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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 exploration of a new strategy to improve the therapeutic index of fluoropyrimidines. Based on the knowledge that uridine phosphorylase (UPase) has a key role in the cancer therapy because of its effects on the metabolism (activation) of fluoropyrimidines and on their biochemical modulators such as uridine, and our recent finding of p53 regulation on UPase expression, we concentrated our research on two main areas: 1) determination of the role of UPase in the metabolism of pyrimidines; and 2) elucidation of the regulation mechanism(s) of UPase expression. The knockout studies of UPase gene have demonstrated that UPase is a key enzyme of activation of 5-FU and its pro-drugs and that UPase-initiated pyrimidine salvage pathway activity obviously affects the PALA anti-proliferative activity. In the investigation of UPase regulation, we have determined that p53 directly regulate UPase by suppressing its expression through specific DNA-binding. The research achievements will help to improve the clinical management of breast cancer patients.

14. SUBJECT TERMS
Experimental therapy; Pyrimidine biosynthesis; Tumor suppressor Gene knockout; and Uridine phosphorylase

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Introduction

This research project has been focused on the exploration of a new strategy to improve the therapeutic index of fluoropyrimidines. Based on the knowledge that uridine phosphorylase (UPase) has a key role in the cancer therapy because of its effects on the metabolism (activation) of fluoropyrimidines and on their biochemical modulators such as uridine, and our recent finding of p53 regulation on UPase expression, we concentrated our research on two main areas: 1) Elucidation of the regulation mechanism(s) of UPase expression; and 2) determination of the role of UPase in the metabolism of pyrimidines. The research achievements will help to improve the clinical management of breast cancer patients.

Body

Two major accomplishments have been achieved in the past scientific year:

A. Knockout of UPase gene has demonstrated that UPase is a key enzyme of activation of 5-FU and its pro-drugs (Please see Appendices for Figures)

1.   Targeted disruption of the murine UPase gene
     We have previously reported the gene structure of the murine UPase characterized by the presence of 9 exons and 8 introns with the translational starting codon located on exon 3 and the putative catalytic region of the protein encoded by exon 5 and 6. Using a BAC clone containing the whole murine UPase gene, we isolated an 8.5 kb fragment covering exon 3 to 6 of the gene. To disrupt the murine UPase gene and nullify its functional products, we replaced with a 1.6 kb neomycin resistance gene expression cassette (Neo, a positive selection marker) a 2.5 kb fragment of the gene, which contains the 3' part of intron 3, the whole exon 4 and intron 4, and the 5' part of exon 5, 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 5' end of 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

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exposed at 5.5 mg/ml G418 for two weeks to select for UPase double allele knockout clones. The resultant 50 clones were subjected to Southern blot analysis, and five of them were found to have the targeted disruption at both alleles (Figure 1).

2.   Nullification of UPase expression in knockout ES cells
     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 were analyzed for mRNA and protein expression. A wild type (WT) parental 129/JV ES clone was also utilized as a control. A 600 bp UPase cDNA probe, corresponding to exon 3 to 7, identified a 1.4 kb UPase RNA in WT and single knockout cells. This RNA fragment was not detectable in the two double knockout clones DL16 and 22. The abundance of UPase mRNA in single knockout cells was approximately 50% of the WT control cells indicating that the UPase gene disruption results in the reduction of the mRNA transcripts (Figure 3). 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 utilizing a anti-UPase polyclonal antibody generated in our laboratory. The data indicate that the expression of the UPase protein was nullified in the two double knockout clones and halved in the single knockout clone DL362 (Figure 2). UPase activity was also assayed in these cell extracts by determining the conversion of uridine into uracil. The results showed that no activity was present in the two double knockout clones and the uridine conversion was halved in single knockout cells compared to the WT control cells (Figure 2). As a whole these data indicate that the UPase gene was specifically disrupted in these clones.

3.   Abrogation of UPase activity does not affect the physiological function of ES cells under normal tissue culture conditions.
     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 media to determine any change in their proliferative rate. As showed in Figure 3, we did not observe any obvious difference in growth rate between WT and knockout cells. Moreover, the size of both pyrimidine and purine ribonucleotide and deoxyribonucleotide pools did not change, the Na^+- dependent active transport of uridine was not affected (data not shown). More interestingly, the intracellular concentration of ribose-1-phosphate, a co-substrate in the phosphorolytic

reaction, was not altered in the knockout clones with an overall concentration of 2.5 nmoles/mg of proteins (Table 1). These data show that the partial or complete abrogation of UPase activity does not obviously affect the cell growth and nucleotide metabolism under normal culture conditions.

4. Nullification of UPase expression results in cell resistance to some pyrimidine analogues and inhibitors of de novo pyrimidine synthesis

To elucidate the effects of the nullification of UPase activity on cell drug sensitivity, double knockout clones DL16 and 22, a single knockout clone DL362, and a WT ES clone were tested against five pyrimidine analogues, a specific inhibitor of pyrimidine de novo synthesis, phosphonacetyl-L-aspartic acid (PALA), and a DNA intercalating anti-tumor agent, doxorubicin. A 72 hour exposure to 5-FU indicated that UPase disruption resulted in a reduced sensitivity to this pyrimidine antimetabolite with a 10-fold increase in IC_{50} from 0.2 uM for the WT cells to 2.0 uM for the two UPase double knockout clones (Figure 5). The single knockout cells still maintained sensitivity to 5-FU with an IC_{50} of 0.35 uM (Figure 4). 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- to 3-fold compared to the WT cells (Table 2). When the cells were exposed simultaneously to 5-FU in the presence of the specific UPase inhibitor BAU (50 uM), we observed a reduction in 5-FU activity in both WT and UPase single knockout ES cells (Figure 5). These data indicated that UPase plays a critical role in activation of 5-FU in this ES cell model. Furthermore, we found that uridine can effectively rescue the cytotoxicity of 5-FU in WT and single knockout cells, but not in the double knockout cells (Figure 6), suggesting that uridine provides its protective rescue effect to ES cells mainly through specific substrate competition for uridine kinase with the 5-FUrd formed via salvage pathway.

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 non-toxic pro-drug that can be selectively activated at tumor level. 5'-Deoxy-5-fluorouridine (5'DFUR) represents one of these examples. 5'DFUR is converted to 5-FU by UPase and TPase in tumor tissues that present a higher expression of the two phosphorylases compared to normal tissues. Our results indicated that WT and UPase single knockout cells were much more sensitive

to 5'-DFUR than the double knockout cells, with IC_{50} of 0.5, 2.5 and 8.0 uM for the WT, single knockout and double knockout cells, respectively (Figure 7), confirming the importance of UPase in the activation of 5'-DFUR. The abrogation of UPase had no effects on the cytotoxicity of 5-fluorouridine, a direct substrate for uridine kinase, 2-deoxy-5-fluorouridine, activated by thymidine kinase, and Ara-C, a deoxycytidine analogue. UPase activity did not also affect the cytotoxic activity of a DNA intercalator and topoisomerase inhibitor such as doxorubicin (data not shown).

PALA was designed as an analogue of the transitional stage, intermediate in the condensation of carbamylphosphate with L-aspartic acid. This compound can efficiently inhibit the pyrimidine de novo synthesis and subsequently deplete the pyrimidine nucleotide pools via inhibition of aspartate transcarbamylase. Our data demonstrate that the nullification of UPase activity causes an increase in the IC_{50} of PALA from 50 uM in WT ES cells to more than 2000 uM for the double knockout cells (Figure 8), indicating the diminished role of the de novo pyrimidine synthesis in the knockout cells. As expected, uridine rescue (50 uM) could efficiently protect both WT and single knockout cells from PALA toxicity (Figure 9).

5.   Effects of UPase disruption on other enzymes regulating pyrimidine metabolism

Considering the potential effects that UPase nullification and changes in uridine metabolism could have on both de novo and pyrimidine salvage pathway, we evaluated the activity of several enzymes involved in the regulation of pyrimidine bio-synthetic pathways. Our data presented in Table 3 indicate that UPase nullification does not induce TPase activity, not found in ES cell model, nor alter the activity of orotate phosphoribosyl transferase or thymidylate synthase. However, we observed a significant increase in the activity of uridine kinase that is more than doubled in the knockout clones. These data confirm previous evidence indicating an increased reliance on the pyrimidine salvage pathway in the UPase knockout ES cells.

B.   We have determined that p53 directly regulate UPase by suppressing its expression through specific DNA-binding.

Please see appendix I

Key research achievements

A. UPase is a key enzyme of fluoropyrimidines therapy
1. UPase is a main enzyme of 5-FU activation.
2. The anabolic reaction of 5-FU catalyzed by UPase is not affected by the intracellular ribose-1-phosphate level. That questioned the traditional concept that ribose-1-phosphate acts as a rating-limiting factor.
3. UPase is also an important enzyme for 5-fluyoro-5-deoxyuridine activation. The nullification of UPase results in a 10-fold increase of the IC50.
4. UPase has no effects on anti-tumor compounds whose metabolism has no involvement of UPase such as Ara-C, doxorubicin, and fluorouridine.
5. Uridine rescue happens at fluorouridine level via specific competition for uridine kinase substrates.

B. P53 suppresses the expression of UPase

1. A p53-binding element present in the UPase promoter region.
2. Wild p53 specifically binds to the element.
3. The p53-binding exerts a suppression function on UPase expression, reducing salvage pathway activity.

Reportable outcomes

1. One manuscripts
2. Two abstracts

Conclusion

In the first scientific year supported by the award, we determined the role of UPase in fluorouridine therapy and elucidated the uridine rescue mechanism. We also found the suppression function of p53 on UPase expression. These data will benefit the design of clinical therapeutic strategy.

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Please Ref. Appendix I

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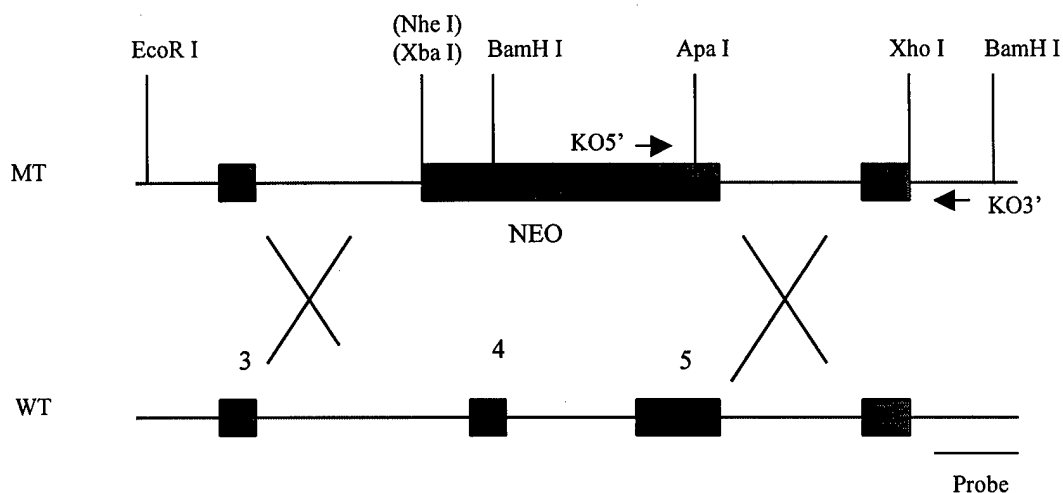
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Appendices

1. Figures and tables
2. Uridine phosphorylase plays a crucial role in 5-fluorouracil metabolism and its anti-proliferative activity (Abstract)
3. Expression and regulation of uridine phosphorylase (UPase) appears altered in human tumors: genomic structure, chromosome mapping and promoter characterization of human UPase gene (Abstract)
4. P53-dependent suppression of uridine phosphorylase gene expression through direct promoter interaction (manuscript)

A. Homozygous Recombination

(Nhe I)
(Xba I)

EcoR I   BamH I   Apa I   Xho I   BamH I

KO5' →

MT

NEO

KO3'

3   4   5

WT

Probe

B. Southern Blot

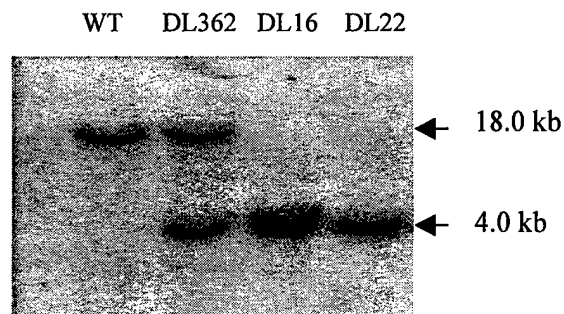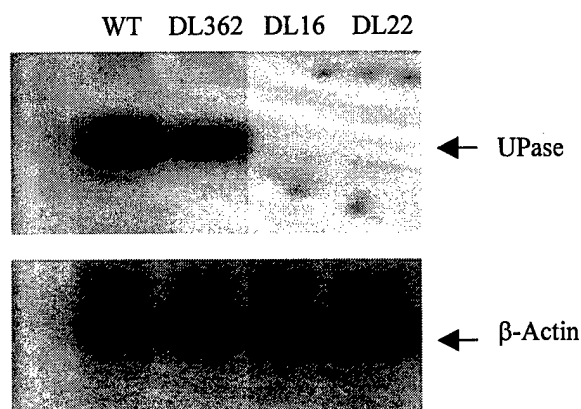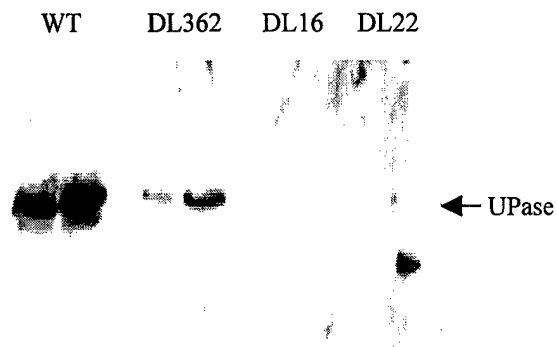WT   DL362   DL16   DL22

← 18.0 kb

← 4.0 kb

Figure 1   Targeted Mutation of Mouse UPase Gene. The wild type UPase gene was disrupted via homozygous recombination (A). The Southern hybridization confirmed the disruption in one (single knockout) or both (double knockout) alleles in the cell (B).

A. Northern Blot   B. Western Blot

WT   DL362 DL16 DL22

← UPase

← β-Actin

WT   DL362   DL16   DL22

← UPase

C. UPase Activity

Cells	UPase Activity (nmoles/mg/min)
WT (+/+)	101.45±5.23
DL362 (+/-)	60.70±4.36
DL16 (-/-)	2.12±1.68
DL22 (-/-)	1.98±1.08

Figure 2   **UPase Expression in Mutant ES Cells.** UPase mRNA (A), protein (B) and enzyme activity (C) are not present in knockout cells and approximately 50% left in single knockout cells, compared to wild type cells.

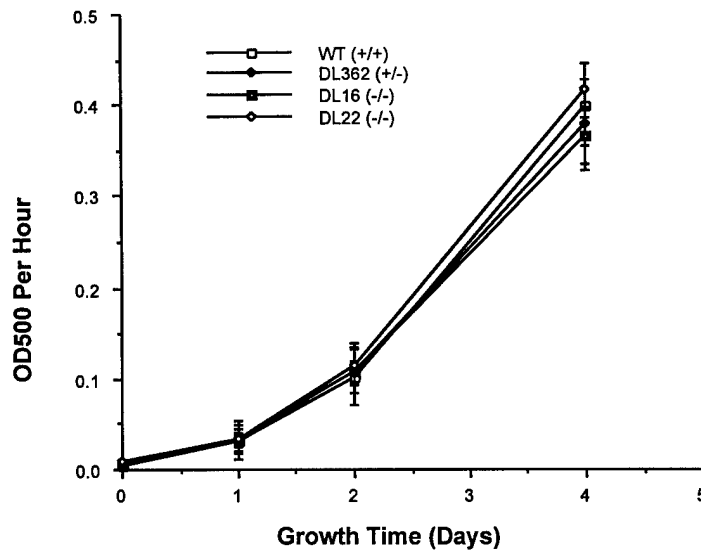**Figure 3**   **The Growth Rate of Various Types of ES Cells in Regular Media.** There is no obvious difference is observed.
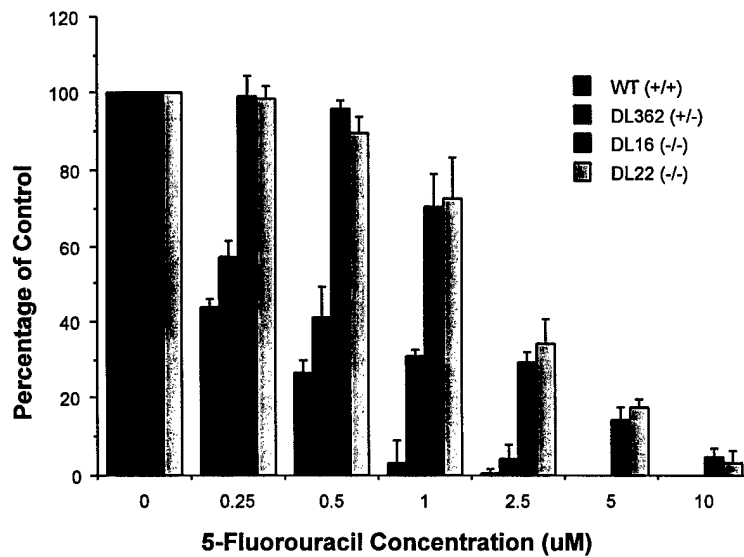
Figure 4   **Response to 5-FU.** UPase knockout ES cells are more resistant to 5-FU than wild type (WT) cells.

5-FU + BAU
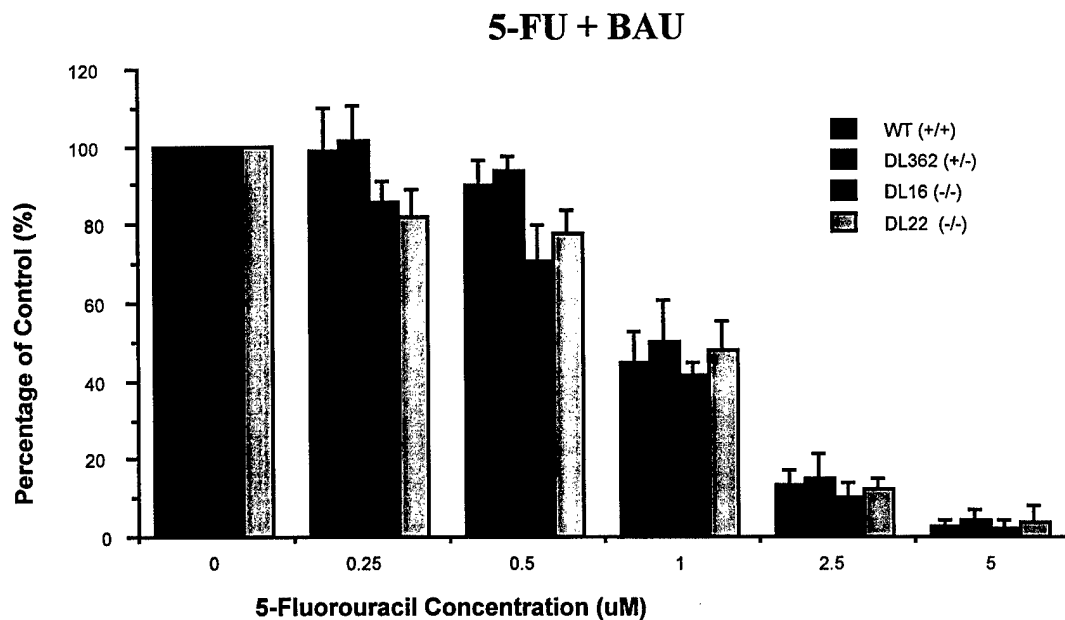
Figure 5 **BAU Protection to 5-FU.** 50 uM BAU, a UPase inhibitor, partially protect WT cells from 5-FU toxicity, but has no effect on mutant cells.
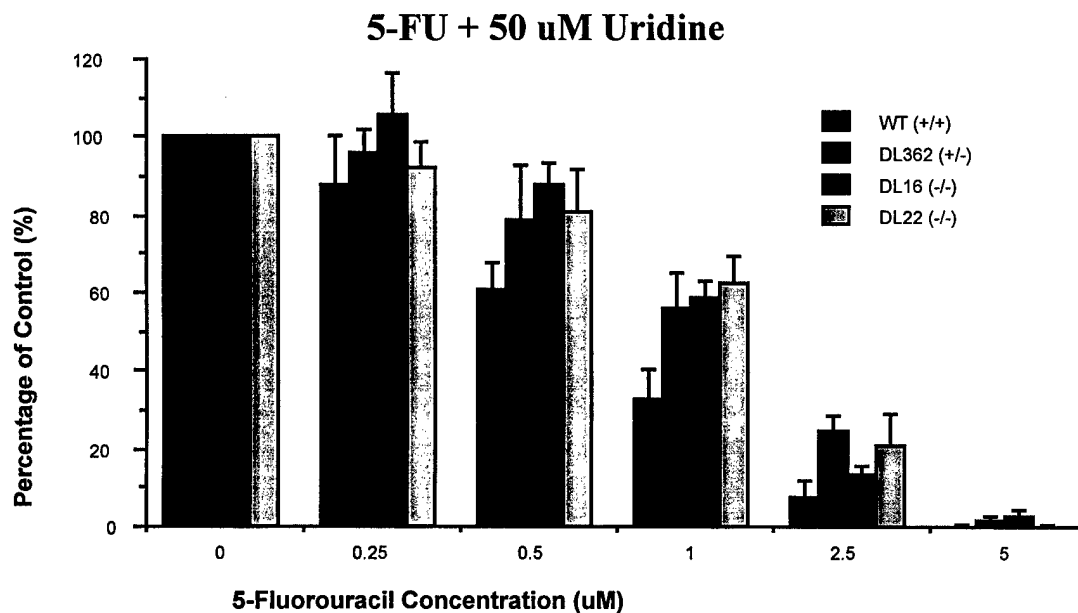
5-FU + 50 uM Uridine

Figure 6 **Uridine Rescue.** 50 uM uridine can partially protect the 5-FU toxicity to WT and single knockout cells, but not to UPase knockout cells
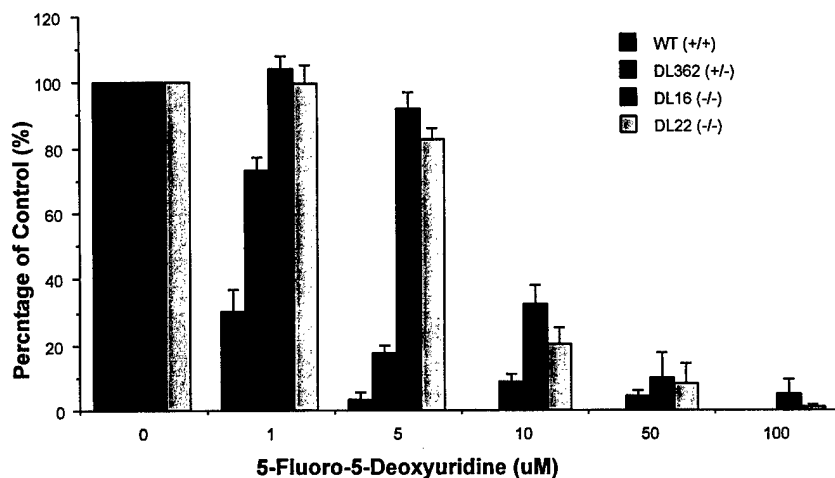
Figure 7 **Activation of 5-Fluoro-5-Deoxyuridine (5-DFUR) Needs UPase Parcitipation.** UPase converts 5-DFUR to 5-FU and then 5-FU is activated to its fluoro-nucleotides mainly by UPase-involved salvage pathway.
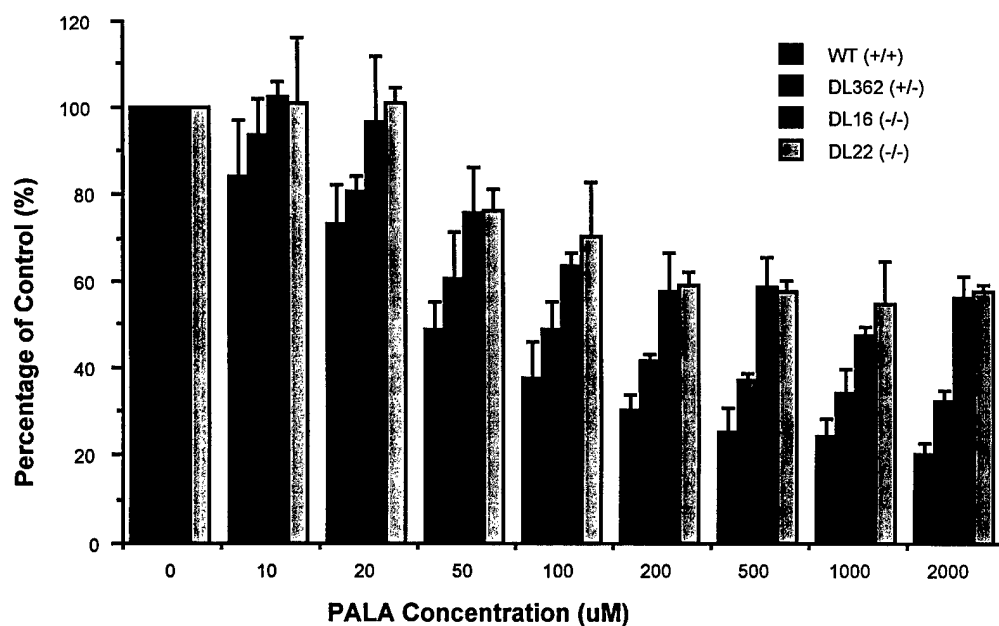
Figure 8 **Response to PALA.** WT ES cells are more sensitive than UPase knockout cells to PALA, an inhibitor of pyrimidine de novo synthesis.
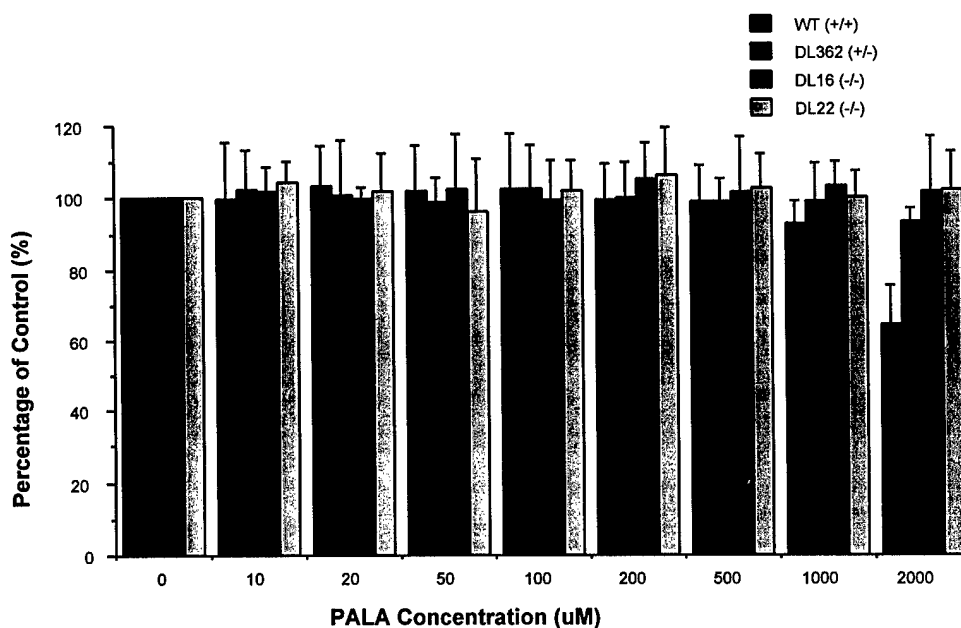
Figure 9 **Uridine Rescue to PALA Toxicity.** The presence of uridine (50 uM), a source of cellular nucleotides via salvage pathway, can effectively "rescue" PALA toxicity.

Table 1 **The rib-1-p concentration**

Samples	Rib-1-P Concentration (nmoles/mg)
ES Cells	
WT (+/+)	2.00 ± 0.15
DL362 (+/-)	2.03 ± 0.24
DL16 (-/-)	2.50 ± 0.18
DL22 (-/-)	2.37 ± 0.21
Cancer Cell Lines	
MDA 231	13.55 ± 0.87
MDA 453	1.95 ± 0.34
NIH/Ras	6.04 ± 0.58
NIH/3T3	7.02 ± 0.49
MCF7	9.11 ± 0.29

Table 2   Incorporation of Radio-Labeled 5-Fluorouracil and Uridine into Nucleic Acid

Cells	5-Fluorouracil Incorporation (pmoles/10E6 Cells/24 hours)		Uridine Incorporation (nmoles/10E6 Cells/24 hours)	
	Normal Condition	Uridine Depleted 24	Normal Condition	Uridine Depleted 24
WT (+/+)	56.67 ± 5.69	46.30 ± 7.81	2.48 ± 0.23	3.55 ± 0.41
DL362 (+/-)	46.88 ± 8.17	50.89 ± 4.93	2.73 ± 0.35	3.78 ± 0.27
DL16 (-/-)	21.18 ± 3.31	30.73 ± 6.58	3.29 ± 0.39	7.32 ± 1.03
DL22 (-/-)	16.88 ± 5.67	24.07 ± 3.79	3.29 ± 0.19	7.15 ± 0.78

Table 3   Activities of Uridine Kinase and OPRTase

Cells	Uridine Kinase (nmoles/mg/hour)	OPRTase (Nmoles/mg/hour)
WT (+/+)	185.9 ± 12.3	103.3 ± 11.5
DL362 (+/-)	188.2 ± 15.9	102.5 ± 9.8
DL16 (-/-)	390.6 ± 11.8	119.5 ± 12.5
DL22 (-/-)	322.4 ± 13.1	117.8 ± 13.5

degree of OSM inducibility was typically affected by the deletions in parallel with effects on the basal promoter activity. This observation suggests a potential link between the key sequences for OSM activation and the basal promoter machinery. Identification of the necessary promoter regions for OSM-mediated activation of the tPA and PAI-1 genes allowed subsequent site-directed mutagenesis and EMSA studies. Utilization of these studies further characterized the specific bases involved in gene regulation. The results of these transfection/expression studies are summarized.

#2646   Expression and Regulation of Uridine Phosphorylase (UPase) Appears Altered in Human Tumors: Genomic Structure, Chromosomal Mapping, and Promoter Characterization of the Human UPase Gene. Dekai Zhang, Deliang Cao, Manjunath A. Nimmakayalu, and Giuseppe Pizzorno. *Yale University School of Medicine, New Haven, CT.*

Our recent studies have illustrated the presence of specific mutations in Uridine Phosphorylase from human breast tumors and the increased enzymatic activity in most of the human tumors compared to matched normal tissues. Furthermore, UPase expression is known to be affected by the c-H-ras oncogene and various cytokines. To better understand the expression and regulation of UPase, we have cloned and characterized its gene. The human UPase gene has a genomic structure similar to the murine one, containing nine exons and eight introns spanning a total of approximately 20 kb. Chromosomal mapping indicates that the human UPase gene is located at 7p12, a position where frequent LOH has been found in human breast cancer. Like the murine gene promoter, the human UPase promoter also lacks canonical TATA and CAAT boxes and presents c- and N-Myc, c- and v-Myb and p53 binding motifs, indicating that UPase expression may be directly regulated by tumor suppression genes and oncogene products. Studies are in progress to determine the relevance of these interactions on uridine phosphorylase activity in human tumors.

#2647   Alternative Promoter Usage and mRNA Splicing in the Regulation of the Expression of the *PCPH* Gene. Ana Rouzaut and Vicente Notario. *Georgetown University, Washington, DC.*

In vitro transformation of Syrian hamster embryo fibroblasts by exposure to 3-methylcholantrene, allowed us to isolate *PCPH* (termed *CPH* in earlier reports), a novel oncogene. We previously described the *PCPH* full cDNA sequence and chromosomal localization in humans and rodents and its intron/exon organization in humans. In order to show the possible role of *PCPH* in the transformation of human cells we analyzed its expression at the protein level in 50 human neoplastic epithelial cell lines. Results showed that PCPH, along with other immunoreactive polypeptides of different masses, was expressed in 42% of the malignant cell lines tested but it was not detectable on western blots from extracts of primary human epithelial cells. It has been reported that injection of cells transformed by the *PCPH* oncogene induced tumors in nude mice and that *PCPH* collaborates with H-ras in the transformation of mouse NIH3T3 fibroblasts. To address the differential regulation of *PCPH* expression during malignant transformation, we have performed experiments to investigate the possible alternative promoter usage in normal versus transformed human breast epithelial cells. We have also searched by RT-PCR for different splicing products in both kinds of cells. To accomplish this, we previously identified and characterized several putative promoter regions by primer extension and RACE 5' on RNA obtained from normal human breast RNA, MCF-10 cells (immortalized human breast epithelial cells) and four human breast carcinoma cell lines, half of which express *PCPH* and the other half do not. We detected different extension products in tumor and/or normal samples. We have subcloned the putative promoter regions into a reporter vector and measured their transcriptional activity. We showed previously that when murine or Syrian hamster cells are subjected to various types of stress *PCPH* expression increases and this enhances cell survival. To study the regulation of *PCPH* expression at the promoter level under stress situations in human cells, we measured the promoter activity of the different promoter constructs in MCF-10 and MCF-7 (human breast carcinoma) cells exposed to serum deprivation, TPA, ionizing radiation, or glucose deprivation. Here we demonstrate a regulation of the promoter activity in normal immortalized cell lines when exposed to different stress stimuli conducive to a gain in *PCPH* expression that could contribute to survival during malignant transformation.

#2648   Investigating Hypoxic Tumor Physiology with Expression Profiling. Nicholas C. Denko, Karen Hudson, and Amato Giaccia. *Stanford University School of Medicine, Stanford, CA.*

Clinical evidence is accumulating that tumor hypoxia represents an independent prognostic indicator of poor patient outcome. Hypoxic tumors have been reported that contain increased levels of angiogenesis, increased local invasion, increased distant metastasis, and altered apoptotic programs. However, the mechanism(s) for these changes have not been elucidated. Because hypoxia is a potent inducer of transcriptional changes, we have used expression profiling of cells treated in vitro with hypoxia to identify genes with altered expression in the hypoxic tumor microenvironment. We have examined normal and transformed epithelial cells and normal stromal cells for changes in expression at the 7000 gene level. We find that approximately 1.5% of the genome is transcriptionally sensitive to hypoxia. There are significant differences in the hypoxic response of the normal and the transformed epithelial cells and the normal stromal cells. We have identified categories of genes that could be involved in the physiologic

changes responsible for the hypoxic tumor phenotype. We have found altered expression in genes involved in angiogenesis, tissue remodeling, apoptotic response and metabolic changes. We are examining the contribution of selected hypoxia-responsive genes to the biology of the hypoxic tumor.

#2649   Modulation of HOX Gene Expression in Human Breast Epithelial Cells by Human Chorionic Gonadatropin. J. Russo, M. H. Lareef, I. H. Russo, and X. Jiang. *Breast Cancer Research Laboratory, Fox Chase Cancer Center, Philadelphia, PA.*

The placental hormone human chorionic gonadotropin (hCG) stimulates mammary gland differentiation and inhibits tumor progression. Because class I homeobox genes (Hox) are involved in mouse mammary gland development and in breast cancer, we hypothesized that hCG's effect is mediated by HOX gene activation. Reverse transcription-polymerase chain reaction (RT-PCR) was used for studying the expression of 39 known HOX genes in MCF-10F, MCF-7, and MDA-MB-231, an immortal and two cancer breast cell lines. Thirty five of the 39 class I homeobox genes analyzed were expressed by the three cell lines; four, HOXA2, B1, C4, and C9 were not detected in any of them. Semi-quantitative RT-PCR technique was used for studying the expression pattern of these genes in cells with μg of recombinant hCG (r-hCG)/ml for 1, 5, 10, 24, 48 and 96 hrs. In MCF-10F cells the 24-hr treatment up-regulated HOXD10 (3.8 folds), D11 (4.2 folds), and D13 (5.8 folds), whereas at 48 hrs of treatment only D8 was upregulated 4.2 folds. The silent gene HOXA2 was transiently detected at 1 hr of treatment. In MCF-7 cells, r-hCG treatments of 10 and 24 hrs resulted in upregulation of HOXB3 (2.5 folds), B8 (1.5 folds) and D8 (2.2 folds). In MDA-MB-231 cells, 1 and 5 hr-treatments upregulated HOXC8 (2.0 folds), C12 (2.0 folds), D8 (2.4 folds) and D11 (3.8 folds). Although HOXD8 was activated in the three cell lines, this effect veried with the time of treatment. The types of genes activated also varied in normal immortalized and in cancer cell lines, indicating that hCG plays a role in the regulation of HOX genes in human breast epithelial cells, but the effect is modulated by the biological characteristics of the cells. (Supported by grants DAMD 17-19-1-9182 and DAMD 17-00-1-0249).

#2650   Development of New Methods for Processing Clinical Samples for Molecular Profiling Studies. John W. Gillespie, Carolyn Best, Kristina Cole, Stephen Hewitt, Mamoun Ahram, Yvonne Gathright, Jonathan Epstein, Stanley Hamilton, Gallya Gannot, Rodrigo Chuaqui, Judi Herring, Verena Bichsel, Valerie Calvert, Emanuel Petricoin, W. Marston Linehan, Paul Duray, G. Steven Bova, and Michael Emmert-Buck. *Center for Prostate Disease Research, Rockville, MD, Food and Drug Administration, Bethesda, MD, Johns Hopkins University, Baltimore, National Cancer Institute, Bethesda, MD, Tel Aviv University, Tel Aviv, Israel, and Univ of Texas M.D. Anderson Cancer Center, Houston, TX.*

Improved methods of tissue fixation and embedding need to be developed in order to facilitate more rigorous and thorough molecular profiling studies of human tissues and associated disease processes. New protocols must produce both excellent histological detail and biomolecule preservation. The present work evaluates the histology and the recovery of DNA, RNA, and proteins from whole-mount prostate specimens. Processing conditions included freezing, ethanol fixation/paraffin-embedding, ethanol-fixation/polyester wax embedding, and standard formalin-fixation/paraffin-embedding. The biomolecules in the specimens were evaluated by a variety of techniques including agarose gel electrophoresis, PCR, RT-PCR, SDS-PAGE, 2D-PAGE, immunoblot, and layered expression scanning. Taken together, the results show that ethanol-fixation and paraffin/polyester-embedding of tissue specimens are useful methods for preparing clinical samples for both diagnosis and molecular profiling studies. Continued refinement of tissue processing methodologies will be a critical step toward ultimately determining the complete molecular anatomy of all normal and cancerous human cell types.

#2651   Detection of Increased Icb-1 Transcription during Cellular Differentiation Processes and in Uterine Tumors. Oliver Treeck, Olaf Ortmann, Klaus Diedrich, and Guenter Vollmer. *Medical University Lubeck, Lubeck, Germany, and Technical University Dresden, Dresden, Germany.*

Human gene icb-1 was initially described as a gene with an increased transcription rate in in vitro differentiated endometrial tumor cells cultured on a reconstituted basement membrane. Icb-1 gene was shown to be widely expressed in a variety of human tissues and cancer cell lines. The gene spans 6.9 kb containing 4 exons and is located on human chromosome #1. We report here the induction of icb-1 transcription in HL60 promyelocytic leukemia cells following granulocytoid differentiation in response to DMSO-treatment, as detected by Northern Blot. This is the second example for up-regulation of icb-1 transcription in in vitro differentiated tumor cells, thus this induction seems to be independent of the stimulus for cellular differentiation. Though the function of icb-1 gene is still unclear, the hypothesis of its involvement in cellular differentiation processes could be validated by detection of an increased transcription rate in human fetal tissues. We further report the identification of a variant transcript synthesized in human spleen, which is not a splice variant but a transcript of a closely related gene. Two additional splice variants of the initially identified gene have been described recently. They were named icb-1 beta and gamma, consequently the originally described transcript is termed icb-1 alpha. Screening of a matched tumor/normal expression array with a probe recognizing all known icb-1 variants resulted in the detection of a highly increased transcription rate in uterine tumor

#2737   T185 Role of p53 and FAS (CD95/APO-1) in Apoptosis and Cyto-toxicity Induced by Antimetabolites in Human Colon Carcinoma Cell Lines. I. Petak, D.M. Tillman, and J.A. Houghton. *St. Jude Children's Research Hospital, Memphis, TN.*

We examined the induction of apoptosis and cytotoxicity in response to treatment of cc to 5-fluorouracil (FUra) + leucovorin (LV) under conditions of both DNA- (HT29, VRC₅/c1, RKO) and RNA- (HCT8, HCT116) directed cytotoxicity, and to the antifolates trimetrexate (TMQ) and methotrexate (MTX). Acute apoptosis was induced in cell lines expressing wtp53 (RKO, HCT8, HCT116). In HT29 that expressed mp53, apoptosis was delayed. Cell lines undergoing RNA-directed FUra toxicity demonstrated dThd-reversible accumulation of cells in S-phase (HT29, RKO). Those lines undergoing DNA-directed FUra cytotoxicity (HCT8, HCT116) were arrested in G2/M, reversible by dThd, that did not influence FUra-LV-induced loss in clonogenic survival. Following TMQ or MTX, cells also arrested in S-phase, independent of p53. TMQ + Hx shifted the cell cycle distribution to G2/M-phase arrest in HCT116 but not in HT29, both partially reversible by dThd. In cells expressing wtp53, Fas was upregulated in response to FUra-LV, TMQ and MTX. FUra-LV sensitized RKO and HCT116 cells to the anti-Fas MoAb CH-11, reversibly by dThd in RKO but not in HCT116. IFN-γ upregulated Fas and sensitized cells to FUra-LV and TMQ (+ Hx), independent of p53. In contrast, MTX cytotoxicity was reversed by Hx +/- IFN-γ. Data demonstrate that p53 influences the kinetics but not the outcome of induction of apoptosis by antimetabolites, and drug-induced elevation in Fas expression in cc. IFN-γ potentiates FUra-LV and TMQ but not MTX cytotoxicity in a Fas-dependent manner, independent of p53. Supported by NCI awards CA 32613, CA 21866 and by ALSAC.

#2738   Interference with Thymidylate Synthase (TS) Activity and DNA Integrity in the HT-29 Human Colon Carcinoma Cell Line not by 5-Fluorouracil (5-FU) Before Irinotecan (CPT-11) but by the Reversed Sequence and Simultaneous Drug Treatment. I. Grivicich, D.R.A. Mans, G.J. Peters, A.B. da Rocha, K. Smid, D.T. Passos, G. Kayser, R. Brentani, and G. Schwartsmann. *Department of Oncology, Free University Hospital, Amsterdam, Netherlands, Ludwig Institute, São Paulo, SP, Brazil, and South-American Office for Anticancer Drug Development (SOAD), Lutheran University of Brazil, Canoas, RS, Brazil.*

Using HT-29 cells, we previously demonstrated synergistic growth-inhibitory and DNA-damaging effects of the CPT-11/5-FU combination with CPT-11 before 5-FU; additivity with both drugs simultaneously; and antagonism with the reversed sequence (Eur J Cancer 35: 1851-1861, 1999). In this study, we tested whether these observations might be due to greater inhibition of TS activity by 5-FU in the presence of CPT-11. Thus, HT-29 cells were exposed for 24 h to IC₅₀ CPT-11 or 5-FU alone; for 24 h to IC₂₀ or IC₅₀ CPT-11 simultaneously with IC₅₀ or IC₂₀ 5-FU; or for 2 h to IC₂₀ CPT-11 or 5-FU, followed by 22 h IC₅₀ 5-FU or CPT-11. IC₂₀ and IC₅₀ values were 1.5 and 2.5 μM, respectively, for CPT-11, and 2.0 and 8.2 μM, respectively, for 5-FU. TS activity was determined by a 3ₕ-release assay, either immediately (day 1), or after two more days in drug-free medium (day 3). In the latter cases, cells were also assessed for DNA integrity by agarose gel electrophoresis. Data were related to those found with untreated controls (TS activity of 700 ± 281 pmoles/h/mg protein, and largely intact DNA). TS activity on day 1 had decreased 2- to 4-fold with all 5-FU-containing treatments, and was not significantly affected by that with CPT-11 alone. On day 3, TS activity was still about 2-fold lower than control values in cultures exposed to CPT-11 before or simultaneously with 5-FU, but not in any of the other cultures. Appreciable oligonucleosomal DNA fragmentation with characteristics of apoptosis was also only apparent in the former, but not in the latter samples. Our results suggest that the use of CPT-11 before or simultaneously with 5-FU led to more efficient inhibition of TS activity in HT-29 cells when compared with the use of 5-FU before CPT-11 or of either agent alone. This might lead to apoptotic cell death, and might explain the above-mentioned greater antitumor efficacy of the former treatments over the latter. The precise role of CPT-11 in the inhibitory effect of 5-FU on TS activity is currently under investigation in our laboratory.

#2739   c-Myc and p53 Interactions Determine Sensitivity of Human Colon Carcinoma Cells to 5-Flourouracil *in Vitro* and *in Vivo*. Diego Arango, Georgia A. Corner, Paul J. Catalano, and Leonard H. Augenlicht. *Albert Einstein Cancer Center, Bronx, NY, and Dana-Farber Cancer Center, Boston, MA.*

We have previously shown that low-level amplification of the *c-myc* gene identifies a subset of stage II and III colon cancer patients with increased disease-free and overall survival in response to 5FU-based adjuvant therapy (Augenlicht *et al* 1997, 57:1769). In the present study we generated a series of cell lines derived from LoVo (wild type p53) and DLD1 (mutant p53) colon carcinoma cells that over-express *c-myc* at different levels relative to the already high levels in the parental lines due to aberrant Wnt signaling. While sensitivity to low serum-induced apoptosis was dependent on high *c-myc* levels, and was eliminated by *c-myc* anti-sense that reduced transactivity, it was independent of p53 function. In contrast, sensitivity to 5FU-induced apoptosis was dependent upon both higher *c-myc* levels as well as the presence of wild type p53. The necessity for a functional p53 protein was further demonstrated using the p53 specific inhibitor pifithrin (PFT)-α. These data predicted that patients whose primary tumors were both *c-myc* amplified and p53 wild type would show the greatest improvement in outcome with 5FU-based adjuvant therapy. This prediction was borne out by analysis of these parameters in patients from EST2284, a phase III multi-institu-tional trial of 5FU-based adjuvant therapy for stage II and III colon cancer patients conducted by the Eastern Cooperative Oncology Group. Results from this study show that only those patients with both amplified *c-myc* and wild type p53 in their primary tumor showed improved outcome in response to 5FU, and long term survival (15 year) for these patients approached 100%. Thus, both *c-myc* and p53 status determine the sensitivity of colonic carcinoma cells *in vitro* and *in vivo* to 5FU. These data have important implications regarding clinical strategies for targeting *c-myc* expression and/or p53 mutations in treatment of colon cancer.

#2740   Role of Thymidine Phosphorylase as Determinant to Colorectal Tumor Cells Sensitivity to Capecitabine: Implication of FAS in Xeloda®-induced Apoptosis? Joseph Ciccolini, Karine Bezulier, Alexandre Evrard, Pierre Cuq, Claude Aubert, Jean-Paul Cano, and Jacques Catalin. *School of Pharmacy, Marseille, France,.*

Because cancer now is more considered as a matter of deficiency of apoptosis rather than proliferative cells, involvement of anticancer drugs in apoptosis is a key issue in today's chemotherapy. In this respect, the aim of this study was to develop an in vitro model dedicated to the exploration of capecitabine molecular pharmacology. Specific work focused on: 1. the role of Thymidine Phosphorylase (TP) as determinant to tumor sensitivity to Xeloda®. 2.the implication of Fas in the subsequent induction of apoptosis. We used a co-culture system mixing hepatoma HepG2 and human colorectal LS174T cells. Thus, both hepatic and tumoral enzymes were available for activating capecitabine to active fluorinated metabolites. Besides, 2 different LS174T sub-lines were tested for sensivity to Xeloda®: wild type LS174T with weak TP activity and TP-transfected LS174T-c2 clone exhibiting strong TP expression. Therefore it was possible to assess the role of TP activity on potentiation of drug response. Results showed that higher tumoral TP yield correlated with higher capecitabine antiproliferative efficacy. Moreover, monitoring of Apo-1 Fas receptor on cell surface by flow cytometry analysis showed an induction of Fas after treatment by capecitabine in overexpressing TP LS174T-c2 line, thus suggesting a Fas-component into apoptosis induction. This implication of Fas was next confirmed by the partial reversal of capecitabine cytotoxicity by anti-FasL and anti-Fas monoclonal antibodies. Further studies will be necessary to fully understand the exact role of Fas in Xeloda® antitumoral activity.

#2741   Uridine Phosphorylase Plays a Crucial Role in 5-Fluorouracil Metabolism and Its Anti-Proliferative Activity. Deliang Cao, Robert E. Handschumacher, and Giuseppe Pizzorno. *Yale University School of Medicine, New Haven, CT.*

Uridine phosphorylase (UPase) is a pyrimidine catabolic enzyme known to regulate the plasma concentration of uridine, a biochemical modulator of the anti-tumor agent 5-fluorouracil (5-FU). However, little is known of the role UPase in the activation and metabolism of 5-FU and its derivatives, mainly because of the lack of an appropriate model system. We have developed a cell panel by specific gene targeting techniques, including wild type, partially nullified (one of the two alleles disrupted) and completely nullified ES cells. The experimental data indicate that the elimination of UPase activity leads to a 10-fold increase in 5-FU ED50 and a reduction in its incorporation into nucleic acids, while no difference in 5-Fluorouridine toxicity was observed in these cell lines. This confirms the anabolic role of UPase on 5-FU in our model system. The reduced incorporation of precursors of de novo pyrimidine biosynthesis into nucleic acids, in UPase nullified cells, indicates a diminished role of de novo synthesis in the knockout cells possibly due to negative feed-back regulation by the accumulated uridine. The reduced dependence of UPase knockout cells on the pyrimidine de novo synthesis is reflected in the apparent resistance to PALA, a specific inhibitor of the pyrimidine pathway, with a 5-fold elevation in its ED50 in UPase nullified cells compared to the wild type clone. Biochemical studies on uridine metabolism, including the effect on the intracellular nucleotide pools, the status of uridine uptake mechanisms, the catalytic activity of other enzymes involved in pyrimidine biosynthesis and metabolism are under investigation. Preliminary evidence indicates a significant increase in uridine kinase activity in UPase double-knockout ES cells.

#2742   Induction of Apoptosis by 5-Fluorouracil through the Suppression of NF-kB Activity in Human Salivary Gland Cancer Cells. Masayuki Azuma, Tsuyoshi Yamashita, Keiko Aota, Tetsuya Tamatani, and Mitsunobu Sato. *Tokushima University School of Dentistry, Tokushima, Japan.*

Activation of the transcription factor NF-kB results in protection against apoptosis, and the chemotherapeutic agent 5-Fluorouracil (5-FU) exerts its cytotoxic effect through the induction of apoptosis. Thus, we examined whether 5-FU could induce apoptosis through the suppression of NF-kB activity. We found that upon treatment of a human salivary gland cancer cell line (cl-1) with 5-FU, the NF-kB activity was suppressed in a time-dependent manner. This inhibition was mediated by a prevention of the degradation of the inhibitory IkBa protein through the inhibition of IKK kinase activity. In addition, the expression of TRAF-2, cIAP-1, and c-FLIP, which function as anti-apoptotic molecules through the interruption of caspase pathway, was also inhibited by 5-FU. Finally, the activity of caspase-8 and caspase-3 showed a significant increase in response to 5-FU. By flow cytometric analysis, 5-FU did not affect the expression level of Fas on the cell

p53-dependent suppression of Uridine Phosphorylase gene expression through direct promoter interaction

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Running Title: Down-regulation of uridine phosphorylase expression by p53

ABSTRACT

Uridine phosphorylase 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. Co-transfection of the UPase promoter constructs (from p-1619 to p-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 construct containing the -445 to -274 bp sequence to suggest the existence in this promoter region of a putative p53 recognition element. Similar co-transfection in murine embryo fibroblasts (MEF) 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. These data indicate the UPase gene is a novel target of p53 and is down-regulated by p53 at the promoter level.

INTRODUCTION

Uridine, a pyrimidine nucleoside essential for the synthesis of RNA and bio-membranes, has also been shown over the years 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 cardio-circulatory 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 re-synthesizing 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 5-fluorouracil (4,5). Uridine in combination with 5-benzylacyclouridine (an inhibitor of UPase) has been also shown to protect mice against the neurotoxic side effects of 5-FU containing drug regimens (6-8). Uridine phosphorylase (UPase) is the key enzyme responsible for the reversible phosphorolysis of uridine to uracil and plays a critical role in the homeostatic regulation of uridine concentration in plasma and tissues.

We have recently shown 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 cytokines: TNF-α, IL-1α and IFN-α and γ, and vitamin D_3 (12). In the treatment of advanced

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colorectal carcinoma, IFN-α in combination with 5-FU has resulted in a significant increase in response rate and patient survival when compared with 5-FU alone (13). In colon 26 tumor cells a mixture of TNF-α, IL-1α and IFN-γ effectively enhanced 5-FU and 5'-dFUrd cytotoxicity 2.7 and 12.4-fold respectively, due to 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 recently reported that the murine UPase gene contains nine exons and eight introns, spanning a total of approximately 18.0 kb (16), we have 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. 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 start 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

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and repress the activity of UPase at the gene promoter level, possibly regulating the pyrimidine salvage pathway following perturbation of the ribonucleotide pools.

EXPERIMENTAL PROCEDURES

Cell Culture- NIH 3T3 fibroblast cells utilized 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 (murine embryo fibroblast) cells were generously provided by Dr. Larry Donehower (Baylor College of Medicine, Houston, TX) (18). Colon 26 cell cultures were established in our laboratory from in vivo growing tumors. All the cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37 ^{0}C in a 5% CO_2 incubator.

Cloning and Sequencing of the 5'-Flanking Region of mUPase Gene- A genomic DNA clone that contains the immediate full-length 5'-flanking UPase sequence was obtained by screening a ES-129/SvJ BAC library with a murine UPase cDNA probe. The 1703 bp XbaI/XbaI 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 by autosequencing by the Protein and Nucleic Acid Chemistry Facility of the Yale Cancer Center.

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 µg) 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 ^{0}C for 5 min in 20 µl of hybridization buffer (50 mM Tris-Cl, pH 8.3, 150 mM KCl, 1 mM EDTA) and followed by incubation at 42 ^{0}C overnight. The DNA-RNA hybrid was then collected by ethanol precipitation and dissolved in 20 µl of reverse transcription buffer (50 mM Tris-Cl, pH 8.3, 75 mM KCl, 3 mM MgCl$_2$, 10 mM dithiothreitol, 0.5 mM dNTP). The primer was extended by SuperScript™ II RNaseH⁻ Reverse Transcriptase (Life Technologies, Inc.) at 42 ^{0}C for 1 hr. 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 XbaI (position -1619 and +84). The promoterless pGL3 luciferase reporter gene vector (Promega) was digested by HindIII. 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 HindIII 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 (AccI), p-1081 (BstEII), p-570 (NheI), p-445 (BglII), p-274 (BstXI), p-212 (PvuII), p-84 (EcoRV). All 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.

Transfection and Luciferase Assays- All transfections were done in triplicate in 6-well plates. Approximately 10^5 cells/well were seeded 24 hours prior to transfection. Plasmids were transfected into cells using LipofectAMINE reagent (GIBCO-BRL). The cells were incubated in transfection buffer for 3 hr, and then harvested after 45 hr 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 Electrophoretic Mobility Shift Assay (EMSA)- Nuclear extracts were prepared according to the method of Lassar (19). To obtain the nuclear extract, cell containing plates were washed three times with Tris-buffered saline solution, and to each 15 cm diameter dish 2.5 ml of lysis buffer (20 mM HEPES [pH 7.6], 20% glycerol, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, 1 mM DTT, 1 mM PMSF, 10 mg/ml leupeptin, 10 mg/ml pepstatin, 100 mg/ml aproteinin) was added. Cell were removed by centrifugation for 5 min at 2000 rpm at 4 ^{0}C. Nuclei were resuspended at 2.5×10^7 nuclei per ml in nuclear extraction buffer (identical to the lysis buffer with the addition of 500 mM NaCl). Nuclei were gently shaked for 1 hr at 4

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^{0}C, centrifuged at 10,000 rpm for 10 min, quickly frozen in liquid nitrogen, and stored at -80 ^{0}C.

EMSA was performed according to the manufacturer's instructions for Gel Shift Assay Systems (Promega). Briefly, 2 µg 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 MgCl$_2$, 50 mM NaCl, 10 mM Tris-HCl, pH 7.5, 0.05 mg/ml poly(dl-dC)·poly(dl-dC) and incubated for 20 minutes at room temperature. To demonstrate the sequence-specific binding, a 100-fold excess of the same unlabeled probe and other unlabeled probes as a specific and non-specific competitor were included in a separate reaction. The reaction mixtures were then separated on a 6% non-denaturing polyacrylamide gel at room temperature in 0.5 × TBE buffer at 100V for approximately 3 hours. The gel was transferred to Whatman 3MM paper, dried and exposed to X-ray film overnight at -70 ^{0}C with an intensifying screen.

Quantitative RT-PCR -Total RNA was extracted from MEF p53 +/+ and p53 -/- cells using TriZol ™ (Life Technologies, Inc.). For reverse transcriptase-PCR analysis, DNase I-treated total RNA was reverse transcribed using oligo(dT) and SuperScript II (Life Technologies, Inc.). The cDNA were amplified using mUPase primers P190 (5'-GAC GAA GTG ATT GAC TGG TGG TC-3') and P720a (5'-CGC CTG AAG TGC CAA TGC G-3'), and 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') were put in the same reaction. PCR products were separated on a 1.0 % agarose gel and stained with ethidium bromide.

RESULTS

Cloning and Sequencing of the Murine UPase Gene 5'-flanking Fragment- We have previously cloned part of the UPase gene promoter with the cloning of the human UPase cDNA (1.2 kb)(16). 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 BAC library was screened using the murine UPase cDNA probe. The 1703 bp XbaI/XbaI 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 present a series of microsatellite and minisatellite repeat bases (Figure 1). A potential p53 binding motif, AGcCTTGTCC, is located in the sequence -303 bp to -294 bp that contains one base difference (the small case c) from the consensus p53 binding sequence, 5'-PuPuPuC(A/T)(A/T)GPyPyPy -3' (20).

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 site of the murine uridine phosphorylase gene was accomplished by primer extension analysis. For the primer extension assay, we used the

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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 Figure 2, lane 1, the primer extension reaction yielded a 133 bp and a 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 (Figure 2). These experiments determined that two transcriptional start sites are located +1, +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 co-transfected 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 (Figure 3). The plasmids containing 5' deletions of various lengths from -1081 to -445 bp produced a very modest decrease in promoter activity. Further 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 (Figure 3).

Suppression of the UPase Gene Promoter Activity by Wild-type p53- Using

transient-expression assays in EMT6 and NIH 3T3 cells, co-transfection of UPase

promoter construct p-1619/+84 with the wild-type p53 construct resulted in significantly

less luciferase activity compared to a co-transfection experiment using an empty vector.

In three independent experiments, we observed an average of 4 fold and 3.5 fold

reduction in luciferase activity with the wild-type p53 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 co-transfection assays (Figure 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 -445 to −274 bp

construct was co-transfected. However, UPase gene promoter activity was repressed

significantly by co-transfection 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

(Figure 5).

p53 Suppresses UPase mRNA Expression- To further confirm our observation of p53 down-regulation of UPase gene expression, we evaluated the UPase mRNA expression in MEF p53 -/- and p53 +/+ cells using quantitative RT-PCR. The data reported in Figure 6 indicate that the level of UPase mRNA expression in MEF p53 -/- cells was elevated 2-3 fold compared to the MEF p53 +/+ control cells.

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 the UPase 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 (Figure 7) due to the formation of a binding complex. The amount of the shifted complex was diminished by increasing the concentration of self-competitor but not by the addition of nonspecific competitor (SP1 and AP2).

DISCUSSION

To study the UPase gene regulation and to understand the alterations in its expression observed in human tumors, we have isolated 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 by progressive 5'-deletion mutations. We found that deletions from -1619 to -445 bp produced a slight decrease in promoter activity. However, further deletion to -274 bp resulted in the elevation of the activity to levels higher that the full promoter. Co-transfection of the UPase promoter constructs with the wild-type p53 construct resulted in significantly less luciferase activity compared to a co-transfection 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, a further 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. Further elucidation of this regulatory element and its function in UPase expression is currently under investigation to clarify the role of cytokines in 5-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') (20). Wild type p53 efficiently binds to this sequence and transactivates expression of the target genes (20-24). p53 can also repress a wide variety of cellular and viral promoters (25). 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 co-transfected 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 "squelching" or sequestering general transcription factors (26). Squelching 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 (27-28).

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 (29), CCAAT-binding factor (30), cAMP response element-binding protein (31), and glucocorticoid receptors (32).

A fourth alternative, which appears to be the most likely in the case of the UPase gene, is that the inhibition is due to the presence of a specific p53 negative response element that is distinct from the core promoter region, as observed previously for the RB

(33), bcl-2 (34), and topoisomerase IIa (35) 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, co-transfection with the wild-type p53 construct resulted in significantly less luciferase activity in the constructs from p-1619 to p-445, whereas down to -274 and more 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. Sequencing analysis of this region found a putative p53 binding motif AGcCTTGTCC 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 showed 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 even in the absence of DNA damage (36). 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 indication of the contribution of p53 to damage repair is quite limited. A recent report (37) has described a p53-induced gene, p53R2 that encodes for a protein similar to one of the two subunit 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 mostly based 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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LEGENDS

Figure 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; the bold and asterisked base represents the only difference from the consensus binding sequence. The larger arrow indicates the major transcription initiation site, the smaller arrow indicates the minor transcription initiation site.

Figure 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 μg of total RNA from Colon 26 tumor cells. *Lane1*, 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. The 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.

Figure 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 Figure 1. The UPase luciferase constructs were co-transfected with a control plasmid pRL-TK and assayed 48 h post-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 represent the standard error from three samples in three independent experiments.

Figure 4. Suppression of UPase promoter activity by wild-type p53 in NIH/3T3 cells. Co-transfection of murine UPase promoter-reporter gene constructs with wild-type p53 in NIH 3T3 cells. The black bars represent the luciferase activity of the UPase constructs co-transfected in the presence of wild-type p53 constructs, the white bars represent the luciferase activity of the constructs co-transfected with empty vectors.

Figure 5. Suppression of UPase promoter activity by wild-type p53 in MEF p53 -/- cells. Co-transfection of UPase promoter constructs p-1619/+84, p-445/+84 and p-274/+84 with the wild-type p53 construct (black bars) and a control vector (empty bars).

Figure 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.

Figure 7. Electrophoretic mobility shift assay. A radioactively labeled 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.

A

```
-1619  -1470            -1081              -570  -445     -274 -212    -84  +1
  |      |                |                  |    |         |    |       |   |
 XbaI   AccI            BstII              NheI BglII    BstXI PvuII  EcoRV XbaI
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B

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-1619  GTTCTAGAAA  GCTGCATGGA  TACTGGTATC  ATCAGAGTTT  CTAGTTCACT
-1569  TTATGCTGCT  TTAGTGATAA  ACACGGAGAC  CGAAGTTTGT  AAAAAAATTA
-1519  TATATATAAT  AATAATAAAA  ATTAAAAAAA  ATAAGGGAAA  TGTTTTTGTA
-1469  TACATTTGAA  GCAGAGCTTT  TTTTTTCTTC  CAGTTTGGCT  TCAGACCCTG
-1419  ACCTCTAGGT  TCTCCCAGCG  TTCCTCAGTC  CGTACCTGGC  ACACTTTATC
-1369  GAAAGGCTTT  TCCCCCATGT  AATCCAGACA  TTTTTGGGTT  TTCCCTTCTT
-1319  CCTTCCTTCC  TTCCTTCCTT  CCTTCCTTCC  TTCCTTCCTT  CCTTCCTTCC
-1269  TTCCTCCCTC  CCTCCCTCCC  TCCCTCCCTC  CCTCCCTCCC  TCCCTCCCTC
-1219  CCTCCCTCCC  TCCCTCCCTC  TCTCTCTCTC  TCTCTCTCTT  TCTTTCTTTC
-1169  TTTCTTTCTT  TCTTTCTTTC  TTTCTTTCTT  TCTTTCTTTC  TTTTCTCTCC
-1119  CCCTCCCCCA  TGGTG------
                      *

 -319  ---------- TTCCCAAGCC  TTGTCCTTCG  -------
                      p53

  -19  CACGGGATAC  TGAAAGGACA  CAGTTTACAA  CAGCCTAGCT  CTGCATTCTT
                       +1

  +39  GACCACGTCC  ACACGGCCAT  TCATCTCTGG  TACATTCCTT  CATCATTTCT

  +89  AGAAAAATGG  ACTTGGACCC  TGGCACTAGA  GGTGACCT
        +92
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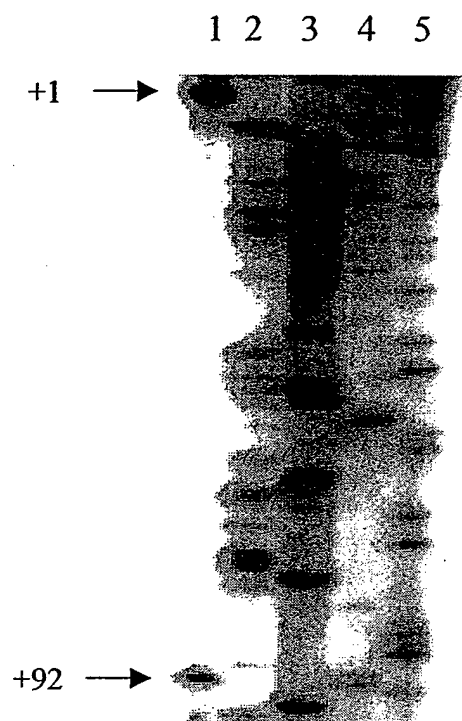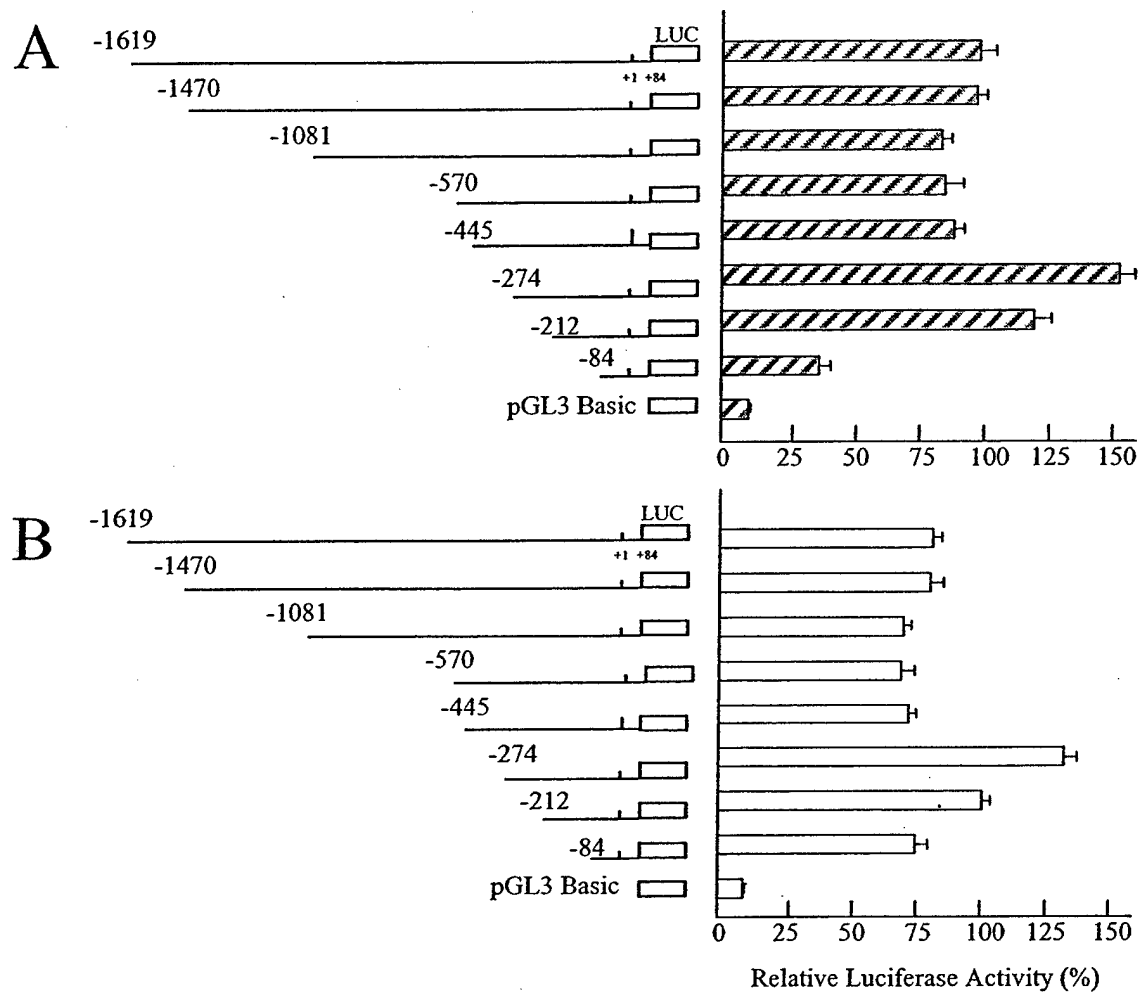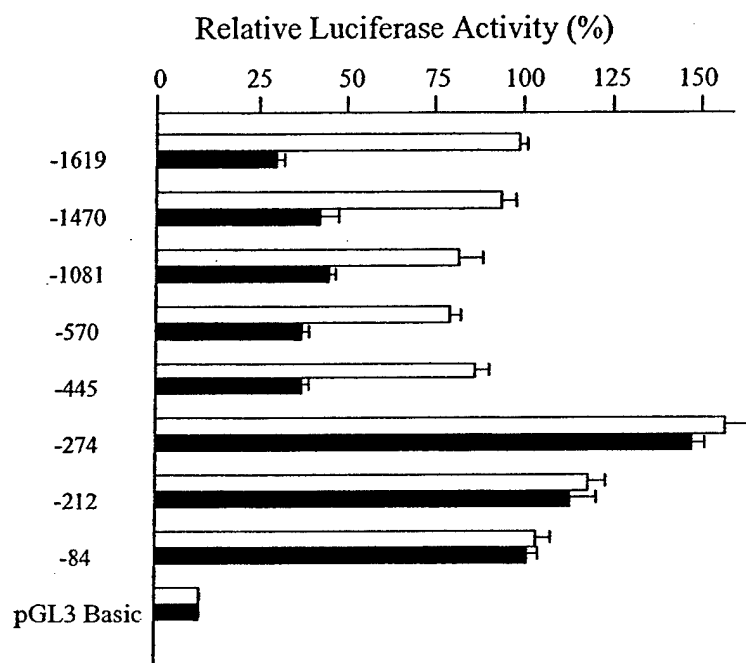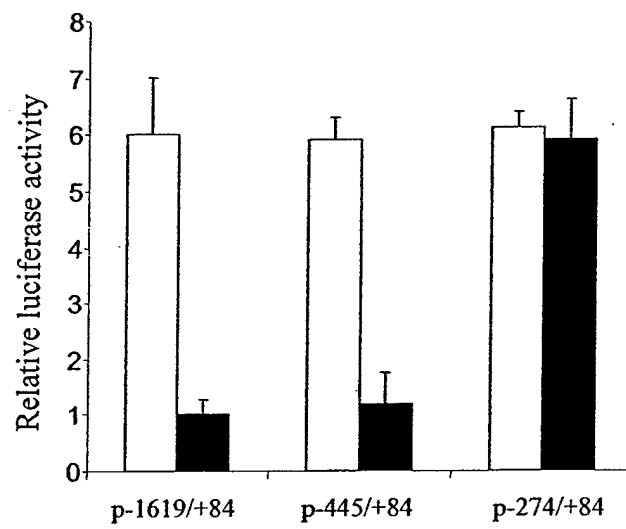
Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5

p53 +/+   p53 -/-

—— UPase(530 bp)

—— mS16 (102 bp)
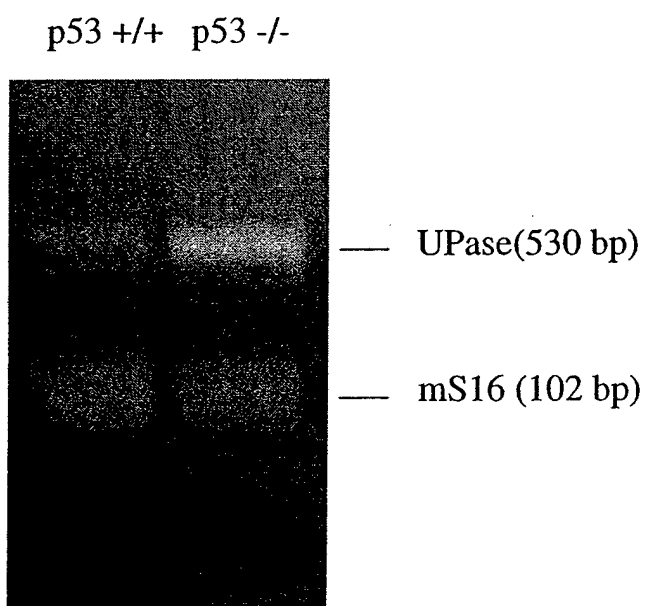
Figure 6

Competitor   -   **p53**   **SP1**   **AP2**

Figure 7