

## Molecular Mechanisms of Cell Cycle Block by Methionine Restriction in Human Prostate Cancer Cells

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**Abstract:** Previous studies have shown that dietary or pharmacological methionine restriction inhibits growth of human prostate cancer cells in vitro or as xenografts in mice. We undertook the present studies to clarify the molecular mechanisms by which methionine restriction inhibits prostate cancer cell growth. We found that PC-3 and DU 145 cells stopped proliferating within six days in growth medium containing homocysteine in place of methionine. In contrast, proliferation of LNCaP cells was only partially inhibited by the absence of methionine. Using flow cytometry, we found that methionine restriction caused PC-3 cells to arrest in all phases of the cell cycle, but predominantly in the G<sub>2</sub>/M phase, whereas LNCaP cells accumulated exclusively in the G<sub>1</sub> phase. Methionine restriction led to accumulation of the cyclin-dependent kinase inhibitors p21 and p27, as determined by Western blot analysis, and inhibited the enzymatic activities of the cyclin-dependent kinases CDK2 and cdc2, as determined by an in vitro kinase assay. However, methionine restriction had little or no effect on CDK2 or cdc2 protein levels. Methionine restriction also induced PC-3 cells to undergo apoptosis, as indicated by the appearance of a typical nucleosomal ladder on gel electrophoresis of genomic DNA. We conclude that methionine restriction has potential as a novel treatment strategy for prostate cancer.

### Introduction

Prostate cancer kills >30,000 American men each year (1). Surgery and radiation treatment save many lives, but far too many men develop metastatic prostate cancer, which is not curable with local treatment measures. Hormonal therapy is effective for a short time, but most patients develop hormone-independent prostate cancer, which responds poorly to conventional chemotherapy and is, therefore, usually lethal. Fortunately, major advances in molecular biology in recent years have led to the discovery of several novel targets for cancer treatment. One such target is the methionine dependence of tumors.

Methionine is an essential amino acid with at least four major functions. First, methionine is a building block for proteins, as are the other amino acids. Second, methionine is a precursor of glutathione, a tripeptide that reduces reactive oxygen species, thereby protecting cells from oxidative stress (2). Third, methionine is required for synthesis of polyamines, which have far-ranging effects on nuclear structure and cell division (3) (Figure 1). Fourth, methionine is the major source of methyl groups for methylation of DNA and other molecules (Figure 1). DNA methylation is one of the mechanisms by which gene expression is regulated (4).

Methionine cannot be synthesized by mammalian cells from any of the other 19 standard amino acids. However, mammalian cells can convert homocysteine to methionine (Figure 1). Normal cells can therefore grow in culture when methionine is replaced by homocysteine in the growth medium (5), and animals fed diets in which methionine has been replaced by homocysteine suffer no ill effects and grow normally (6,7). In contrast, most human and rodent cancer cells growing in culture are methionine dependent, since they fail to grow in the absence of methionine, even when provided with homocysteine (8–11). Methionine dependence is thought to be due to increased rates of transmethylation in cancer cells compared with normal cells (12–14). Tumors are also methionine dependent *in vivo*. Dietary methionine restriction causes regression of animal tumors, including human prostate cancer xenografts in nude mice (15,16), and inhibits metastasis in animal models (7,17). Methioninase, an enzyme that specifically degrades methionine, also inhibits growth of solid tumors and leukemia in animals (18–22). One clinical trial of dietary methionine restriction combined with chemotherapy has shown preliminary evidence of activity against gastric cancer (23).

Methionine restriction induces cell cycle arrest in cancer cells (16). Simian virus 40 (SV40)-transformed human fibroblasts (24) and androgen-independent human prostate cancer cells (15,25) growing in culture arrest in the late S/G<sub>2</sub> phase when deprived of methionine, as do Yoshida sarcomas in nude mice (16). We undertook the present studies to clarify the molecular mechanisms by which methionine restriction induces cell cycle arrest in prostate cancer cells.



through a 12% sodium dodecyl sulfate-polyacrylamide gel, which was exposed to X-ray film.

### Fluorescence-Activated Cell Sorter Analysis

Two million cells were suspended in 2 ml of 0.9% NaCl and then fixed at room temperature for 30 minutes by the addition of 5 ml of 95% ethanol. Cells were then spun down and resuspended in 1 ml of propidium iodide solution (50  $\mu\text{g/ml}$ ) containing 10  $\mu\text{l}$  of ribonuclease at a concentration of 10 mg/ml and incubated at 37°C for 30 minutes. DNA content per cell was measured by flow cytometry using an EPICS XL-MCL apparatus (Coulter, Hialeah, FL).

### DNA Fragmentation Assay

Genomic DNA was isolated using a kit purchased from Qiagen (Chatsworth, CA). Ten micrograms of DNA per lane were electrophoresed through a 1.8% agarose gel in Tris-acetate-0.001 M EDTA buffer, and the gel was stained with ethidium bromide. Fluorescent DNA bands were visualized with an ultraviolet transilluminator and photographed.

## Results

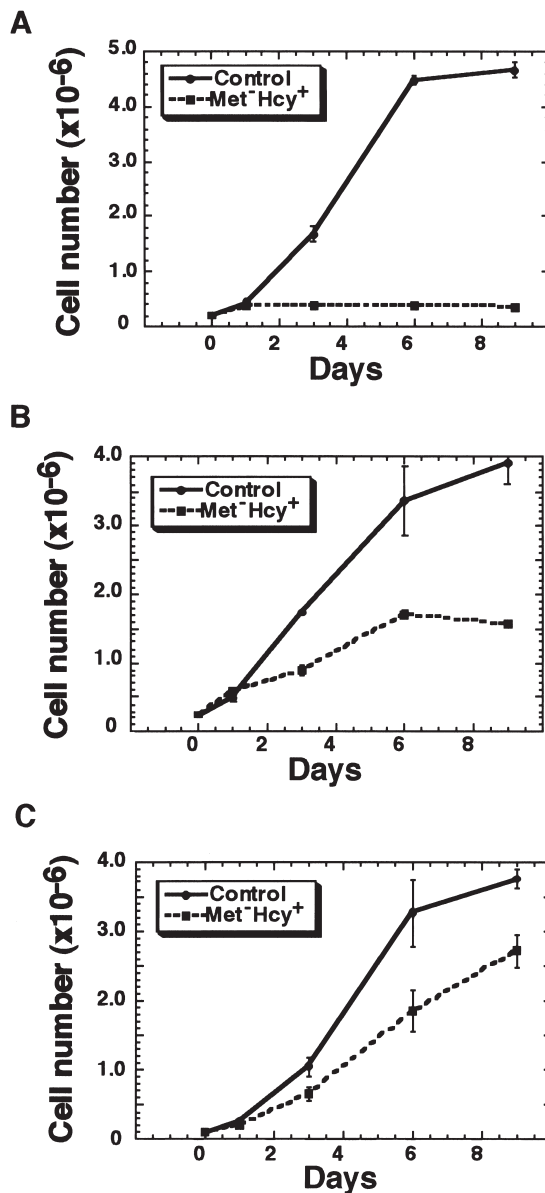
### Three Human Prostate Cancer Cell Lines Vary in Their Sensitivity to Methionine Restriction

Previous studies have shown that methionine restriction inhibits growth of PC-3 cells *in vitro* (11,25,27) and *in vivo* (15). In the present studies, we compared three different human prostate cancer cells lines with respect to their methionine dependence/independence. LNCaP cells originated from a human lymph node metastasis, are androgen dependent, express prostate-specific antigen, and have normal p53 and retinoblastoma genes (28–30). DU 145 and PC-3 cells are less differentiated than LNCaP cells, since they originated from distant metastases, express only mutant p53, are androgen independent, and do not express prostate-specific antigen (28,29).

We compared the growth rates of each of the cell lines in standard medium with their growth rates in methionine-free medium. We found that PC-3 cells were entirely methionine dependent (Figure 2A), in sharp contrast to LNCaP cells, which were nearly methionine independent (Figure 2C). DU 145 cells were intermediate in their response to methionine restriction (Figure 2B).

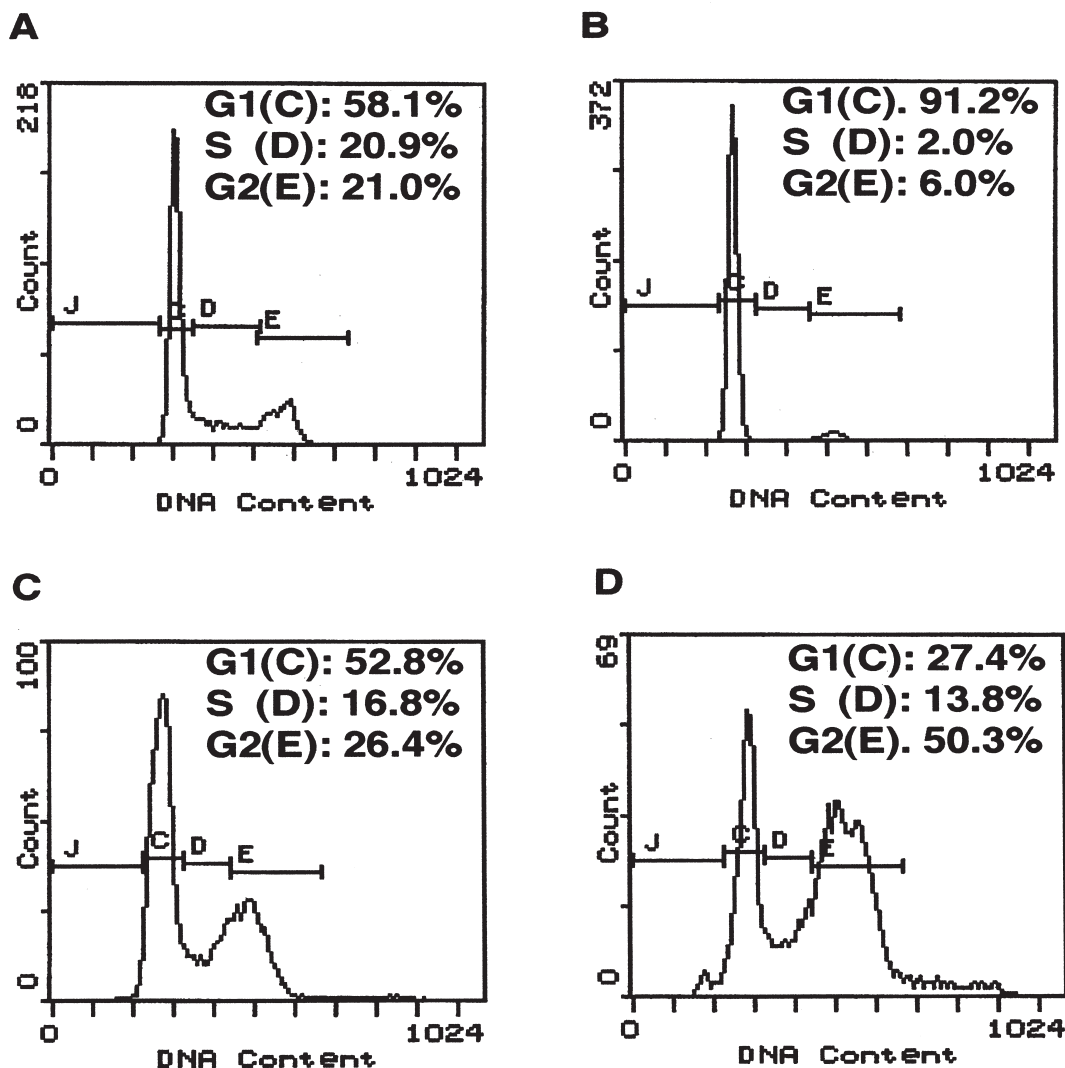
### Methionine Restriction Slows Cell Cycle Progression and Induces Apoptosis in Prostate Cancer Cells

We next used fluorescence-activated cell sorter analysis to determine how methionine affects the distribution of human prostate cancer cells in different phases of the cell cycle. We found that LNCaP cells grown in methionine-free medium for six days accumulated almost exclusively in the G<sub>1</sub> phase, with only 2% of cells remaining in the S phase and 6% remaining in the G<sub>2</sub>/M phase after methionine restriction



**Figure 2.** Methionine restriction inhibits growth of 3 human prostate cancer cell lines. PC-3 (A), DU 145 (B), and LNCaP (C) cells were grown for up to 9 days in methionine-free RPMI medium containing 100  $\mu\text{M}$  homocysteine (Met<sup>-</sup>Hcy<sup>+</sup>) or in RPMI medium containing 100  $\mu\text{M}$  methionine + 100  $\mu\text{M}$  homocysteine (control). All media contained 10% fetal calf serum. At indicated times, cells were trypsinized and counted with a hemocytometer. Values are means  $\pm$  SD of 3 separate wells. In some cases, SD bars are not visible, since they were smaller than corresponding symbol.

(Figure 3, A and B). In contrast, PC-3 cells accumulated in multiple phases of the cell cycle. The G<sub>2</sub>/M fraction increased from 26.4% to 50.3% in response to methionine restriction, but 27.4% of PC-3 cells remained in the G<sub>1</sub>/S phase, and 13.8% remained in the S phase (Figure 3, C and D). These results, in combination with the growth curves in Figure 2, suggest that methionine-deprived LNCaP cells continue to traverse the cell cycle, although at reduced rates compared with control conditions, whereas PC-3 cells undergo cell cycle arrest.



**Figure 3.** Methionine restriction slows cell cycle progression in human prostate cancer cells. LNCaP (A and B) and PC-3 (C and D) cells were grown for 6 days in RPMI medium containing 100  $\mu$ M methionine + 100  $\mu$ M homocysteine (A and C) or in methionine-free RPMI medium containing 100  $\mu$ M homocysteine (B and D). All media contained 10% fetal calf serum. Cells were then prepared as described in **Methods** and analyzed with a fluorescence-activated cell sorter. Cell numbers are indicated on vertical axes, and relative proportions of cells in different phases of cell cycle are indicated at top right. In D, a small hypodiploid peak representing apoptotic cells is present to left of  $G_1$  peak. J, C, D, and E, manually set regions corresponding to hypodiploid,  $G_0/G_1$ , S, and  $G_2/M$  cell populations, respectively.

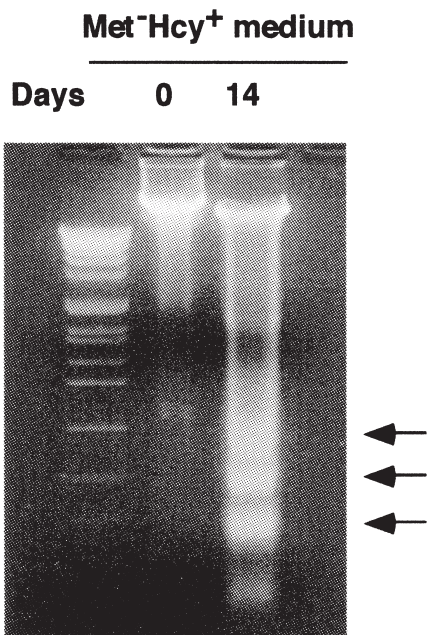
PC-3 cells also began to undergo apoptosis within six days of methionine restriction, as indicated by a hypodiploid peak in the DNA histogram (Figure 3D). By 14 days of methionine restriction, PC-3 cell apoptosis was readily apparent by gel electrophoresis of genomic DNA, which revealed a typical nucleosomal ladder after ethidium bromide staining (Figure 4).

#### **Methionine Restriction Increases Levels of Cyclin-Dependent Kinase Inhibitors and Inhibits Activities of Cyclin-Dependent Kinases**

We next determined whether methionine restriction alters the levels or activities of several key cell cycle regulatory molecules that have been implicated in tumorigenesis and/or

cancer progression. These included p21, a cyclin-dependent kinase inhibitor (31,32) that blocks  $G_1$ -to-S transition (33) and  $G_2$ -to-M transition (34); p27, a cyclin-dependent kinase inhibitor in the same family as p21 that blocks  $G_1$ -to-S transition; p16 and p15, two members of the INK4 family of cyclin-dependent kinase inhibitors that block progression through the early  $G_1$  phase; and the cyclin-dependent kinases CDK2 and cdc2 (CDK1). CDK2 primarily regulates the  $G_1$ -to-S transition, whereas cdc2 regulates the  $G_2$ -to-M transition (35).

We found that p21 levels rose in LNCaP cells within one day of methionine restriction and remained elevated for at least six days (Figure 5). p27 also accumulated in LNCaP cells deprived of methionine for three days and remained elevated for at least six days (Figure 5). Levels of p16 were unaffected by methionine restriction, and p15 was undetect-



**Figure 4.** Methionine restriction induces apoptosis in human prostate cancer cells. PC-3 cells were grown for 14 days in RPMI medium containing 100  $\mu$ M methionine + 100  $\mu$ M homocysteine or in methionine-free RPMI medium containing 100  $\mu$ M homocysteine. Both media contained 10% fetal calf serum. Genomic DNA was then analyzed by gel electrophoresis. Arrows, fragments of DNA that differ in size by  $\sim$ 200 bp, consistent with a typical nucleosomal ladder. Leftmost lane contains DNA molecular weight standards.

able by Western blot analysis in LNCaP cells under control conditions and remained undetectable after methionine restriction for up to six days (not shown).

Similar results were obtained with PC-3 cells. p27 accumulated in PC-3 cells within three days of methionine restriction and remained elevated for at least six days (Figure 5). p21, p16, and p15 were undetectable by Western blot analysis of PC-3 cells at baseline or after methionine restriction (not shown).

The observed increases in p21 and/or p27 levels were associated with a commensurate inhibition of cyclin-dependent kinase activities in LNCaP and PC-3 cells (Figure 6). Methionine restriction eliminated or greatly reduced the kinase activities of CDK2 and cdc2 in both cell lines. The observed inhibition of enzymatic activities was not simply due to reduction in cyclin-dependent kinase protein levels, since those levels remained constant, except in the case of cdc2 in LNCaP cells (Figure 6).

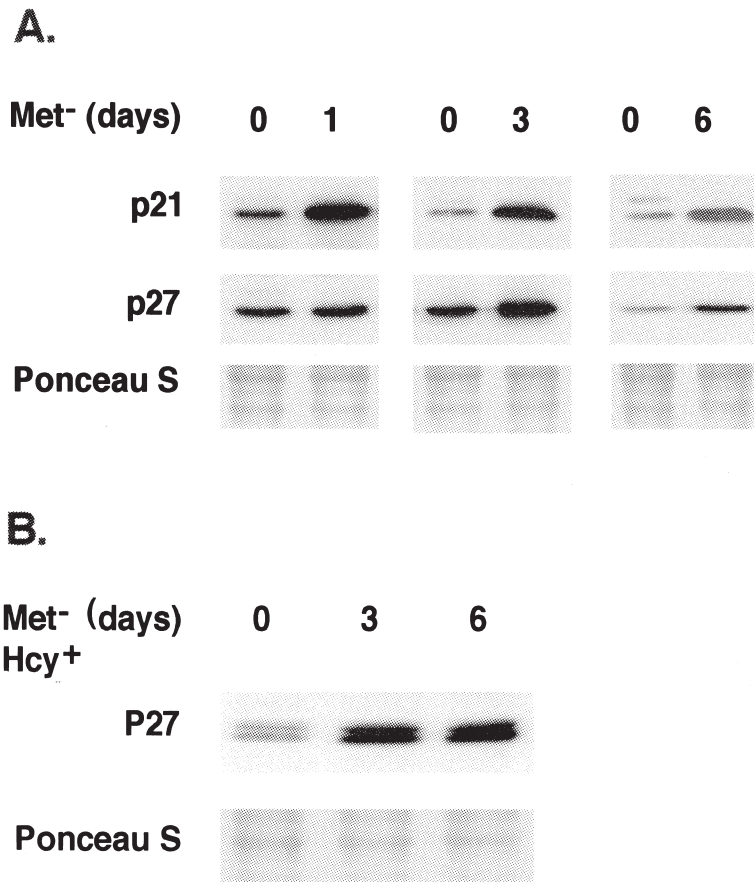
## Discussion

The present studies show that three human prostate cancer cell lines differ with regard to their responses to methionine restriction. Methionine restriction inhibited growth of PC-3 and DU 145 cells to a greater extent than it did LNCaP cells, which are relatively well differentiated, al-

though all three cell lines were significantly inhibited by methionine restriction. PC-3 cells arrested in all phases of the cell cycle, with the greatest accumulation of cells in the G<sub>2</sub>/M phase, whereas LNCaP cells accumulated primarily in the G<sub>1</sub> phase but continued to traverse the cell cycle at a reduced rate. Accumulation of PC-3 cells predominantly in the G<sub>2</sub>/M phase is similar to the effect of DNA-damaging drugs, such as doxorubicin, on other types of cancer cells (36,37). "Methionine-free" medium used for these experiments actually contained trace amounts of methionine and homocysteine due to the presence of 10% nondialyzed FBS (see **Methods**). Stringent elimination of all methionine and homocysteine, by methioninase treatment or by use of dialyzed serum for cell culture experiments, could possibly lead to different parameters of cell cycle arrest. PC-3 cells also began to undergo apoptosis within six days of methionine restriction, as noted previously (15). In contrast, LNCaP cells were relatively resistant to the proapoptotic effects of methionine restriction for  $\geq$ 14 days. These results suggest that dietary or pharmacological methionine restriction may be active against poorly differentiated prostate cancer, which responds poorly to conventional cytotoxic chemotherapy. However, future studies, including clinical trials, are required to test this possibility.

The present studies identify some of the components of the cell cycle machinery that are regulated by methionine. We found that methionine restriction increased levels of cyclin-dependent kinase inhibitors p21 and p27 in LNCaP cells, with a commensurate inhibition of CDK2. These results are consistent with the observed accumulation of LNCaP cells in the G<sub>1</sub> phase. cdc2, which promotes the G<sub>2</sub>-to-M transition, was also inhibited in LNCaP cells. It was therefore surprising that LNCaP cells did not accumulate in the G<sub>2</sub>/M phase. We hypothesize that other cell cycle regulatory molecules promote the G<sub>2</sub>-to-M transition in methionine-restricted LNCaP cells, despite the lack of cdc2 activity. Future experiments will test that hypothesis. We also found that methionine restriction increased levels of p27, but not p21, in PC-3 cells. The molecular mechanisms underlying cdc2 inhibition and the associated G<sub>2</sub>/M block in PC-3 cells, therefore, remain unclear in light of the established role of p27 in blocking the G<sub>1</sub>-to-S transition. p27 and p21 share a common NH<sub>2</sub>-terminal domain for binding to and inhibiting the kinase activities of CDK-cyclin complexes (38,39). We therefore hypothesize that p27 inhibits cdc2 and CDK2 in methionine-restricted PC-3 cells, thereby leading to arrest in multiple phases of the cell cycle. Future experiments are required to test that hypothesis.

Future studies are also required to identify the "upstream" biochemical events that ultimately slow or halt the cell cycle machinery and initiate proapoptotic programs in cancer cells deprived of methionine. The effects of methionine restriction may be related to methionine's role as a methyl donor for methylation of DNA, RNA, proteins, and other molecules. Several growth inhibitory and proapoptotic genes become transcriptionally silenced in tumors as a result of focal DNA hypermethylation (4,40). RNA methylation



**Figure 5.** Methionine restriction increases levels of cyclin-dependent kinase inhibitors. A: LNCaP cells were grown in standard RPMI medium containing 100  $\mu$ M methionine or in methionine-free RPMI medium for up to 6 days and were then analyzed for p21 and p27 levels by Western blot. B: PC-3 cells were grown in standard RPMI medium containing 100  $\mu$ M methionine or in methionine-free RPMI medium containing 100  $\mu$ M homocysteine for up to 6 days and analyzed for p27 by Western blot. All media contained 10% fetal calf serum. Ponceau S staining indicated equal protein loading.

regulates mammalian ribosomal RNA processing and messenger RNA transport, translation, and stability (41,42). Cancer cells generally have higher rates of transmethylation and, therefore, higher methionine requirements than normal cells (12–14). Methylation of nucleic acids may therefore be the rate-limiting step that prevents growth of cancer cells in the presence of low methionine concentrations (43). However, it remains to be seen whether methionine restriction will restore expression of growth inhibitory genes by reversing focal DNA hypermethylation or will regulate RNA function in cancer cells.

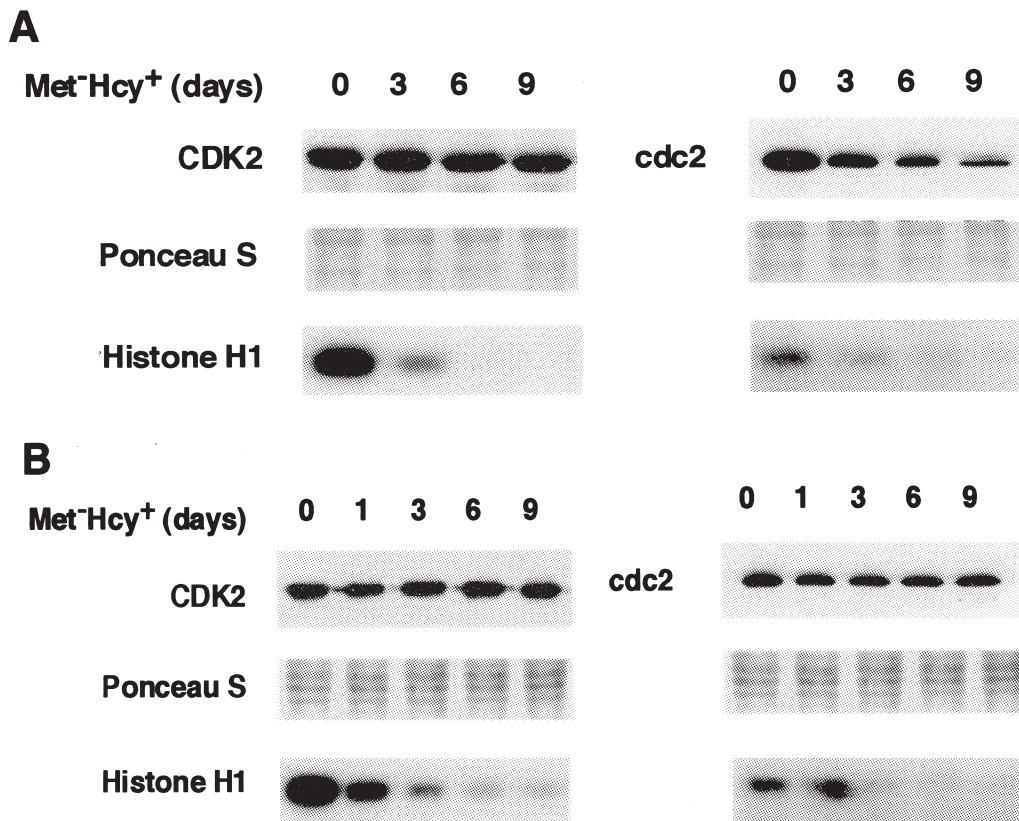
The methionine dependence of tumors may also relate to the role of methionine in glutathione homeostasis. Glutathione is a ubiquitous tripeptide ( $\gamma$ -glutamylcysteinylglycine) that protects cells against oxidative stress (2). Many tumors contain elevated levels of glutathione that confer resistance to certain chemotherapy drugs, namely, cisplatin and alkylating agents (44,45). Previous studies have shown that methionine restriction lowers glutathione levels in cancer cells by increasing glutathione efflux or inhibiting its synthesis (46–48). Methionine restriction thereby sensitizes tumors to chemotherapy (44,45,49,50) and radiation therapy (47,48). Methionine restriction may therefore have the greatest

promise as an adjunct to chemotherapy or radiation therapy instead of as monotherapy (5,23,51).

Most of the clinical studies concerning dietary methionine restriction have involved patients who do not have cancer. Two studies showed that plasma methionine levels fell by  $\sim$ 70% in healthy adults fed a methionine-free diet for eight days (52,53). Several studies have shown that patients with homocystinuria, a hereditary metabolic disorder, tolerate dietary methionine restriction chronically (54–56). All these studies support the feasibility of dietary methionine restriction for treatment of cancer. We are testing dietary methionine restriction for adults with refractory solid tumors in a phase I trial at our institution. It is hoped that our trial will provide preliminary evidence in support of this novel treatment strategy.

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**Figure 6.** Methionine restriction inhibits activities of cyclin-dependent kinases. LNCaP (A) and PC-3 (B) cells were grown for up to 9 days in methionine-free RPMI medium containing 100  $\mu$ M homocysteine + 10% fetal calf serum. CDK2 and cdc2 protein levels (top bands) were measured by Western blot analysis. Kinase activities (bottom bands) were measured using an *in vitro* kinase assay with histone H1 as substrate. Ponceau S (middle bands) indicated equal protein loading.

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