in the LE phase (except for the grains). This suggests that these protrusions should be regarded as noncrystalline residues. The modulus of elasticity in the protrusions was about 10^8 Pa (that is, one order higher than that of rubber), whereas the modulus in the LC domains was substantially higher.

Monolayers transferred at the onset of the main phase-transition region (position 1 in Fig. 1; all other conditions are the same as for Fig. 2, A and C) exhibited a few bigger domains (>2 μm in diameter) surrounded by many smaller domains (~0.5 μm in diameter, Fig. 4A). In the case of a freshly prepared sample imaged within 1 hour after deposition, the shape of these small domains is similar to that of the large domains (compare Fig. 4B and Fig. 2A). However, the domains change their shape spontaneously on the solid substrates (Fig. 4C) within several hours during storage (humidity, 42 ± 1%; temperature, 21° ± 0.5°C). This phenomenon can thus only be detected by applying the AFM technique within a short time after film deposition. It is likely that, in the case of a high compression speed, the small domains consolidated and merged into the LC domains with visible boundaries as shown in Fig. 2D; in the case of a low compression speed, the domains grow at the expense of smaller ones, leading to better packed LC domains, as shown in Fig. 2C.

In fluorescence microscopy studies it is necessary that trace amounts of fluorescence dyes be added to the system under investigation. The influence of the dye molecules on the phase behavior of the monolayers is generally thought to be negligible in low concentrations (<3 mol%). With the AFM we were able to investigate the potential influence of dye molecules in even very low concentrations (here 0.25 mol%) as an impurity on the domain structures on transferred films. Figure 5, A and B, shows domain structures without and with fluorescence dye, respectively [all other conditions were kept exactly the same (V_c = 5.68 Å^2 molecule^{-1} min^{-1} and V_d = 20 mm min^{-1})]. Obviously, the presence of the fluorescence dye does affect the domain structures. In addition, the domains of the dye-containing systems change their shapes drastically from "star-like" (Fig. 5C) to "fiber-like" (Fig. 5D) during the film deposition, and the changes are very sensitive to the transfer conditions (in Fig. 5C, V_c = 8.52 Å^2 molecule^{-1} min^{-1} and V_d = 50 mm min^{-1}; in Fig. 5D, V_c = 2.84 Å^2 molecule^{-1} min^{-1} and V_d = 4 mm min^{-1}). These results indicate that care must be taken in extrapolating the results obtained from dye-labeled monolayers at the air-water interface to the transferred films.

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22. We dissolved stearic acid (Fluka) in chloroform (HPLC quality, Baker) to get a spreading solution with a concentration of 5 × 10^{-3} M. Branched poly(ethyleneimine) with an average molecular weight of M_n = 1800 (Polysciences) was dissolved in deionized water (water resistance, 19 megohms; Christ System), providing an aqueous solution with a concentration of 4 × 10^{-3} M.
23. The 250-μm scanner allows inspections from 0.25 mm to the submicrometer scale, thus providing a wide overlap with the resolution of a light microscope. The 10-μm scanner allows imaging down to the molecular scale.
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25. We thank M. Lösche for stimulating discussions.

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An Inhibitor of p34^{CDC28} Protein Kinase Activity from *Saccharomyces cerevisiae*

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The p34^{CDC28} protein from *Saccharomyces cerevisiae* is a homolog of the p34^{cdc2} protein kinase, a fundamental regulator of cell division in all eukaryotic cells. Once activated it initiates the visible events of mitosis (chromosome condensation, nuclear envelope breakdown, and spindle formation). The p34^{CDC28} protein also has a critical role in the initiation of DNA synthesis. The protein kinase activity is regulated by cycles of phosphorylation and dephosphorylation and by periodic association with cyclins. An endogenous 40-kilodalton protein (p40) originally identified as a substrate of the p34^{CDC28} protein kinase was purified. The p40 protein bound tightly to p34^{CDC28} and inhibited the activity of the kinase. The p40 protein may provide another mechanism to regulate p34^{CDC28} protein kinase activity.

The p34$^{CDC28/cdc2}$ protein kinase (p34) is a universal regulator of mitosis in eukaryotes (*1*). Its activity is required for entry into the DNA replicative phase (S phase) and for the initiation of mitosis (M phase) (*2*). Deactivation of p34 is required for exit from mitosis (*3*). The regulation of p34 protein kinase activity, subcellular localization, and substrate interaction is complex. Three primary modes of control have been described: (i) activation resulting from association with particular members of a family of cyclins (*4*), the synthesis and stability of which are tightly controlled (*5*); (ii) activating and deactivating cycles of phosphorylation and dephosphorylation at multiple sites on the p34 polypeptide (*6*); and (iii) controlled synthesis of p34 itself (*7*). Here, evidence is presented for another regulatory mechanism—the binding of an inhibitory protein.

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The protein kinase activity of p34, which is encoded by the *CDC28* gene of the budding yeast *Saccharomyces cerevisiae*, was originally demonstrated in immunoprecipitates from crude cell extracts (*8*). In these experiments, a 40-kD substrate (p40) coimmunoprecipitated with p34. Phosphorylation of p40 by p34 was cell cycle–dependent (*9*). Protein extracts taken from cells arrested in S phase or M phase had active p34 protein kinase (*10*), but phosphorylation of p40 was not detected in p34 immunoprecipitates (*9*). Extracts from cells arrested in the prereplicative phase (G_1) by mating pheromone treatment or nutrient deprivation had no detectable p34 protein kinase activity, but when these extracts were mixed with p34 immunoprecipitates from S or M phase–arrested cells, p40 phosphorylation was observed. These results suggested that p40 is only present in cells arrested in G_1 or is modified in S and M phase cells such that it cannot bind to, or be phosphorylated by, p34.

We purified p40 from cells arrested in G_1 by nutrient deprivation (Fig. 1A). Only small amounts of p40, typically 40 to 80 μg of stationary cells, were obtained. The low yield was due in part to the instability of p40 in the early stages of purification despite the use of protease inhibitors, but it also reflects the small amount of this protein in yeast cells.

Because it was possible that the binding of p40 to the p34 protein kinase complex could affect the activity of p34 toward other substrates, the p34-dependent phosphorylation of histone H1 (HH1) was assayed in vitro with and without added p40. In the presence of p40 a diminution of HH1 phosphorylation was observed (Fig. 2A). The reduction in HH1 phosphorylation was not due to depletion of [γ-^{32}P]adenosine triphosphate (ATP) as a result of p40 phosphorylation because total incorporation of ^{32}P into protein precipitable with trichloroacetic acid (TCA) also declined (*11*). Inhibition of kinase activity was also seen in a soluble assay with partially purified p34 and a 1.6-kD synthetic peptide substrate (*12*) containing the p34 consensus phosphorylation site. Increasing amounts of p40 added simultaneously with the peptide and [γ-^{32}P]ATP resulted in decreasing amounts of total acid-precipitable counts (Fig. 3). The amount of p40 required to inhibit phosphorylation by 50% (IC_{50}) under these conditions was 1.6 nM. Acid-precipitable counts were similar when either p34 or the peptide was omitted from the reaction (<5000 cpm). When p40 was incubated with p34 for 1 hour before the addition of ATP and the peptide to allow time for complexes of p34 and p40 to form, the initial rates of incorporation of ^{32}P into the peptide were lower (IC_{50} = 0.68 nM) than when p40 and peptide were added simultaneously with the ATP (*11*). Kinetic analysis with a range of concentrations of peptide substrate indicated that inhibition by p40 was complex because both the maximal velocity (V_{max}) and the Michaelis constant (K_m) for the peptide substrate decreased with increasing p40 concentration (*11*).

To provide independent confirmation of the identity of the protein kinase being inhibited, we immobilized p34 through its interaction with Cks1, using a modification of the microtiter plate assay procedure of Ducommun and Beach (*13*). Cks1 is the *S. cerevisiae* homolog of the *Schizosaccharomyces pombe* protein p13^{suc1} (*14*). The p13^{suc1} protein has been used to specifically immobilize complexes containing p34 (*15*). Partially purified p34 protein kinase was added to a 96-well microtiter dish to which bac-

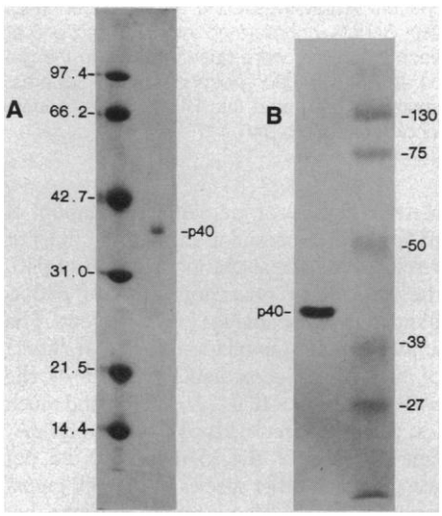

Fig. 1. Characterization of purified p40. Coomassie-stained gel after SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (A) and immunoblot (B) of p40 preparation. (**A**) Left lane, Bio-Rad molecular size standards; right lane, purified p40 (1 μg). The p40 protein was purified from stationary D4 yeast cells (*MATa ade1 his2 cdc28-4 leu2-3, 112 trp1-1^a*) by PEI precipitation, ammonium sulfate fractionation, and sequential chromatography on phosphocellulose, MonoQ, and ProRPC columns (*19*). This preparation was used for all experiments described in this report. (**B**) Left lane p40 (0.1 μg); right lane, Bio-Rad prestained molecular size standards. After transfer to Immobilon membrane the blot was treated with MAb63 (mouse monoclonal antibody to purified p40) (1 μg/ml), washed, and then treated with a 1:1000 dilution of Sigma alkaline phosphatase–conjugated sheep antibody to mouse immunoglobulin G. Immunoreactive bands were visualized with nitroblue tetrazolium (NBT) and bromochloroindoylphosphate (BCIP). The MAb63 was purified from mouse ascites fluid by hydroxyapatite and diethylaminoethyl-cellulose chromatography (*20*).

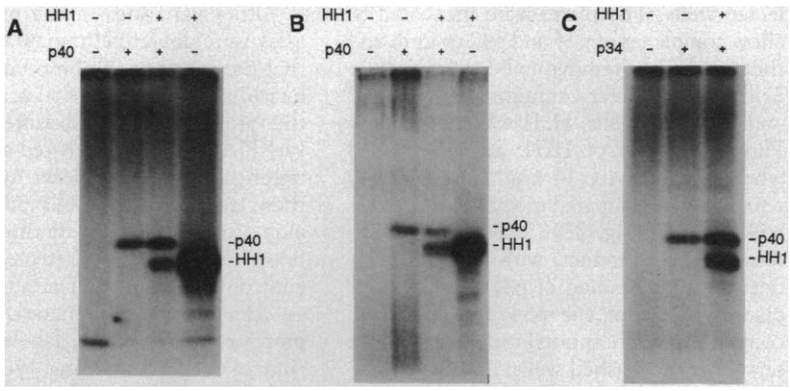

Fig. 2. Inhibition of HH1 phosphorylation by p40. (**A**) Protein A–Sepharose beads (30 μg) were incubated on ice for 1 hour with a rabbit polyclonal antibody (0.1 μl) to the NH_2-terminal eight amino acids predicted by the *CDC28* coding sequence. The beads were washed once with 50 mM tris-HCl (pH 7.5), 0.2% Tween-20, and 150 mM NaCl [enzyme-linked immunosorbent assay (ELISA) wash)]. A protein preparation (5 μg) enriched for p34 (*21*) was added, and the mixture was incubated on ice for 1 hour. The protein A–Sepharose beads were washed three times with ELISA wash and twice with 25 mM tris-HCl (pH 7.5). Excess buffer was then removed, and a volume of about 20 μl remained. The p40 protein (8 ng) was then added as indicated, and the mixture was incubated for 1 hour on ice. Beads were then washed three times with ELISA wash and twice with reaction buffer [20 mM tris-HCl (pH 7.5), 7.5 mM $MgCl_2$, 0.1 mM EDTA, and 0.1 mM EGTA]. Reaction buffer (40 μl) containing 5 μCi of [γ-^{32}P]ATP (ICN; 5000 Ci/mmol) was then added. Where indicated, HH1 (1 μg) was also added at this time. The reaction was allowed to proceed for 30 min at room temperature and was stopped by the addition of 3× SDS-PAGE (20 μl) sample buffer. The samples were boiled for 5 min. Proteins were separated by SDS-PAGE and detected by autoradiography. (**B**) Cks1-bound p34 was assayed by a modification of the procedure of Ducommun and Beach (*13*). Bacterially produced Cks1 protein (4 μg) in 40 μl of 100 mM tris-HCl (pH 8.3) was added to the bottom of a 96-well enzyme-linked immunoassay or radioimmunoassay plate and allowed to bind for several hours. Tween-20 (0.2%) in 100 mM tris-HCl (pH 7.5) was added, and the plates were incubated at 4°C overnight. The wells were then washed with ELISA buffer and the p34 preparation (5 μg) in 100 mM tris-HCl (pH 7.5) was added. The plates were incubated for 1 hour on ice and then treated in the same way as the protein A–Sepharose beads in (A). (**C**) Mouse MAb to p40 (1 μg) was used instead of Cks1 [as in (B)], and the order of binding of p34 and p40 was reversed. The protein preparation enriched for activated p34 was made from H17C1A1 (*MATa cdc17-1 his7 ura1*) cells arrested in S phase. Arrest in S phase was accomplished by shifting a logarithmically growing culture to 37°C for 5 hours. At this temperature the mutant DNA polymerase encoded by *cdc17-1* is nonfunctional, and the cells arrest uniformly in S phase. Protein extracts from these cells were then enriched for active p34 by PEI precipitation, Q-Sepharose chromatography, ammonium sulfate fractionation, and gel filtration chromatography (*21*). More than 90% of the protein kinase activity in this preparation was immunoprecipitated by rabbit polyclonal antibody to an NH_2-terminal peptide from Cdc28. Cks1 was prepared from bacteria overproducing the protein by PEI precipitation (0.3%) and ammonium sulfate fractionation (0 to 45% precipitate).

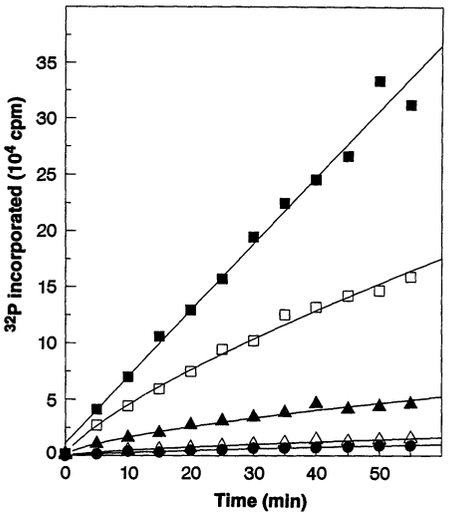

Fig. 3. Inhibition of phosphorylation of a peptide substrate by p40. A solution containing [γ-^{32}P]ATP (250 μCi/ml), 500 μM ATP, synthetic peptide substrate (500 μg/ml) (*12*), and various amounts of p40 was added at time 0 to the p34 preparation (5 μg/ml) (Fig. 2) in a reaction buffer containing 10 mM tris (pH 7.5), 7.5 mM $MgCl_2$, 0.1 mM EDTA, and 0.1 mM EGTA. Portions (20 μl) were removed at 5-min intervals, added to 10 μl of glacial acetic acid, and spotted onto phosphocellulose filter discs. The filter discs were washed (*22*) and then dried. The acid-precipitated ^{32}P was quantitated in a scintillation counter. Filled squares, no p40 added; open squares, p40 (66 ng/ml); filled triangles, p40 (134 ng/ml); open triangles, p40 (200 ng/ml); filled circles, p40 (266 ng/ml).

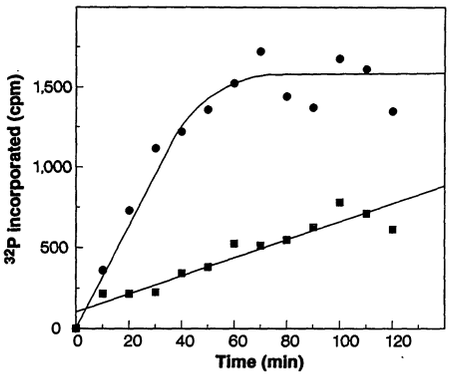

Fig. 4. Time course of p40 and HH1 phosphorylation. Assay was done as described (Fig. 2B). Counts incorporated into HH1 and p40 at each time point were quantitated from the gel on an AMBIS 4000 phospho-imager. Squares, counts incorporated into HH1; circles, counts incorporated into p40.

terially produced Cks1 had previously been bound. The p40 protein was added to selected wells. The plates were incubated to allow complexes of p34 and p40 to form and then washed to remove unbound p40 (Fig. 2). Reaction buffer containing [γ-^{32}P]ATP and, for some wells, HH1 was then added. Phosphorylation of HH1 was diminished when the activity of the Cks1-p34-p40 complex was compared to that of the Cks1-p34 complex (Fig. 2B). These results confirm that p40 interacts with p34 and demonstrate that binding of p40 does not displace Cks1 from the p34 protein kinase complex (p40 phosphorylation was still observed in the washed wells).

To address the possibility that the inhibition of HH1 phosphorylation was caused by a contaminant that copurified with p40, we used a mouse monoclonal antibody (MAb) to purified p40 to bind p40 to a microtiter dish (Fig. 2C). The wells were washed with a detergent-containing buffer, and p34 was added. The wells were then washed again, and [γ-^{32}P]ATP and HH1 were added. Although some phosphorylation of HH1 was detected in lane 3 (Fig. 2C), the phosphorylation of p40 is similar to that seen in lane 3 of Fig. 2A. Because increasing the amount of p40 in the experiment in Fig. 2A or increasing the amount of p34 in the experiment in Fig. 2C did not result in additional phosphorylation of p40, the amount of phosphorylation of p40 is indicative of the amount of p34 present. The amount of phosphorylation of HH1 in lane 3 of Fig. 2C is comparable to that of the p40-p34 complex (Fig. 2A, lane 3) and much less than that seen with p34 alone (Fig. 2A, lane 4). Because the MAb to p40 did not recognize any other species in the p40 preparation (Fig. 1B), these results indicate that p40 inhibits the activity of p34. Phosphorylation of p40 was dependent on the addition of p34, indicating that under these conditions p40 does not autophosphorylate.

Other variations of the microtiter dish assay were done to eliminate the possibility that the apparent inhibition of p34 activity by p40 was an artifact of an action of p40 on the protein kinase substrates. HH1 and [γ-^{32}P]ATP were incubated in wells containing p34-p40 complexes for 30 min and then transferred to wells containing p34 alone for 30 min. No diminution of HH1 phosphorylation was detected relative to controls (*11*). This indicates that p40 is not an adenosine triphosphatase or a histone protease. When HH1, labeled by incubation in wells containing [γ-^{32}P]ATP and immobilized p34, was transferred to wells containing immobilized p34-p40 complexes, no reduction in the amount of ^{32}P incorporation was detected (*11*). This indicates that p40 is not a histone phosphatase. The possibility that p40 causes dephosphorylation of p34 or the displacement or degradation of cyclin cannot be ruled out. However, preincubation of p34-p40 complexes at room temperature or at 37°C for 30 min before the addition of [γ-^{32}P]ATP resulted in no diminution of p34-dependent p40 phosphorylation (*11*). Binding to cyclin and phosphorylation on threonine are thought to be required for p34 histone kinase activity (*16*), but the human apoenzyme does have activity toward casein (*17*).

We have been unable to determine conditions that will release p40 from p34 complexes and still preserve p34 protein kinase activity. The p34-p40 complex is resistant to washes with 5 M NaCl, 3 M urea, or solutions containing 0.1% sodium dodecyl sulfate, 1% Triton X-100, 1% deoxycholate, or 1% Tween-20 (*11*). Small biological effectors, such as adenosine 3′5-monophosphate (1 μM) and guanosine triphosphate (1 mM), also had no effect on binding or phosphorylation of p40 or on the inhibition of HH1 phosphorylation. Phosphorylation of p40 by p34 also appeared not to affect the inhibition of phosphorylation of HH1 because phosphate incorporation into HH1 proceeded linearly during a long time course and at a low rate long after phosphorylation of p40 reached a plateau (Fig. 4). These results, and the p34-dependent phosphorylation of p40, suggest that p40 inhibits p34 by forming a tight complex that prevents access of other substrates, even peptides as small as 1.6 kD, to the active site of the protein kinase. ATP must still enter the active site, however, because p40 is specifically labeled when [γ-^{32}P]ATP is added after the p34-p40 complex has formed.

Although the 13-kD protein encoded by the *S. pombe suc1*$^+$ gene (p13^{suc1}) is a tightly binding inhibitor of p34 (*14*, *17*), several distinctions can be made between the actions of p40 and p13^{suc1}: (i) p40 inhibits the active kinase, whereas binding of p13^{suc1} inhibits the activation of p34 but has no effect on the activated protein kinase (*18*); (ii) p40 binds only to the activated kinase (*10*), whereas p13^{suc1} recognizes both the active and inactive forms (*16*, *17*); (iii) the binding sites of p40 and p13^{suc1} on the active p34 complex appear to be distinct and not mutually exclusive because p40 can bind without displacing p34 complexes immobilized through interaction with the p13^{suc1} homolog, Cks1. The p40 protein might function to prevent premature commitment to the cell division cycle when cell size or growth conditions are unfavorable.

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The Prevention of Thymic Lymphomas in Transgenic Mice by Human O^6-Alkylguanine-DNA Alkyltransferase

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Nitrosoureas form O^6-alkylguanine-DNA adducts that are converted to G to A transitions, the mutation found in the activated *ras* oncogenes of nitrosourea-induced mouse lymphomas and rat mammary tumors. These adducts are removed by the DNA repair protein O^6-alkylguanine-DNA alkyltransferase. Transgenic mice that express the human homolog of this protein in the thymus were found to be protected from developing thymic lymphomas after exposure to *N*-methyl-*N*-nitrosourea. Thus, transgenic expression of a single human DNA repair gene is sufficient to block chemical carcinogenesis. The transduction of DNA repair genes in vivo may unravel mechanisms of carcinogenesis and provide therapeutic protection from known carcinogens.

DNA damage is an initiating event in chemical carcinogenesis that leads to mutations in proto-oncogenes and tumor suppressor genes (*1*). Subsequent induction of error-prone DNA repair and cell proliferation may increase the rate of secondary mutations, thereby accelerating neoplastic transformation (*2*). These early events in chemical carcinogenesis have been extensively studied in animal tumors induced by *N*-methyl-*N*-nitrosourea (MNU) (*3*). Like other *N*-nitroso compounds, MNU methylates DNA at a number of sites, including the O^6 position of guanine (*4*). The resulting O^6-methylguanine-DNA adduct is converted to a G → A point mutation during DNA synthesis (*5*). MNU also induces other events that may be carcinogenic, including DNA strand breaks, sister chromatid exchanges (*6*), and cell proliferation within the target tissue (*7*, *8*). Although any of these forms of genotoxic damage could act as the initiating event, persistent O^6-methylguanine adducts have been proposed to be the critical lesion in a variety of MNU-induced experimental tumors, such as mouse lymphomas and rat mammary tumors, because these tumors frequently display G → A activating mutations in K-*ras* or H-*ras* (*9*).

O^6-alkylguanine-DNA adducts are removed by the DNA repair protein O^6-alkylguanine-DNA alkyltransferase (E.C. 2.1.1.63; alkyltransferase) (*10*), which transfers the alkyl group from the O^6 position of guanine to a cysteine residue in its own active site and becomes irreversibly inactivated in the process (*11*). The capacity for O^6-methylguanine adduct repair by this mechanism is therefore directly proportional to the number of alkyltransferase molecules in the cell. A correlation exists between alkyltransferase activity and nitrosourea-induced cytotoxicity and mutagenicity in vitro (*6*, *12*). Manipulation of alkyltransferase concentrations alters cellular resistance to nitrosoureas. Thus, specific alkyltransferase inhibitors increase the cytotoxicity and the frequency of mutations and sister chromatid exchanges induced by nitrosoureas (*13*), whereas transduction of the alkyltransferase gene *MGMT* into cells without endogenous alkyltransferase activity reduces cytotoxicity and the frequency of G → A mutations (*13*, *14*). Tissues susceptible to the carcinogenic effect of nitrosoureas, such as thymus, breast, bone marrow, kidney, and brain, have 3 to 18 times less alkyltransferase activity than tissues that are not susceptible to these agents (*3*, *15*).

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