

# Cell cycle regulation of the glyceraldehyde-3-phosphate dehydrogenase/uracil DNA glycosylase gene in normal human cells

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Received October 7, 1992; Revised and Accepted January 7, 1993

EMBL accession no. X53778

## ABSTRACT

**The cell cycle regulation of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH)/uracil DNA glycosylase (UDG) gene was examined in normal human cells. Steady state RNA levels were monitored by Northern blot analysis using a plasmid (pChug 20.1) which contained the 1.3 kb GAPDH/UDG cDNA. The biosynthesis of the 37 kDa GAPDH/UDG protein was determined using an anti-human placental GAPDH/UDG monoclonal antibody to immunoprecipitate the radiolabeled protein. Increases in steady state GAPDH/UDG mRNA levels were cell cycle specific. A biphasic pattern was observed resulting in a 19-fold increase in the amount of GAPDH/UDG mRNA. The biosynthesis of the 37 kDa GAPDH/UDG protein displayed a similar biphasic regulation with a 7-fold increase. Pulse-chase experiments revealed a remarkably short half life of less than 1 hr. for the newly synthesized 37 kDa protein, comparable to that previously documented for a number of oncogenes. GAPDH/UDG mRNA levels were markedly reduced at 24 hr. when DNA synthesis was maximal. These results define the GAPDH/UDG gene as cell cycle regulated with a characteristic temporal sequence of expression in relation to DNA synthesis. The cell cycle synthesis of a labile 37 kDa monomer suggests a possible regulatory function for this multidimensional protein. Further, modulation of the GAPDH/UDG gene in the cell cycle may preclude its use as a reporter gene when the proliferative state of the cell is not kept constant.**

## INTRODUCTION

The glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH, E.C. 1.2.1.12) is a tetramer of four identical 37 kDa subunits. Although intensively studied for its role in cellular metabolism, i.e., the conversion of 3-phosphoglyceraldehyde to 3-phosphoglycerol phosphate, recent

evidence suggested that the 37 kDa monomer may be a multifunctional protein capable of a number of diverse activities. These include a close association of GAPDH with red cell membranes (1-4); its ability to act as a protein-kinase in phosphorylation of transverse-tubule protein (5); a role in the assembly of junctional triads from transverse tubules in skeletal muscle cells (6); the ATP-dependent bundling/unbundling of microtubules in the brain (7); its identification as the DNA binding protein P8 (8); and, the observation that it may play a role in transcriptional regulation (9).

In this laboratory, a 1.3 kb human uracil DNA glycosylase (UDG) cDNA was isolated by immunoscreening a lambda gt11 human placental cDNA library (10). Hybrid selection demonstrated a 1.6 kb transcript which encoded a 37 kDa protein. Sequence analysis revealed an open reading frame of 335 amino acids containing a protein of Mr=36,050 with a pI of 8.7 (11). A search of the GenBank data base determined no significant homology to other uracil DNA glycosylase cDNAs. Surprisingly the cDNA had complete homology to the 37 kDa GAPDH subunit. Subsequently, the GAPDH monomer was electroeluted from SDS/PAGE and shown to exhibit uracil DNA glycosylase activity.

The observation that the GAPDH/UDG gene may encode a multifunctional protein suggests that unique molecular mechanisms may be necessary to regulate its expression. In particular, in vivo, a number of critical cellular pathways may depend on the modulation of GAPDH/UDG gene transcription and the biosynthesis of the 37 kDa protein. To begin to define these control mechanisms, we examined whether GAPDH/UDG gene expression was dependent on the cell cycle in a defined temporal sequence relative to the induction of DNA replication. We now report that the GAPDH/UDG gene may be classified as a cell cycle regulated. Steady state levels of GAPDH/UDG mRNA were increased in a biphasic manner and were decreased in S phase. Further, the biosynthesis of the 37 kDa GAPDH/UDG protein displayed an identical cell cycle regulation. This newly synthesized protein was labile with a half life of less than 1 hr.

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This striking regulation of the GAPDH/UDG gene in the cell cycle suggests a potential regulatory role for this multifunctional protein in human cells. It also may preclude its use as a reporter gene.

## MATERIALS AND METHODS

### Cell culture

Normal human fibroblasts, GM 06112 and GM 06167, were obtained from the Human Genetic Mutant Cell Repository in Camden, N.J. Cells were maintained at 37°C in a sterile and humidified atmosphere of 5% CO<sub>2</sub> in air. The cells were grown as monolayers in Dulbecco's Modified Eagle's Medium (DMEM), 5.5 mM glucose (Gibco BRL, Life Technologies Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum, 2mM L-glutamine, 100 µg/ml streptomycin, and 100 units/ml penicillin in 150 cm<sup>2</sup> tissue culture flasks. Cells were used between passages 10 and 18. For synchronization, confluent cells were collected by treatment with 0.25% trypsin in Hank's balanced salt solution; pelleted by centrifugation at 800×g for ten minutes at 4°C; then resuspended in 0.3% fetal bovine serum supplemented media and plated into 100×20 mm culture dishes at 0.5–1.0×10<sup>6</sup> cells/dish. The cells were maintained in 0.3% serum for 5 days. To initiate synchronous growth, complete media containing 20% fetal bovine serum was added (12). Cells were harvested by trypsinization; washed twice in ice-cold PBS; then collected by centrifugation at 800×g for 10 minutes at 4°C. Crude lysates were prepared by resuspending cell pellets in 20 mM Tris-HCl, pH 8.0, 1mM DTT, and 20% glycerol (Buffer I) and sonicated at 60 watts for 20 seconds on ice using a Braunsonic needle probe. Unbroken cells were removed by centrifugation at 2300×g for 10 min. at 4°. Protein was determined by the method of Bradford (13).

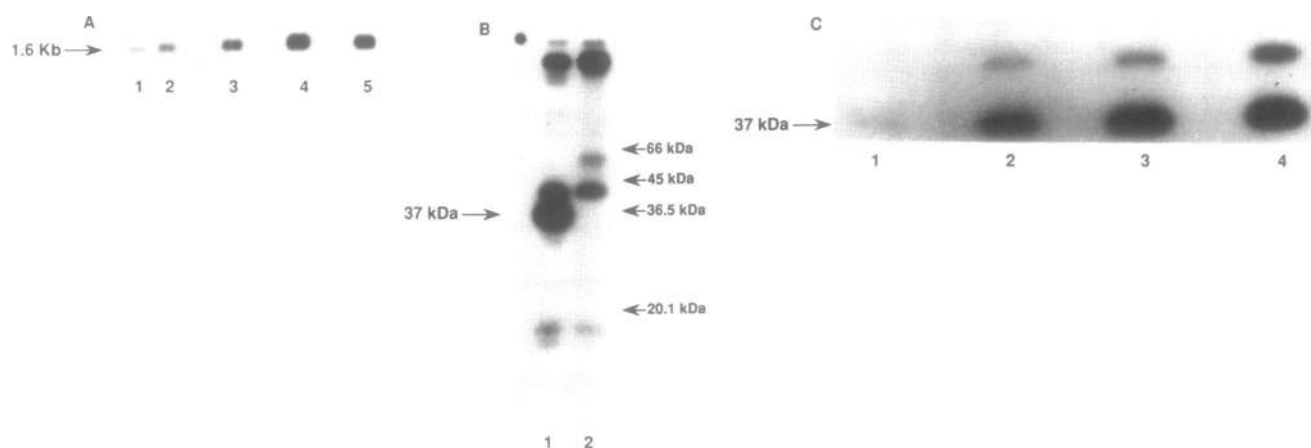
### Determination of GAPDH/UDG RNA

Total cellular RNA was extracted with ice-cold RNAzol<sup>™</sup> (0.2 ml/1×10<sup>6</sup> cells, Tel-Test Inc., Friendswood, Texas) as described (14). The RNA pellet was redissolved in 1 mM EDTA,

pH 7.0, and stored at –20°C for further use. RNA was quantitated by measuring absorbance at 260 nm while purity of the samples was estimated by measuring the ratio between the readings at 260 nm and 280 nm. RNA (10 µg) was separated on a 1.5% agarose-6.6% formaldehyde gel at a constant voltage of 30 V for 16–18 hours at room temperature; transferred onto a nylon membrane and cross-linked by UV transillumination. Hybridization at moderate stringency was performed using nick-translated (<sup>32</sup>P)-labeled pChug-20.1 cDNA (5×10<sup>5</sup> cpm/lane). Two 20-minute washes in 1×SSC/1% SDS at room temperature were followed by three cycles of 15-minute duration at 55°C with 0.1% SSC/1% SDS. Membranes were then dried in air and autoradiographed on Kodak XAR film with Dupont Cronex Lightening Plus screens at –70°C. The extent of hybridization was determined after autoradiography by densitometric scanning.

### Biosynthesis of the 37 kDa GAPDH/UDG monomer

Cell proteins were labeled by incubating cells with (<sup>35</sup>S)methionine (500 µCi/culture; 1150 Ci/mmmole; Amersham) in methionine-free media. Immunoprecipitations were performed (15) using negative control monoclonal antibody 1.05 or anti-human placental GAPDH/UDG monoclonal antibody 40.10.09 (16) to immunoprecipitate radiolabeled 37 kDa protein (17). Aliquots of 100 µg of total cellular protein were normalized to an equal volume with Buffer I and diluted with 100 µl of ice-cold buffer containing 100 mM Tris-HCl (pH 7.6), 0.6 M NaCl, 2 mM EDTA, 2% Triton X-100, 1% deoxycholate, 0.2% SDS, and 2 mM PMSF. Monoclonal antibody 1.05 or 40.10.09 (50 µg) was added to appropriate samples and allowed to incubate for 1 hour at 4°C with gentle shaking. The protein- antibody aggregate was precipitated by adding 2.8 mg rabbit anti-mouse immunoglobulin (Sigma Chemical Co., St Louis, MO) and again incubating the mixture for 2 hours at 4°C with gentle shaking. Immune complexes were collected by centrifugation at 10,000 rpm for 10 minutes. Precipitates were resuspended in 50 µl of Laemmli buffer then electrophoresed through an 8% SDS/PAGE gel (18). The gels were exposed to Kodak X-OMAT AR-5 film (Eastman Kodak, Rochester, NY) at –70°C then quantitated by densitometry.

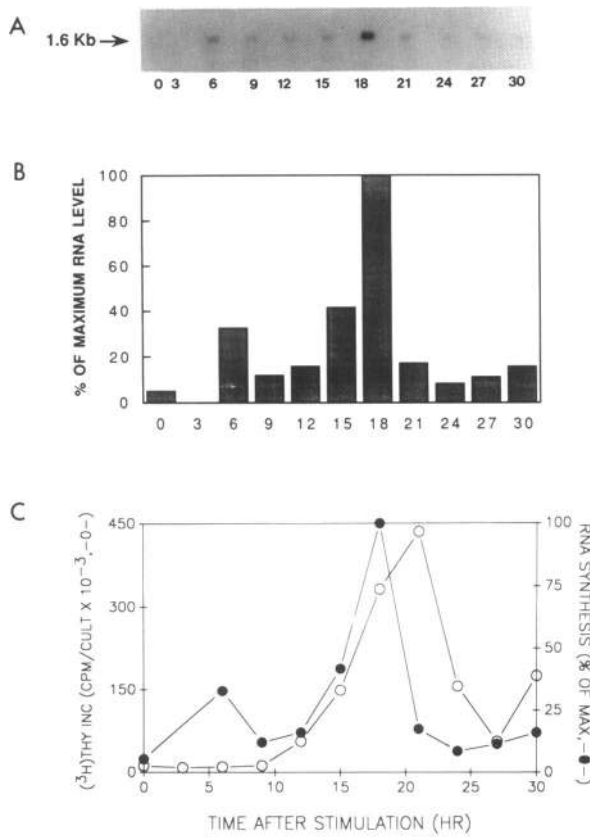


**Figure 1.** Characterization of the GAPDH/UDG gene and protein. Northern blot analysis was performed as described in Materials and Methods. Increasing concentrations of total RNA (Fig. 1A, lanes 1–5, 2–10 µg, respectively) were probed with nick-translated <sup>32</sup>P labeled pChug 20.1. Immunoprecipitation of (<sup>35</sup>S)methionine labeled proteins were performed with monoclonal antibodies 40.10.09 (Fig. 1B, lane 1) or negative control antibody 1.05 (Fig. 1B, lane 2) using 100 µg of crude cell protein and 50 µg of antibody. The rate of incorporation of (<sup>35</sup>S)methionine into the 37 kDa GAPDH/UDG protein was determined by incubating cells for 0, 30, 60 and 120 min. (Fig. 1C, lanes 1–4 respectively) prior to collection.

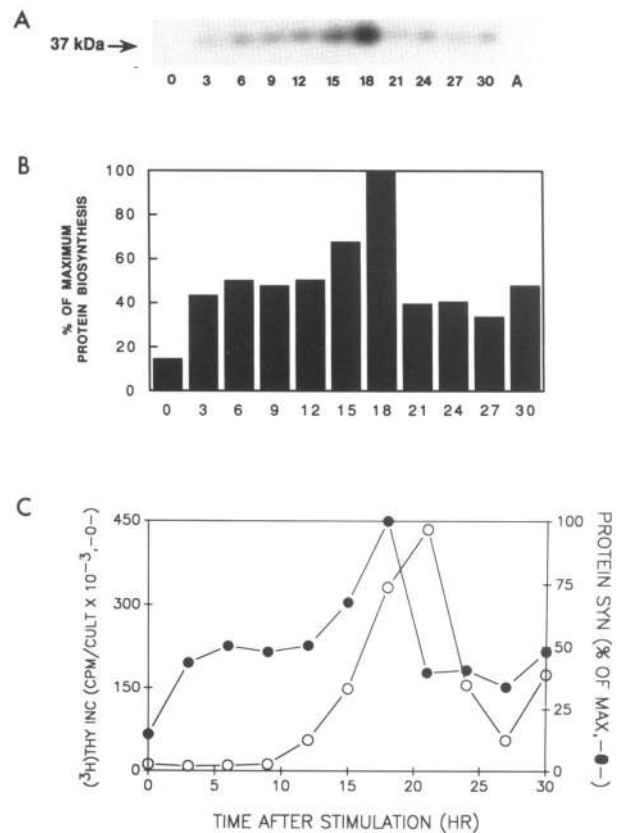
**RESULTS**

The pChug 20.1 plasmid containing the 1.3 kb GAPDH/UDG cDNA was used in Northern blot analysis to quantitate steady state mRNA levels in confluent normal human cells. As shown in Figure 1A, a single RNA of 1.6 kb was observed comparable to that detected in other human cell samples (10, 11). Increasing the amount of RNA demonstrated that the extent of hybridization was proportional to the amount of RNA added. The immunoprecipitation of (<sup>35</sup>S)methionine radiolabeled protein in confluent cells was monitored using negative control monoclonal antibody 1.05 and anti-human GAPDH/UDG monoclonal antibody 40.10.09. As shown in Figure 1B, the 40.10.09 antibody selectively immunoprecipitated the 37 kDa protein. The 45 kDa protein immunoprecipitated by both antibodies was identified as actin using an anti-actin antibody in the immunoprecipitation reaction (results not shown). As shown in Figure 1C, radiolabeling of both the 37 kDa protein and actin was linear up to a labeling time of 120 min. enabling the use of a 30 min. incubation to examine the rate of synthesis of the 37 kDa protein.

Cell cycle regulation of steady state GAPDH/UDG mRNA levels were examined in two different normal human skin fibroblast cell strains. Cells were synchronized by serum depletion then stimulated to traverse the cell cycle by the addition of complete medium. Northern blot analysis (normalized to total RNA concentration) was performed at 3 hr. intervals using the pChug 20.1 plasmid containing the 1.3 kb human GAPDH/UDG cDNA. As shown in Figure 2A, in the GM-6112 cell strain, the level of hybridization of the nick-translated plasmid to the 1.6 kb GAPDH/UDG RNA increased dramatically during the cell cycle. Similar results were observed in other human cell strains (results not shown). Relative levels of the 1.6 kb GAPDH/UDG RNA were determined by densitometry (Fig. 2B). Little RNA was observed at 0 hr. and no RNA at 3 hr. A 6-fold increase in the 1.6 kb RNA was observed at 6 hr. GAPDH/UDG RNA synthesis then diminished until a further increase began at 12–15 hr. At 18 hr. after serum addition, GAPDH/UDG RNA levels increased 19-fold relative to 0 hr. At subsequent times, the extent of hybridization diminished drastically approaching basal levels at 27–30 hrs. after serum addition. The temporal increase in steady state GAPDH/UDG mRNA levels was examined in



**Figure 2.** Regulation of steady state GAPDH/UDG mRNA in the cell cycle. Cells were synchronized by serum depletion then stimulated to enter the cell cycle by the readdition of serum as described in Materials and Methods. Northern blot analysis was performed using total RNA (normalized to 10 µg RNA/lane) extracted from cells incubated with complete media (zero time) and at 3 hr. intervals thereafter (Fig. 2A). The extent of hybridization to the pChug 20.1 probe was determined by densitometry (Fig. 2B). DNA synthesis was determined by pulsing parallel cultures with (<sup>3</sup>H)thymidine (2 Ci/mMole; 30 µCi/culture) at the indicated intervals (Fig. 2C) for 30 min. prior to collection. Cell sonicates were prepared as described in Material and Methods. Acid precipitable radioactivity was determined by liquid scintillation spectroscopy.



**Figure 3.** Translational regulation of the GAPDH/UDG protein in the cell cycle. Cell cycle analysis was performed as described in Materials and Methods and in the legend to Figure 2. Crude cell extracts were prepared from parallel cultures pulsed with (<sup>35</sup>S)methionine 30 min. prior to their collection at the identical intervals described in the legend to Figure 2. Immunoblot analysis was performed using 50 µg of monoclonal antibody 40.10.09 (Fig. 3A, 0–30 hr.) or 50 µg of negative control antibody 1.05 (lane A, zero time interval). The rate of biosynthesis in the cell cycle was determined by densitometry (Fig. 3B). The relationship between GAPDH/UDG biosynthesis and DNA replication (Fig. 3C) was determined as described in the legend to Fig. 2.

relation to the induction of DNA synthesis (Fig. 2C). No detectable DNA synthesis was observed during the first phase of GAPDH/UDG expression (0–9 hr.). In the second phase (15–21 hr.) the rate of DNA synthesis continued to increase. However, the 19-fold increase in the amount of GAPDH/UDG mRNA preceded DNA replication. In particular, at 21 hr., representing the height of genomic replication, this second phase was completed. Thus, irrespective of function, these results demonstrate that steady state levels of the 1.6 kb GAPDH/UDG RNA were specifically regulated in the cell cycle prior to DNA replication.

The biosynthesis of the GAPDH/UDG protein was then examined at identical intervals in parallel cultures of GM-6112 cells. Immunoprecipitation of (<sup>35</sup>S)methionine labeled proteins was performed normalized to equal amounts of total protein. The 37 kDa protein was identified after SDS/PAGE. As shown in Figure 3A, although basal levels of the 37 kDa protein could be detected at 0 hr., the extent of immunoprecipitated radiolabeled protein increased dramatically during the cell cycle. The relative rate of biosynthesis was determined by densitometry (Fig. 3B). A biphasic increase in the translation of the GAPDH/UDG gene was observed. In the first phase (0–9 hr.), the rate of GAPDH/UDG protein synthesis increased 3-fold compared to zero time. In the second phase (15–21 hr.), a 7-fold increase was observed as compared to basal levels observed at zero hr. The regulation of GAPDH/UDG biosynthesis was identical to that observed for gene transcription. The relationship between the

translation of the GAPDH/UDG gene and the induction of DNA synthesis was determined (Fig. 3C). The temporal regulation of GAPDH/UDG protein synthesis preceded DNA replication and was indistinguishable to that observed for GAPDH/UDG gene transcription. A similar pattern was observed in the GM-6167 normal human cell strain (results not shown). Again, irrespective of function, these studies document that normal human cells actively regulate the biosynthesis of the 37 kDa GAPDH/UDG protein.

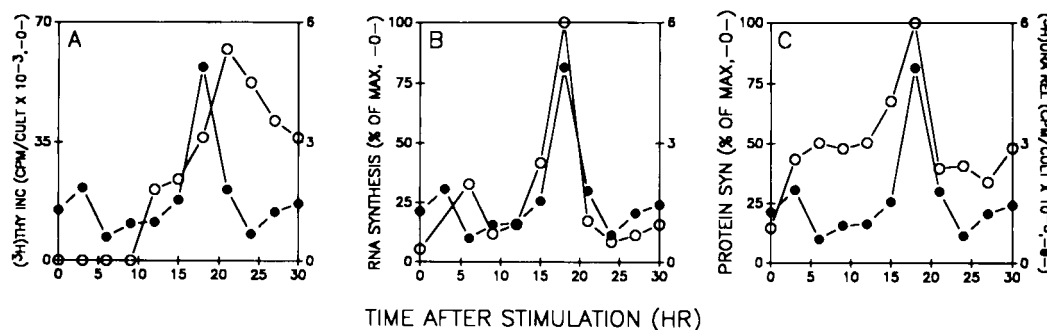
The half-life of the 37 kDa protein in the cell cycle was then examined. Cells were pulsed with (<sup>35</sup>S)methionine for 30 min. at 0, 18 and 24 hr. after serum addition then collected or incubated further for 1, 2 or 4 hr. in complete media containing unlabeled methionine. As shown in Figure 4, Lanes 1–4, no radioactivity was detected in the 37 kDa protein when cells at 0 hr. were pulsed with (<sup>35</sup>S)methionine prior to collection. The 0 hr. time point represents an interval at which time GAPDH/UDG gene expression and the production of the 37 kDa protein were at basal levels. A similar result was observed when (<sup>35</sup>S)methionine was added to cells at 24 hr. after serum addition (Fig. 4, lanes 9–12). The 24 hr. time point represents an interval at which time both steady state levels of GAPDH/UDG RNA and the biosynthesis of the 37 kDa protein were diminished. However, at each interval significant radioactivity was observed in the 45K actin band.

In contrast, at 18 hr. after serum addition, significant radioactivity was detected in the 37 kDa protein when cells were incubated with (<sup>35</sup>S)methionine for 30 min. (Fig. 4, lane 5). Incubation for 1 hr. in media containing unlabeled methionine resulted in a significant decrease in the extent of labeling (Fig. 4, lane 6). Similar results were observed at the 2 and 4 hr. chase intervals. (Fig. 4, lanes 7,8). The 18 hr. time point represents that interval at which time the transcription and translation of the GAPDH/UDG gene was its highest level. The relative rate of GAPDH/UDG protein synthesis and degradation at 18 hr. after serum addition was determined by densitometry. Radioactivity in the 37 kDa protein decreased by over 80% at the 1 hr. chase interval then remained relatively constant at 2 and 4 hr. In contrast, the extent of radioactivity in the 45 kDa actin band remained relatively constant with a decrease of approximately 20% after 4 hr.

To begin to consider the relationship between GAPDH/UDG gene transcription and the reported activities of the 37 kDa



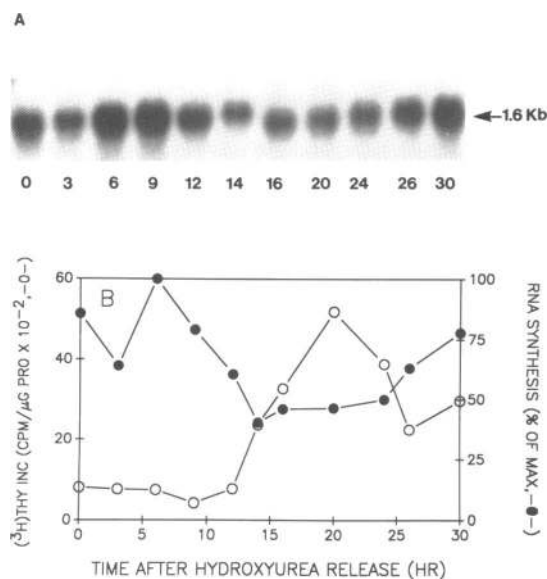
**Figure 4.** Biosynthesis of the GAPDH/UDG protein during the cell cycle. Cells were pulsed by incubation with (<sup>35</sup>S)methionine for 30 min. Cells were then collected or the radiolabel chased by incubation in complete media for 1, 2 or 4 hr. prior to collection. Cells were pulse-chased at zero time (lanes 1–4), 18 hr. (lanes 5–8), or 24 hr. (lanes 9–12) after the addition of serum containing medium to synchronized cells.



**Figure 5.** Regulation of the uracil DNA glycosylase and GAPDH/UDG gene expression in the cell cycle. Crude cell extracts were prepared at the indicated intervals in the cell cycle as previously described (12). Glycosylase activity was determined by in vitro biochemical assay which quantitated the release of (<sup>3</sup>H)uracil from a poly (dA)-poly (<sup>3</sup>HdU) substrate (29). Data for GAPDH/UDG transcription and biosynthesis are derived from Fig. 2 and 3, respectively. **A:** Regulation of glycosylase activity in relation to DNA synthesis; **B:** Regulation of GAPDH/UDG gene transcription in relation to glycosylase activity; **C:** Regulation of GAPDH/UDG protein synthesis in relation to glycosylase activity.

protein, uracil DNA glycosylase activity was determined at each interval in the cell cycle. As shown in Figure 5A, UDG activity was increased after serum addition. Basal UDG levels were observed at 0–9 hr. while maximal enzyme stimulation was detected at 18 hr. after serum stimulation. UDG activity then decreased and returned to basal levels. The relationship between UDG activity and steady state levels of GAPDH/UDG RNA was then examined. As shown in Figure 5B, little or no increase in UDG activity was detected during the first increase in GAPDH/UDG RNA levels. In contrast, the temporal pattern of UDG activity was virtually identical to that observed for the second phase of GAPDH/UDG gene regulation. Similarly, there was no relationship between UDG activity and the first phase of GAPDH/UDG protein synthesis (Fig. 5C) while UDG levels and the second phase of GAPDH/UDG biosynthesis were indistinguishable. The identical regulation of uracil DNA glycosylase activity and the major peak of GAPDH/UDG gene expression suggests a strict correlation between that regulation and an activity reported for this multifunctional protein.

Synchronization with hydroxyurea (HU) was then used to distinguish between changes in GAPDH/UDG mRNA levels mediated by growth factors or other factors in serum and a specific cell cycle effect. Confluent normal human cells were replated at a lower density in complete medium and incubated for 4 hr. Hydroxyurea (0.5 mM) was then added and cells were incubated for an additional 18 hr. Hydroxyurea was removed through media change to allow for subsequent cell growth. Maximal levels of GAPDH/UDG mRNA were observed at 0–6 hr. after HU release (Fig. 6). The extent of hybridization decreased steadily thereafter. Lowest mRNA levels were observed at 15–24 hr. after drug release, intervals which were characterized by the induction of DNA synthesis. Subsequently,



**Figure 6.** Regulation of GAPDH/UDG mRNA in hydroxyurea synchronized normal human cells. Cells were synchronized by treatment with 0.5 mM hydroxyurea then permitted to progress through the cell cycle as previously described (30). Steady state GAPDH/UDG mRNA levels through the cell cycle were monitored by Northern blot analysis while the rate of DNA synthesis examined by pulsing cells with (<sup>3</sup>H)thymidine 30 min. prior to collection. **A:** Northern blot analysis; **B:** Hybridization levels as determined by densitometry (closed circles); DNA synthesis (open circles).

as DNA synthesis diminished, the amount of GAPDH/UDG mRNA again started to increase at 28–30 hr. These results demonstrate that the regulation of steady state GAPDH/UDG mRNA levels did not appear to be a serum-induced event but instead was intrinsic to the cell cycle. Noteworthy, in contrast to serum arrested cells, the increase in GAPDH/UDG mRNA had already occurred presumably due to cell synchronization by HU between G<sub>1</sub> and S. However, irrespective of the method of synchronization (i.e., serum depletion or hydroxyurea), the increase in GAPDH/UDG mRNA was observed before DNA synthesis demonstrating that the expression pattern relative to S phase was comparable.

## DISCUSSION

Numerous studies over the past three decades appeared to establish glyceraldehyde-3-phosphate dehydrogenase as a classical glycolytic enzyme. It has been used in numerous studies as a model system not only to examine mechanisms of enzyme action but also to determine the relationship between protein structure and function. Further, the GAPDH gene itself provided a model system for initial studies in gene structure and was used as a reference for subsequent other studies of unique genes. In toto, conventional wisdom indicated only limited utility and interest in this housekeeping gene. However, a series of studies continued to discover new and surprising activities (1–9) including the recent report that this 37 kDa protein may function as a uracil DNA glycosylase in DNA repair (11).

The investigations described here provide the first indication of a unique and striking regulation of this gene in human cells. They provide documentation that the expression of the GAPDH/UDG gene is cell cycle dependent with a defined temporal sequence in relation to the induction of DNA synthesis. This is in accord with previous results demonstrating the proliferative-dependent regulation of the GAPDH/UDG gene (19). This study documents a biphasic increase in steady state RNA levels which was paralleled by an identical increase in the biosynthesis of the 37 kDa GAPDH/UDG protein. Accordingly, these studies demonstrate that human cells tightly couple increases in steady state GAPDH/UDG mRNA levels gene in the cell cycle to its translation.

Initial studies localized the GAPDH/UDG gene to chromosome 12 (20). Southern blot hybridization using GAPDH/UDG cDNA probes indicated the possible existence of a multigene family with a complex pattern of cross-hybridizing sequences on a number of chromosomes, including 1, 2, 4, 6, 12 and X (21). These were considered to be processed pseudogenes. Studies in human liver suggested the presence of multiple GAPDH/UDG genes (22) while a subsequent report identified a single human GAPDH/UDG gene (23). That analysis would suggest that only one functional mRNA would be present in human cells. In this study we observed a unique pattern of expression of the GAPDH/UDG gene. In particular, a biphasic increase in steady state RNA levels was detected. This would infer that a mechanism must exist through which a human cell sequentially stimulated the production of a single mRNA species from one functional gene at different intervals in the cell cycle.

Immunocytochemical studies using the 40.10.09 GAPDH/UDG antibody demonstrated that the subcellular localization of the 37 kDa protein displayed a defined pattern as a function of cell growth (24). In non-cycling normal human fibroblasts, the 37 kDa protein was present solely in the cytoplasm

with no detectable fluorescence in the nucleus. This was in contrast to its nuclear or perinuclear localization in randomly proliferating non-transformed human cells. This study demonstrates that the biosynthesis of the GAPDH/UDG 37 kDa protein was regulated with a specific biphasic increase in the cell cycle. In concert with the immunocytochemical studies, this temporal pattern of protein synthesis may be indicative of the multifunctional activities catalyzed by the 37 kDa protein in vivo. In particular, it is very difficult to reconcile the presence of this protein in the nucleus if its sole physiological function is that of GAPDH in glycolysis. In this regard, it is interesting to note that pulse-chase studies indicated that the 37 kDa protein had a half-life less than 1 hr. This is remarkably similar to previous reports documenting that oncogene proteins display a comparable lability (25–28).

The biphasic pattern of GAPDH/UDG biosynthesis may be indicative of selective functions of this protein at specific intervals in the cell cycle. This study documents a strict correlation of UDG activity with the second phase of GAPDH/UDG transcription and biosynthesis. In contrast, no detectable increase in UDG activity was observed during the first phase of GAPDH/UDG gene regulation and translation. Thus, human cells may display different temporal patterns in the cell cycle for the other activities which were reported to characterize the 37 kDa GAPDH/UDG protein. Alternatively, the first phase may simply reflect a non-specific serum response. The results described in this report provide a model system to distinguish between these two possibilities and to consider the molecular mechanisms through which human cells couple gene transcription to the biosynthesis and function of a multidimensional protein.

## ACKNOWLEDGEMENTS

The generous counsel of Dr Kenneth Soprano is gratefully appreciated. This research was supported by a grant to M.A.S. from the National Institutes of Health (CA-29414) and by grants to the Fels Institute for Cancer Research and Molecular Biology from the National Institutes of Health (CA-12227) and the American Cancer Society (SIG-6).

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