UPase and vimentin present in each fraction depicted in Fig. 4A.

Confirmation of the association of UPase with the soluble pool of vimentin was also confirmed using a combination of gel

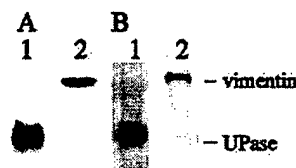


FIG. 3. UPase was purified from NIH 3T3 (A) and colon 26 cells (B) using a BAU affinity column. *Lane 1* (A and B) are the Western blots of the UPase isolated from the affinity column for NIH 3T3 and colon 26, respectively. *Lane 2* (A and B) are the same Western blots re-analyzed for vimentin using clone V9 monoclonal antibody as described.

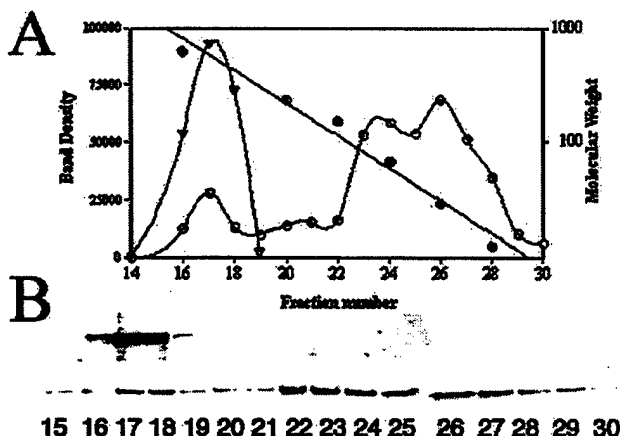


FIG. 4. A, all lysates were prepared as described under "Experimental Procedures" and applied to a 27- × 1.5-cm² Sephacryl-s300 column. Fractions were collected at 0.5-min intervals and analyzed using Western blot for UPase and vimentin. The *solid circles* indicate the molecular mass calibration standards (thyroglobin, 634 kDa; ferritin, 440 kDa; catalase, 199 kDa; aldolase, 177 kDa; bovine serum albumin, 67 kDa; carbonic anhydrase, 29 kDa; and cytochrome c, 12.9 kDa). The *open circles* represent the densitometric analysis of UPase isolated from each fraction, and the *solid triangles* represent the densitometric analysis of the vimentin isolated from each fraction as determined by Western blot of 5% of the effluent. B, the Western blot of vimentin (*upper row*) and UPase (*lower row*) present in each of the fractions 15–30 as shown in A.

filtration chromatography and immune precipitation (data not shown).

The stoichiometry of the UPase-vimentin complex, was estimated using a slot blot binding assay to measure direct protein-protein interactions. Purified recombinant human UPase was directly coupled to horseradish peroxidase and used to probe known concentrations of purified recombinant vimentin (*inset*,

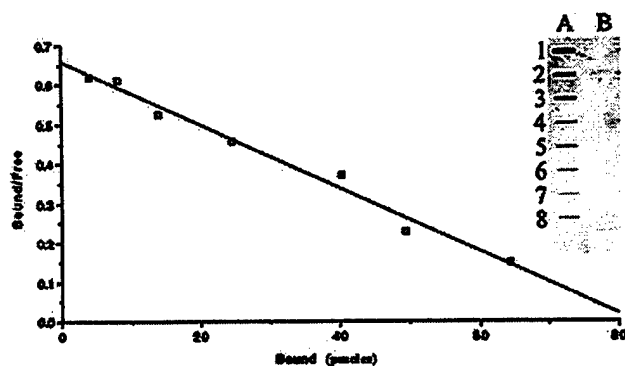


FIG. 5. Human recombinant UPase (5 μ g/ml) directly conjugated to horseradish peroxidase was used to probe a nitrocellulose membrane to which purified recombinant vimentin (inset, lane A) or BSA (inset, lane B) was affixed. Vimentin and BSA concentrations of 25, 12.5, 6.25, 3.2, 1.7, and 0.85 μ g are shown in lanes 1–8, respectively. ECL was used to develop the blots, and the optical density of the resulting film was converted to picomole values of UPase using a standard curve containing known quantities of UPase as described. Data transformation was performed, and the Scatchard analysis is shown.

Fig. 5, lane A) or BSA (inset, Fig. 5, lane B) and the Scatchard transformation is shown in Fig. 5. Using this technique, the stoichiometry of UPase binding to vimentin is calculated at ~1:2 and the K_d of the complex is 120 pM.

We wanted to determine whether UPase could be found in combination with the polymeric form of vimentin, so NIH 3T3 cells and colon 26 cells were sequentially extracted using Tris-buffered saline, followed by 1% Triton X-100, and finally RIPA buffer. The detergent extracts were utilized to solubilize cytoskeleton (including vimentin) and associated proteins while maintaining optimum enzyme activity (Table I) and UPase-vimentin association (Fig. 6). Extracts from each of these conditions were affinity-purified using anti-vimentin chromatography. The results from these experiments demonstrate that UPase and vimentin exist together in a complex in each of these fractions. Fig. 6B shows a Western blot of UPase isolated from colon 26 cell lysates purified using anti-vimentin affinity column and indicates the presence of a UPase-vimentin complex in each of these fractions. Although the extraction of vimentin from both the Tris and Triton X-100 fractions are similar (Fig. 6A, lanes 1 and 2), less vimentin was retained on the affinity column in the RIPA solubilized fraction, probably a function of lower antibody efficiency in the presence of detergents contained in the RIPA buffer. The predominant form of UPase exists in the Tris-soluble fraction in combination with the soluble tetrameric vimentin. Further solubilization of the cell pellet with Triton X-100 liberates a smaller percentage of UPase (~15–30% based on enzymatic activity shown in Table I) suggesting less UPase is associated with the polymeric membrane-associated form of vimentin liberated using this technique. Finally, there is a very small percentage of UPase released by the final RIPA solubilization of the pellet, which is between 10–20% based on enzymatic activity (Table I) and is difficult to visualize on the Western blot shown in Fig. 6. This additional pool of UPase is also associated with the polymeric form of vimentin. Taken together, both the Triton X-100 and RIPA buffer extracted UPase represent between 25–50% additional UPase enzymatic activity found in association with the polymeric and membrane-associated vimentin pool.

Western blots of NIH 3T3 (lanes 1–3) and colon 26 cells (lanes 4–6) sequentially extracted without detergent (lanes 1 and 4), using 1% Triton X-100 (lanes 2 and 5) and RIPA buffer (lanes 3 and 6) are shown in Fig. 7. The distribution pattern of vimentin in these fractions is shown in the upper panel (A), and the

TABLE I
Fraction of total UPase associated with the soluble and detergent insoluble cytoskeleton

Cell line	Tris	Triton X-100	RIPA
	% of total UPase enzyme activity		
NIH 3T3	62.1 (6.0)	22.7 (8.2)	15.3 (2.3)
Colon 26	59.3 (2.9)	24.8 (4.1)	15.8 (4.9)



FIG. 6. Colon 26 tumor homogenates were prepared by sequentially extracting the cell pellet in 50 mM Tris-HCl (lane 1), followed by a 1% Triton X-100 extraction of the pellet (lane 2), and a final extraction of the residual pellet using RIPA buffer (lane 3). The lysates were chromatographed on an anti-vimentin column and eluted using 0.1 M glycine, pH 3.0. The upper panel (A) is the Coomassie Blue-stained Western blot of each of these extraction protocols eluted from a vimentin affinity column. B is a Western blot for the UPase present in each of the fractions.



FIG. 7. NIH 3T3 cell homogenates were sequentially extracted using 50 mM Tris HCl (lane 1), followed by 1% Triton X-100 (lane 2), and finally RIPA buffer (lane 3). Equal protein was loaded for each extraction and analyzed by Western blot for UPase (B) and vimentin (A). Colon 26 cell homogenates were sequentially extracted using 50 mM Tris HCl (lane 4), followed by 1% Triton X-100 (lane 5) and finally RIPA buffer (lane 6). Equal protein was loaded for each extraction and analyzed by Western blot for UPase (B) and vimentin (A).

distribution of UPase in the same fractions is shown in the lower panel (B). Although UPase and vimentin copurify in all three fractions as determined by affinity chromatography (Fig. 6), there is an inverse relationship between the relative abundance of each protein in these fractions. Although the majority of UPase exists in the Tris buffer-soluble fraction (lanes 1 and 4) for NIH 3T3 and colon 26 cells, respectively, less than 1% of the total vimentin has been shown to exist in this pool (41) (Fig. 7A, lanes 1 and 4). Although 99% of the vimentin exists in the polymeric form and is extractable using detergents (Fig. 7A, lanes 3 and 6), less than 20% of the total UPase is present in this fraction (RIPA) based on enzyme activity (Table I).

Further evidence for the association of UPase with the polymeric form of vimentin was demonstrated using NIH 3T3 cells from which soluble proteins were extracted in the presence of cytoskeleton stabilizing agents (36). We extracted NIH 3T3 cells grown on glass slides with cytoskeleton-stabilizing buffer containing 1% Triton X-100 and 4% polyethylene glycol as described (36) before fixing and processing them for immunofluorescent microscopy. Subcellular localization of UPase in NIH 3T3 demonstrated a distinctly filamentous pattern (Fig. 8A). The staining for UPase was particularly intense in the perinuclear area of the cell and extends outward toward the cell periphery in a filamentous network, which is identical to the intermediate filament vimentin as shown in Fig. 8B. Regions of the cells where vimentin and UPase colocalize are shown in Fig. 8C and appear yellow.

Because the intermediate filament network is found in close

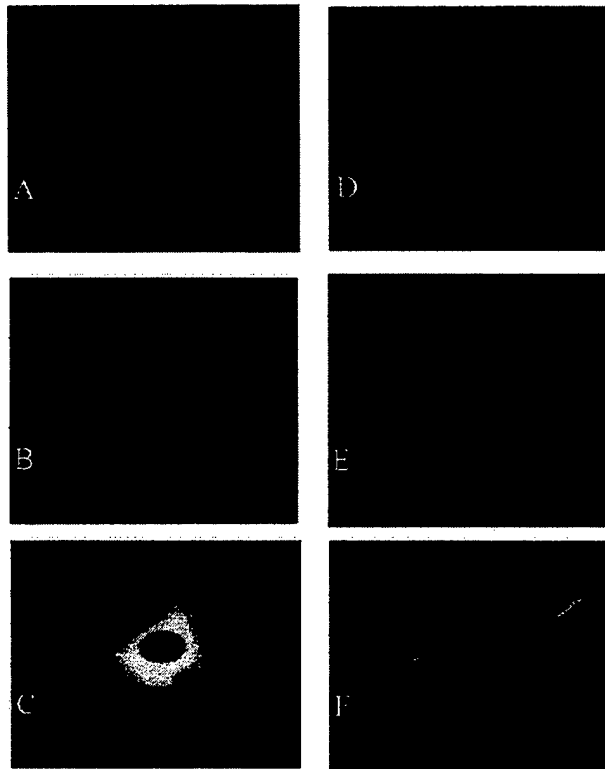


Fig. 8. NIH 3T3 cells were grown overnight on glass cover slides and processed for immunofluorescent antibody detection as described, using cytoskeleton-stabilizing buffers to maintain microtubule integrity. In *A*, UPase-staining is shown as red fluorescence. Cells were double-labeled for vimentin, as shown in *B*, and appear green. Areas where UPase and vimentin are colocalized appear yellow using double filters (*C*). NIH 3T3 cells were grown overnight on glass cover slides and on the second day, 0.125 nM nocodazole was added for 24 h (*D-F*). UPase staining following nocodazole is shown in *D*, vimentin staining is shown in *E*, and areas of colocalization are shown in *F* and appear yellow.

association with the microtubule system, we used nocodazole to depolymerize the microtubules as a means of dissociating the microtubule and intermediate filament networks (42, 43). This treatment is characteristically associated with a perinuclear collapse of the intermediate filament network away from the cell periphery. In Fig. 8 (*D-F*), changes in the UPase staining that result from treating NIH 3T3 cells with nocodazole are shown. The UPase is no longer associated with the filamentous network, which extends toward the cell membrane, but is surrounding the nucleus in a dense network (Fig. 8*D*) that is coincidental with the vimentin intermediate filament as shown in Fig. 8*E*. Areas of colocalization are shown in Fig. 8*F* and appear yellow.

DISCUSSION

We have shown that UPase and the intermediate filament protein vimentin are colocalized using immunofluorescent antibody techniques, affinity chromatography, gel filtration, and immunoprecipitation. We have further shown these proteins interact *in vitro* using binding assays. The difficulty is in determining the physiological relevance of this observation. The role of uridine phosphorylase in the salvage pathway of pyrimidine nucleoside biosynthesis does not readily translate into a role for this enzyme in association with the cytoskeleton. Additional difficulty in interpreting these data lies in the inability to clearly establish a functional role for the intermediate filament vimentin. Although a number of theories have been proposed for the function of this network, the data are not

conclusive. Cellular processes as diverse as differentiation, motility, signal transduction, cell division, cytoskeletal stability, and vesicular trafficking have been associated with alterations in the dynamics of the intermediate filaments (reviews in Refs. 44–48, and references within). Deletion of the vimentin protein in mice had no detrimental characteristics, and the mice apparently developed and reproduced normally (49). It has recently been shown that vimentin null mice exhibit neurological defects and impaired motor coordination (50). Further *in vitro* analyses of fibroblasts from vimentin null and vimentin null mouse embryos show that vimentin null cells exhibit reduced mechanical stability, decreased growth factor-directed and random motility, and reduced capacity to cause contraction of collagen fibrils (51), a process necessary in wound healing.

In recent years, a number of proteins have been shown to be associated with the vimentin intermediate filament scaffold, including p53 (52), protein kinase C (53), Yes and cGMP kinase (54, 55), glycolytic enzymes pyruvate kinase, creatine kinase, and glyceraldehyde-3-phosphate dehydrogenase (56–58), and nucleoside diphosphate kinase (NDPK) (56, 59) as well as the cross-linking proteins plectin, IFAP-300, and filamin, which link intermediate filaments to other cytoskeletal elements and membranes (59–63). It is particularly interesting to note the number of proteins involved in signal transduction and energy metabolism that have been associated with vimentin. Although the phenotype of the vimentin knockout mice is not evident under “normal” conditions, the recent observations of reduced mechanical strength and the cellular response to motility stimulating growth factors in fibroblasts isolated from these animals supports a role for the vimentin three-dimensional network in the coordination of these responses.

The proposed role for nucleoside diphosphate kinase (NDPK, nm23) in nucleotide channeling (59), production of most cellular non-ATP nucleoside triphosphates (64), and copurification with vimentin and enzymes involved in ATP formation/regeneration (56), together with our observation of UPase colocalization with this same cellular machinery, suggests that such observations are biologically relevant. If vimentin serves a largely structural role in cellular homeostasis, the localization of the vimentin-associated proteins within the milieu of the cell may represent a mechanism for “docking” these proteins to the cytoskeletal scaffold. In this case, proteins associated with glycolytic processes and signal transduction may be bound to vimentin as a mechanism of sequestration of enzymatic activity or signal transduction. If the vimentin network serves as a functional scaffold that directs mRNA and vesicular trafficking, as proposed (reviewed in Ref. 45), the association of glycolytic, UPase, NDPK, and signal transduction molecules with this filamentous network may be under dynamic control. In the case of UPase, it may be relevant that a large fraction of this enzyme is associated with the soluble pool of vimentin. Because soluble vimentin represents the fraction of this protein that is added to existing filaments in response to changing cell dynamics, it seems relevant that this form of the filament is associated with UPase. Particularly, if newly synthesized vimentin targets areas of mRNA translation, having a pyrimidine degradation enzyme in close proximity to areas of high mRNA translation would seem reasonable.

It is possible that the UPase complex with vimentin represents the biologically active form of this enzyme. It has been shown by Vita *et al.* (65) that in *Escherichia coli* B., enzymatically active UPase exists as a tetramer. From our observations in colon 26 cells, the majority of soluble UPase (55–60%) exists as a monomer and the remaining UPase is found in association with the soluble vimentin tetramer, possibly in a 1:2 stoichiometry as suggested by *in vitro* binding assays. It is possible

that the biologically active species of UPase is the UPase: vimentin multimer. Alternatively, it is possible that in mammalian cells UPase could exist predominantly either as a monomer or an easily dissociated tetramer not detected with our techniques.

The association of proteins with the cytoskeleton may serve different functions, including activation or inactivation (reviewed in Ref. 66) of enzymatic activity, localization of a particular enzymatic activity to multiprotein complexes (67-69), or sequestration of proteins from the soluble or nuclear pool (52, 69). Whether any or all these possibilities are true for UPase sequestered to the vimentin filaments remains to be proven. *In vitro* enzymatic analyses of the detergent-extractable pool of UPase demonstrated that this source of enzyme retains enzymatic activity. It is difficult to say whether this is true in the intact cell where it exists in an insoluble form. The UPase found in association with the polymeric vimentin network may represent a pool of enzyme able to mobilize that is only active when liberated from its three-dimensional network, possibly regulated by variations in the intracellular concentrations of uridine. The association with the insoluble vimentin network could also represent a way of localizing enzymatic activity to a particular area within the cell.

It is possible that the interaction between UPase and vimentin is a function of nonspecific interactions between two relatively hydrophobic molecules or is mediated by an intermediate element. Based on the variety of different biochemical analyses that demonstrate the colocalization of these proteins, this seems unlikely. Experiments are in progress to further analyze the specificity and nature of the interactions between these two proteins.

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