

A Novel Protein Complex Distinct from Mismatch Repair Binds Thioguanylated DNA

EUGENE Y. KRYNETSKI, NATALIA F. KRYNETSKAIA, AMY E. GALLO, K. GOPAL MURTI, and WILLIAM E. EVANS

Departments of Pharmaceutical Sciences (E.Y.K., N.F.K., A.E.G., W.E.E.) and Virology and Molecular Biology (K.G.M.), St. Jude Children's Research Hospital, Memphis, Tennessee; and University of Tennessee, Memphis, Tennessee (E.Y.K., N.F.K., W.E.E.)

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ABSTRACT

To elucidate molecular mechanism(s) of cellular response to mercaptopurine, a widely used antileukemic agent, we assessed mercaptopurine (MP) sensitivity in mismatch repair (MMR) proficient and MMR deficient human acute lymphoblastic leukemia (ALL) cells. Sensitivity to thiopurine cytotoxicity was not dependent on MMR (i.e., MutS α) competence among six cell lines tested. Using electrophoretic mobility shift assay analysis, we found that the incubation of nuclear extracts from ALL cells with synthetic 34-mer DNA duplexes containing deoxythioguanosine (G^S) within either G^S-T or G^S-C pairs, resulted in formation of a DNA-protein complex distinct from the DNA-MutS α complex and unaffected by ATP. Isolation and sequence analysis of proteins involved in this DNA-protein complex identified glyceraldehyde 3-phosphate dehydrogenase

(GAPDH) as a component. Western blot analysis of nuclear extracts from a panel of human lymphoblastic leukemia cell lines revealed markedly different basal levels of GAPDH in nuclei, which was significantly related to thiopurine sensitivity ($p = 0.001$). Confocal analysis revealed markedly different intracellular distribution of GAPDH between nucleus and cytosol in six human ALL cell lines. Redistribution of GAPDH from cytosol to nucleus was evident after MP treatment. These findings indicate that a new DNA-protein complex containing GAPDH and distinct from known MMR protein-DNA complexes binds directly to thioguanylated DNA, suggesting that this may act as a sensor of structural alterations in DNA and serve as an interface between these DNA modifications and apoptosis.

Mercaptopurine (MP) is one of the most widely used antileukemic agents and is an important medication in essentially all modern acute lymphoblast leukemia (ALL) treatment regimens (Elion, 1989; Pui and Evans, 1998). Elucidation of the precise mechanism(s) through which thiopurines exert their antileukemic effects should provide new insights into the basis of resistance among patients who are not cured with chemotherapy (Evans and Relling, 1999.)

Mercaptopurine, as well as several other antileukemia drugs (e.g., thioguanine, cytarabine, gemcitabine, and 2-chlorodeoxyadenosine), require incorporation of their nucleotide metabolites into DNA to exert cytotoxic effects (LePage, 1963; Kufe et al., 1980; Szondy, 1995; Gandhi et al., 1997). However, the widely held view that these agents are cytotoxic by impairing DNA polymerization is probably an oversimplification (Lipp and Bokemeyer, 1999), because incorporation of many nucleoside analogs does not abrogate DNA elongation. It was recently reported that the DNA mismatch

repair system (MMR) participates in recognition and processing of deoxythioguanosine (dG^S) incorporated into DNA (Swann et al., 1996) and that MSH2/MSH6 (MutS α)-deficient cell lines are more resistant to thiopurines (Aquilina et al., 1989; Branch et al., 1993; Hawn et al., 1995; Glaab et al., 1998).

We hypothesized that other proteins in addition to MMR may be involved in recognition of structural alterations caused by incorporation of deoxythioguanosine into DNA. To this end, we compared cytotoxic effects of MP in MMR-proficient and MMR-deficient cells and assessed dG^S-DNA binding activity in nuclear extracts prepared from these cells. These experiments revealed that MMR-competence is not essential for leukemia cells to be sensitive to thiopurines and identified a protein complex, distinct from MutS α , that binds dG^S-DNA.

Materials and Methods

Cell Culture and Preparation of Cell and Nuclear Extracts.

The human T-lineage leukemia cell lines CCRF-CEM and Molt4 and the human B-lineage WIL2NS (B lymphoblast) cells were obtained from ATCC (Manassas, VA); the human T-lineage leukemia P12,

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ABBREVIATIONS: MP, mercaptopurine; ALL, acute lymphoblastic leukemia; MMR, mismatch repair; MutS α , MSH2/MSH6 mismatch repair complex; dG^S, deoxythioguanosine; PAAG, polyacrylamide gel; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PCNA, proliferating cell nuclear antigen; FITC, fluorescein isothiocyanate; EMSA, electrophoretic mobility shift assay.

EMSA Assay and Nucleoprotein Complex Isolation. DNA-protein binding assays were performed as described earlier, in 25 to 30 μ l of buffer A (10 \times incubation buffer; Stratagene, La Jolla, CA) or buffer B (10 \times 200 mM Tris-Cl, pH 8.0, 50 mM NaCl, 3 mM MgCl₂, 1 mM dithiothreitol, 10% glycerol) (Griffin et al., 1994). DNA-protein complexes were isolated by 5% (w/v) denaturing polyacrylamide gel (PAAG) at 4°C. Fractions A, B, C were extracted by electroelution and analyzed in 7.5% SDS-polyacrylamide gels. N-terminal sequencing was performed in the Hartwell Center for Biotechnology and Bioinformatics at St. Jude Children's Research Hospital, after electrotransfer to the polyvinylidene difluoride membrane.

Confocal Microscopy. To assess GAPDH distribution between nucleus and cytosol and the translocation of GAPDH from nucleus to cytoplasm at various times after incubation with MP, immunofluorescence analysis was performed on ALL cells using a monoclonal antibody to GAPDH and FITC (green)-coupled second antibody. Cells were also stained with propidium iodide to identify the nucleus. Cells were examined in a Leica confocal laser scanning microscope (model TCS NT SP; Leica, Wetzlar, Germany) to derive optical sections of cells to unambiguously localize the nuclear and cytoplasmic labels. Cells were centrifuged onto glass slides using the Cytospin-1 cyto-centrifuge (Shandon Southern Instruments, Inc., Sewickley, PA). Slides bearing cells were rinsed in PBS, fixed in 3% paraformaldehyde and permeabilized with acetone as per our published procedures (Murti et al., 1993). The cells were incubated with monoclonal GAPDH antibody, followed by FITC-coupled secondary antibody. Slides processed per the above were also stained with propidium iodide (after incubating the cells with RNase) to label the nuclei. Differences in median intensity in the nucleus at 0 and 48 h were assessed by the Exact Wilcoxon rank-sum test.

Thiopurine Sensitivity and MMR Status of Human Lymphoblastoid Cells.

Abbreviation	Length	Sequence
1	13/42	5'-d (GTACCACTGCAGT) 3'-d (CATGGTGACGTGAGGTGCGACCCACTGTTTCGTTTTGTGACA)
2	13/42	5'-d (GTACCACTGCAGT) 3'-d (CATGGTGACGTGAG ^S GTCGGACCCACTGTTTCGTTTTGTGACA)
3GT	34/34	5'-d (AGCTTGGCTGCAGGT G GACGGATCCCCGGAATT) 3'-d (TCGAACCGACGTCCA T CTGCCTAGGGGCCCTTAA)
3GC	34/34	5'-d (AGCTTGGCTGCAGGT G GACGGATCCCCGGAATT) 3'-d (TCGAACCGACGTCCA C CTGCCTAGGGGCCCTTAA)
3G ST	34/34	5'-d (AGCTTGGCTGCAGGT G ^S GACGGATCCCCGGAATT) 3'-d (TCGAACCGACGTCCA T CTGCCTAGGGGCCCTTAA)
3G ^{SC}	34/34	5'-d (AGCTTGGCTGCAGGT G ^S GACGGATCCCCGGAATT) 3'-d (TCGAACCGACGTCCA C CTGCCTAGGGGCCCTTAA)
4GT	34/34	5'-d (ACTCTTGCCTTTAAG G AAAGTATCTAAATGCTTC) 3'-d (TGAGAACGAAATTC T TTTCATAGATTACGAAG)
4GC	34/34	5'-d (ACTCTTGCCTTTAAG G AAAGTATCTAAATGCTTC) 3'-d (TGAGAACGAAATTC C TTTCATAGATTACGAAG)
4G ST	34/34	5'-d (ACTCTTGCCTTTAAG G ^S AAAGTATCTAAATGCTTC) 3'-d (TGAGAACGAAATTC T TTTCATAGATTACGAAG)
4G ^{SC}	34/34	5'-d (ACTCTTGCCTTTAAG G ^S AAAGTATCTAAATGCTTC) 3'-d (TGAGAACGAAATTC C TTTCATAGATTACGAAG)

icity, DNA modification and mismatch repair protein expression was assessed in human T- and B-lineage leukemia cells (CEM, Molt4, P12, WIL2NS, 697, Nalm6), after treatment with 10 μ M MP. Nuclear extracts from CEM, Molt4, P12, 697, WIL2NS cell lines demonstrated G-T-mismatch binding by the MSH2/MSH6 (MutS α) complex (Fig. 1A). In contrast, this G-T-binding activity was absent in Nalm6 cells. Western blot analysis of nuclear extracts revealed the absence of both components of MutS α , namely hMSH2 and hMSH6, in Nalm6 cells (Fig. 1B) and the absence of hMLH1 in CEM (not shown), in contrast to the other cell lines that had these MMR proteins.

As we have reported previously in detail (Krynetskaia et al., 1999), after 24 h exposure to mercaptopurine, there was essentially no cytotoxicity. Only after 48 h did the viability of cells decline, with even greater cytotoxicity after 72 h except for the most resistant cells. CEM, Molt4, and Nalm6 cells were the most sensitive to MP (<50% viability after 72 h exposure to MP), whereas P12 and 697 cells were more resistant (75–90% viability after 72 h). Using terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay, it was shown that Nalm6 cells were highly sensitive, with up to 30% of cells exhibiting apoptotic changes after 24 h incubation, in contrast to P12 cells, which did not show evidence of apoptotic changes after 24 h exposure to 10 μ M MP. Parallel experiments assessing BrdU incorporation established that MP did not inhibit DNA synthesis (data not shown). With the exception of the most resistant cells (697 and P12), the nuclei are too fragile after 72 h exposure for isolation and characterization; therefore, post-treatment nuclei were harvested at 24 and 48 h.

MutS α Binds Duplex DNA with dG^S·T Mismatch. Incubation of either the G-T- or G^S·T containing DNA duplexes (3GT/3G^ST or 4GT4/G^ST) with nuclear extracts from MutS α -proficient cells (Molt4, CEM, 697, WIL2NS, P12) resulted in formation of a nucleotide-protein complex with MutS α (G-T binding shown in Fig. 1A; G^S·T in Fig. 2A), that was abrogated by ATP (fraction B in Fig. 2A, lanes 1 and 2). In contrast, no such protein-DNA complex was formed in the MSH2/MSH6 deficient Nalm6 cells (Fig. 1A lanes 1 and 2; Fig. 2A, lanes 3 and 4). However, we observed formation of a second DNA-protein complex in each of these cell lines when nuclear extracts were incubated with DNA duplexes containing either a G^S·T- or a G^S·C, and this complex was not abrogated by ATP (Fig. 2A, fraction A). Similar DNA-protein complexes were formed with G-T- and G-C-containing DNA (duplexes 3GT/3GC or 4GT/4GC), but with lower affinity than its binding to dG^S-containing duplexes (Fig. 3C). Biochemical isolation of the G^S-DNA-protein complexes gave

fractions A, B, and C as depicted in Fig. 2A. Individual bands isolated by EMSA were further analyzed by SDS electrophoresis and Western blot analysis using hMSH2- and hMSH6-specific antibodies. The MSH2 and MSH6 proteins constituting the MutS α heterodimer were documented by Western blot analysis in fraction B but not in A or C, as exemplified with P12 nuclear extract in Fig. 2B.

Identification of GAPDH in dG^S-DNA-Protein Complex. The DNA-protein complex formed between dG^S-containing oligonucleotide duplexes and nuclear extracts in the presence of ATP was eluted (fraction A) and characterized by SDS electrophoresis. Multiple protein bands ranging from 30 to 200 kDa were detected by silver staining, with four to five major components (Fig. 3A). N-terminal sequencing of the 37-kDa protein band identified GAPDH as one of the components of the dG^S-binding complex. GAPDH was documented in fraction A, but not in C by Western blot analysis, as exemplified with Nalm6 nuclear extract in Fig. 2C. As depicted in Fig. 3, B (lane 2) and C (lanes 2 and 4), anti-GAPDH monoclonal antibody added to the reaction mixture effectively dissociated this complex. Control experiments with anti-PCNA monoclonal antibodies revealed no effect on this protein complex (Fig. 3B, lane 3).

Nuclear Localization of GAPDH in ALL Cells. Western blot analysis revealed similar levels of GAPDH in total cell lysates of these six human lymphoblastic leukemia cell lines (Fig. 4A). In contrast, the intranuclear level of GAPDH differed markedly in these cells (Fig. 4B), with the lowest level of intranuclear GAPDH in P12 cells and the highest in Nalm6 cells. Furthermore, immunostaining of cells with anti-GAPDH antibody and confocal microscopy revealed differences in the distribution of GAPDH between nucleus and cytosol in these cell lines (Fig. 4C). Sequence analysis of RT-PCR amplified RNA isolated from Nalm6, WIL2NS, P12, and 697 cells did not reveal any mutations in the GAPDH open reading frame among these cell lines, indicating other mechanisms for the heterogeneity in nuclear GAPDH levels.

As depicted in Fig. 5C, there was a statistically significant relationship between the amount of GAPDH in nuclear extracts of human ALL cells before treatment and the extent of cytotoxicity after 72 h of incubation with 10 μ M MP. Cells with higher GAPDH were more sensitive to MP and are localized in the lower right part of the chart, whereas more resistant cells are grouped in the upper left part, reflecting decreased intranuclear content of GAPDH ($p = 0.001$; Pearson coefficient).

Interestingly, MP treatment resulted in intranuclear accumulation of GAPDH, as exemplified by Western blot analysis of nuclear extracts from Molt4 and P12 cell lines (Fig. 6A).

TABLE 2

Efficiency of incorporation of dCTP, dTTP, dGTP, and dATP opposite deoxyguanosine or 6-thiodeoxyguanosine in duplexes 1 and 2 by Klenow (exo⁻)

dNTP	G-template			G ^S -template		
	V_{\max}^a	K_m^b	V_{\max}/K_m^c	V_{\max}^a	K_m^b	V_{\max}/K_m^c
dCTP	5.46 \pm 0.78	0.06 \pm 0.02	91	7.84 \pm 0.33	0.8 \pm 0.19	9.8
dTTP	3.51 \pm 0.706	44.1 \pm 6.02	0.08	1.83 \pm 0.706	60.03 \pm 0.61	0.03
dGTP	3.66 \pm 0.422	63.8 \pm 22.43	0.06	2.94 \pm 0.612	130 \pm 86.2	0.022
dATP	14.07 \pm 5.06	947 \pm 477	0.01	4.90 \pm 1.684	361 \pm 244	0.014

^a nmol/min/U polymerase.

^b μ M.

^c per min/(U polymerase/ml).

Visual confirmation of this was obtained by direct evaluation of intranuclear GAPDH level by confocal microscopy. Figure 6B depicts intracellular redistribution of GAPDH in P12 cells at different time points after MP treatment, evaluated by immunostaining and confocal microscopy. Confocal micros-

copy revealed that the treatment of human leukemia cells with 10 μ M MP resulted in translocation of GAPDH from the cytosol to the nucleus, evident by 24 h and increasing further at 48 h of MP exposure, as shown for P12 cells (Fig. 6). Before MP exposure essentially all of the GAPDH was cytoplasmic in these cells, as indicated by the green color at the periphery of cells (Fig. 6B, panel 1) and the paucity of yellow color in the nuclei (Fig. 6B, panel 3). At 24 h after incubation with MP (Fig. 6B, panels 4–6), modest translocation of GAPDH into the nucleus was evidenced by the presence of green label in some nuclei (panel 4) and yellow in the merged image (panel 6). After 48 h of exposure to MP (Fig. 6B, panels 7–9), a significant fraction of GAPDH translocated into the nucleus, as revealed by the central distribution of green label (panel 7) and yellow in the merged image (panel 9). The mean fluorescence intensity in nuclei of these cells was 26.9 ± 5.8 at zero hour, 53.7 ± 20.4 at 24 h and 79.2 ± 28.0 at 48 h ($p = 0.03$). It is noteworthy that even after nuclear translocation of GAPDH, the relatively MP-resistant P12 cells had substantially lower nuclear GAPDH levels compared with the MP sensitive cells, [e.g., Molt4 (Fig. 6A)].

Discussion

Although thioguanine incorporation into DNA is considered a *sine qua non* for thiopurine cytotoxicity, we have shown that the level of dG^S incorporation is not directly related to the extent of cytotoxicity in human lymphoblastic leukemia cells (Krynetskaia et al., 1999). We therefore postulated that heterogeneity in cellular recognition and response to DNA modified by dG^S incorporation plays a critical role in thiopurine sensitivity and resistance. Previous findings from our lab and others indicate that the alterations in DNA structure caused by dG^S incorporation into DNA likely contribute to the pharmacological effects of thiopurines (Ling et al., 1992a; Krynetskaia et al., 1999; Tendian and Parker, 2000). There-

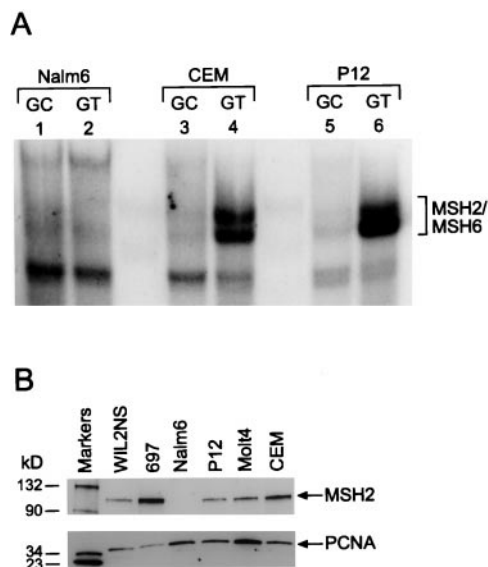


Fig. 1. MutSα (MSH2/MSH6) mismatch repair proteins in human leukemia cells. A, EMSA analysis of G-C and G-T-binding activity in nuclear extracts of MutSα-proficient (CEM, P12) and MutSα-deficient (Nalm6) leukemia cell lines. The 34-mer duplex DNA 3GC (Table 1) was used in lanes 1, 3, and 5 (labeled GC), and duplex DNA 3GT (Table 1) was used in lanes 2, 4, and 6 (labeled GT). Bracket indicates the specific complex formed by MutSα heterodimer with G-T-containing oligonucleotide duplex. The lower band corresponds to a consistently present, nonspecific nucleoprotein complex. Sequence of duplex DNA provided in Table 1. B, Western blot analysis of proteins from nuclear extracts of human lymphoblastic leukemia cell lines (WIL2NS, 697, Nalm6, P12, Molt4, CEM) developed with anti-MSH2 antibody or anti-PCNA antibody (marked arrows).

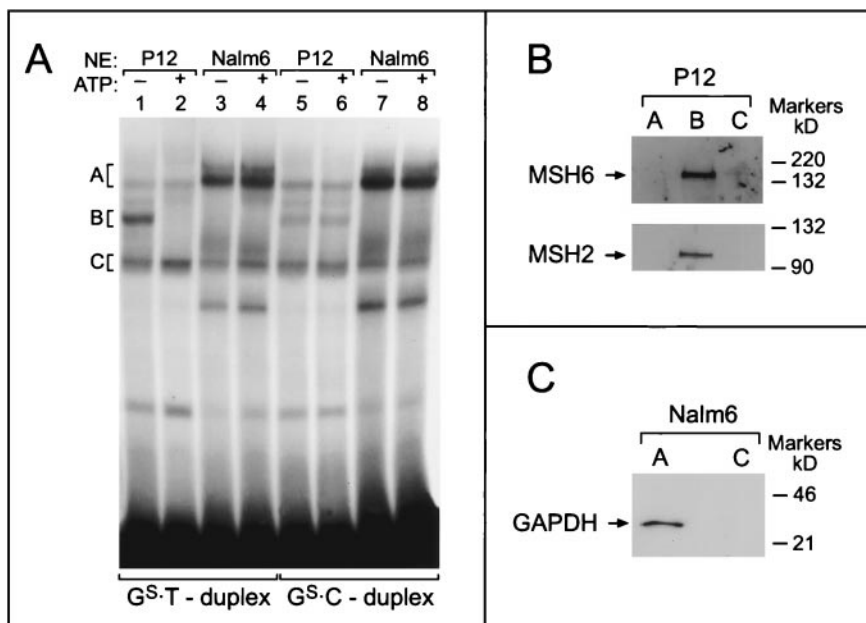


Fig. 2. Proteins involved in dG^S-DNA recognition. A, EMSA analysis of nuclear extracts from P12 and Nalm6 cells with G^S-T-containing duplex 4G^S-T (lanes 1–4) or G^S-C-containing duplex 4G^S-C (lanes 5–8) in the presence (+) or absence (–) of ATP (34-mer duplex DNA sequences are shown in Table 1). B, Western blot analysis of P12 proteins in fractions A, B, and C (as designated on A) developed with anti-MSH6 or anti-MSH2 antibodies. C, Western blot analysis of Nalm6 proteins in fractions A (as designated on A) developed with anti-GAPDH antibody.

fore, we hypothesized that specific cellular proteins detect subtle changes in DNA structure, thereby triggering a chain of biochemical events that culminates in cytotoxicity in sensitive cells. The present work provides new insights into the proteins that participate in these cellular events, after thiopurine treatment.

It was previously shown that dG^STP is utilized efficiently in DNA-dependent DNA polymerase reactions, as well as RNA-dependent DNA polymerase reactions catalyzed by viral, bacterial, and mammalian DNA polymerases (Ling et al., 1991; Ling et al., 1992b; Rappaport, 1993). The present experiments showed that the Klenow fragment of *E. coli* DNA polymerase I efficiently elongated a 13-mer DNA primer using a thioguanylated template. The presence of dG^S in the template resulted in the formation of G^S·T-pairs, but formation of G^S·C-pair was preferred 300-fold (Table 2), in contrast to published data for *S*-methyl deoxythioguanosine template (Spratt and Levy, 1997), yet consistent with published reports on polymerization using a dG^S-template (Ling et al., 1992; Rappaport, 1993).

Recently, mismatch repair proteins were reported to be major mediators of the cytotoxic effects of thiopurines (Karran and Bignami, 1996; Swann et al., 1996; Armstrong and Galloway, 1997). Our experiments confirmed that the MutS α complex is able to bind G^S·T-containing DNA in MutS α -expressing cells (Molt4, P12, 697, Wil2NS, CEM) as previously reported (Griffin et al., 1994), but not in MutS α -deficient Nalm6 cells. The involvement of MutS α in G^S·T binding was also demonstrated by abrogation of the DNA-protein complex by ATP (Drummond et al., 1995). Consistent with this observation, there was markedly lower binding of MutS α with DNA containing a G^S·C mismatch (Fig. 2A, lanes 5 and 6, fraction B). Final proof for MSH2/MSH6 involvement in G^S·T recognition was obtained after isolation of fraction B and Western blot analysis of proteins interacting with G^S·T-containing DNA (Fig. 2B).

In contrast to previous reports that MMR-deficient colorectal carcinoma, endometrial carcinoma, and Burkitt's lymphoma cell lines are resistant to thioguanine (Branch et al., 1993; Hawn et al., 1995; Glaab et al., 1998), we have shown that two of the most MP-sensitive human lymphoblastic leukemia cell lines, CEM and Nalm6, lack one or more components of MMR. Western blot analysis demonstrated absence of both components of MutS α , namely hMSH2 and hMSH6, in Nalm6 cells (Fig. 1), and CEM cells were deficient in hMLH1 (E. Y. K., unpublished observations). This latter protein (MLH1) is not directly involved in binding to DNA mismatches (Kolodner and Marsischky, 1999).

These findings indicate that MMR protein expression is not the only determinant of MP sensitivity in human leukemia cells, and that MMR-deficient leukemia cells can be highly sensitive to thiopurines. EMSA experiments with nuclear extracts from human leukemia cells revealed protein complexes distinct from the MMR complex that interacted with duplex DNA containing either G^S·T- or G^S·C-pairs, leading to the identification of a new protein complex involved in recognition of thioguanylated DNA (Fig. 2A, fraction A, lanes 3, 4, 7, 8). This complex is distinct from MutS α (fraction B) because it is present in MMR-deficient as well as MMR-proficient cells, and its interaction with DNA is not sensitive to ATP (Fig. 2A, lanes 3,4,7,8). This protein complex interacted predominantly with G^S·T- and G^S·C-containing duplexes (Fig. 2A), rather than duplexes with G·T or G·C-pairs (Fig. 3C). Another important difference between this complex (fraction A) and MutS α (fraction B) is that the new complex is formed in the presence of both G^S·T and G^S·C duplexes with similar affinity, whereas MutS α predominantly binds the G^S·T-containing duplex DNA (Fig. 2A, lanes 1, 5 and 3,7). Western blot analysis established the presence of MSH2 and MSH6 in fraction B, and established their absence in the new protein complex in fraction A (Fig. 2B).

Subsequent biochemical isolation of this new protein com-

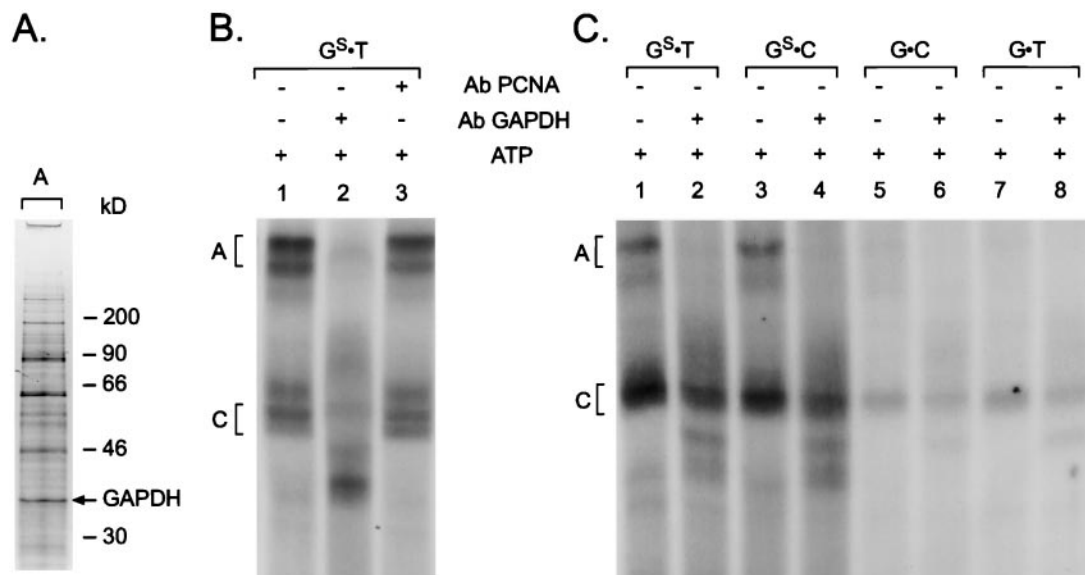


Fig. 3. Nalm6 nuclear extract proteins involved in G^S·C and G^S·T-binding. Band A and C designation as in Fig. 2A. A, Electrophoretic analysis of fraction A by 7.5% PAAG with SDS and silver staining. The arrow indicates position of GAPDH. B, EMSA assay with 34-mer duplex DNA 4G^S·T (Table 1) in the presence of ATP. GAPDH-containing duplexes dissociate upon addition of anti-GAPDH mAb (lane 2), but not anti-PCNA mAb (lane 3). C, EMSA assay with 34-mer duplexes 3G^S·T (lane 1,2); 3G^S·C (lane 3,4); 3G·C (lane 5,6) and 3G·T (lane 7,8). GAPDH-containing complex (fraction A) was formed with G^S-duplex, but not G·C or G·T duplexes. Fraction C is a nonspecific band observed with all duplex DNA tested, under all conditions evaluated.

plex (fraction A, from Nalm6 nuclear extract) and N-terminal protein sequencing identified GAPDH as one of its components. The presence of GAPDH in the complex was further confirmed by Western blot analysis after electroelution of fraction A (Fig. 2A line 4) and SDS electrophoresis (Fig. 2C).

EMSA experiments with anti-GAPDH mAb caused dissociation of this complex (Fig. 3, B and C); it is not unusual for an antibody to block or disrupt complex formation rather than to cause a supershift. Because GAPDH readily dissociates from the complex, we speculate that GAPDH is localized on the periphery of the core protein-DNA complex. SDS gel electrophoresis of fraction A revealed at least four other components of the dG^S-binding complex (Fig. 3A), in line with other reports demonstrating the presence of GAPDH in nuclear multiprotein complexes. For example, the presence of GAPDH in nuclear bodies containing promyelocytic leukemia protein was recently reported (Carlile et al., 2000), as well as physical association of GAPDH with DNA-polymerase α and

primase (Grosse et al., 1986; Seal and Sirover, 1986). Enzymes participating in structural functions unrelated to their principal enzymatic activity have been recognized, as exemplified by several plant and mammalian proteins (Hendriks et al., 1988; Mulders et al., 1988; Cooper et al., 1993; Mori-

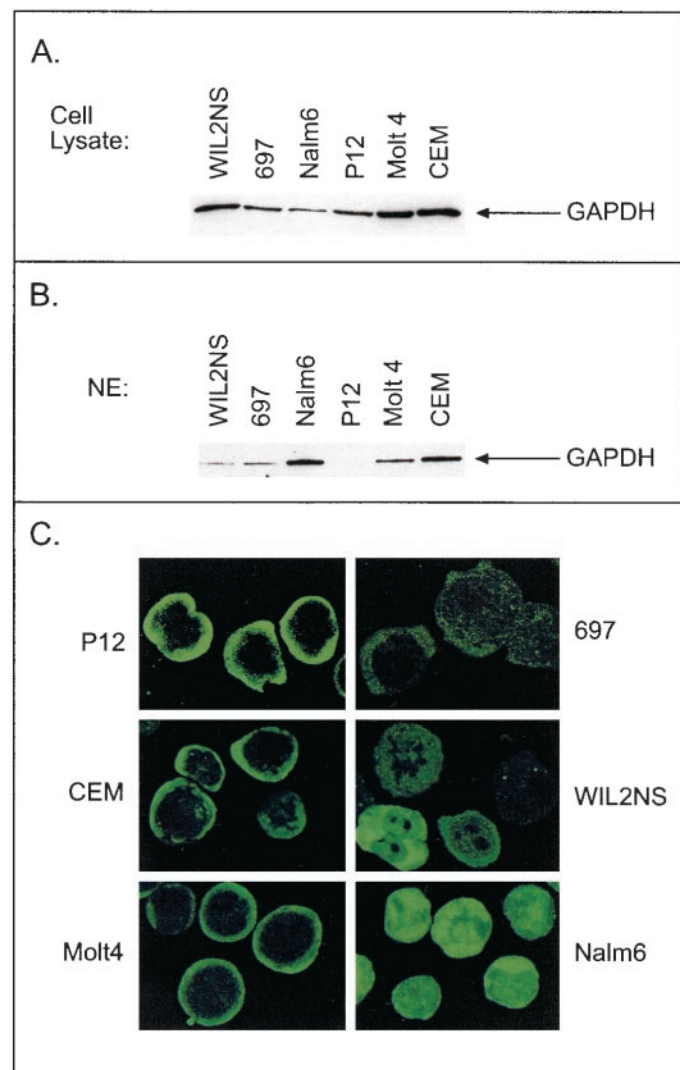


Fig. 4. Basal level of GAPDH in ALL. A, Western blot analysis of total cell lysates from six human leukemia cell lines (WIL2NS, 697, Nalm6, P12, Molt4, CEM) using monoclonal anti-GAPDH antibody. B, Western blot analysis of nuclear extracts (NE) from the same cells as in A using monoclonal anti-GAPDH antibody. GAPDH levels are normalized to total protein loaded per lane. C, confocal laser scanning micrograph of GAPDH distribution between nucleus and cytosol in the six human ALL cells before MP treatment, using monoclonal anti-GAPDH antibody.

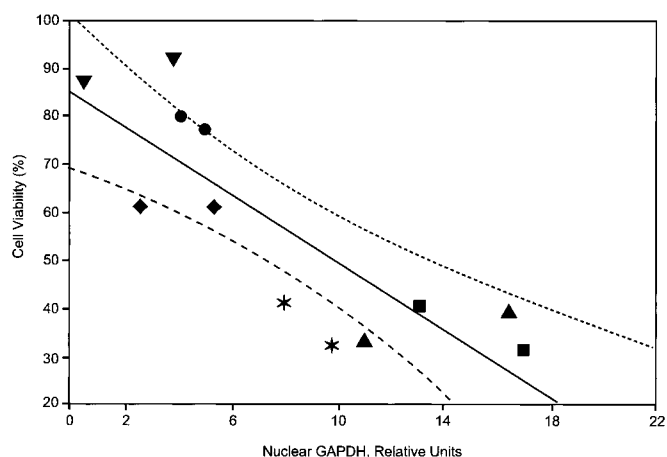


Fig. 5. Relation between the intranuclear level of GAPDH in nuclear extracts of ALL cells before exposure to MP and viability of human leukemia cells after 72 h treatment with 10 μ M MP ($p = 0.001$). Solid line depicts best fit linear regression and broken lines depict upper and lower boundaries of 95% confidence interval ($r^2 = 0.78$). Symbols depict 697 (\bullet), P12 (∇), CEM (\blacktriangle), Nalm6 (\blacksquare), WIL2NS (\blacklozenge), and Molt4 ($*$) cells, each characterized in two separate experiments.

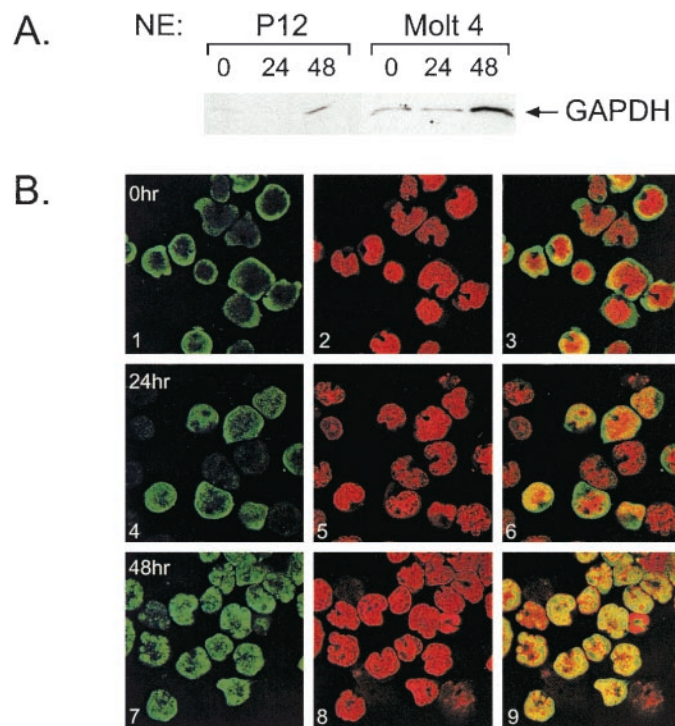


Fig. 6. Nuclear GAPDH translocation after MP treatment in T-lineage human leukemia cells. A, accumulation of GAPDH in nuclear extracts (NE) from P12 (relatively resistant) and Molt4 (sensitive) cells, at 24 h and 48 h after treatment with 10 μ M MP. B, confocal laser scanning micrograph of optical sections of P12 leukemia cells incubated with MP for 0 h (1, 2, and 3), 24 h (4, 5, and 6) and 48 h (7, 8, and 9), labeled for immunofluorescence with a monoclonal antibody to GAPDH. 1, 4, and 7 show the FITC (green) labeling of GAPDH; 2, 5, and 8 show propidium iodide (red) staining of nuclei; and 3, 6, and 9 show the merged image of GAPDH and propidium iodide staining, with yellow depicting colocalization of GAPDH and DNA.

moto et al., 1999). Identification of other components of this complex should provide additional insights into the biological role of this protein complex, work that is ongoing in our lab.

GAPDH is a well known example of a multifunctional enzyme, with DNA repair as one of its functions (Sirover, 1999). Previous reports demonstrated intranuclear translocation of GAPDH and its participation in apoptotic pathways after cytotoxic treatment with cytosine arabinoside (Sawa et al., 1997), although the upstream events and points of intersection with the known apoptotic pathways remain to be elucidated. Consistent with this report, we observed translocation of GAPDH into the nucleus after MP treatment; the phenomenon was noted in both resistant (P12) and sensitive (Molt4) cells. The current work establishes GAPDH as a part of the protein complex interacting with DNA modified by thioguanine incorporation, indicating a role of this protein in a broader spectrum of cytotoxic agents.

Western blot analysis of nuclear extracts from six human leukemia cell lines, as well as confocal microscopy of intact cells, demonstrated marked heterogeneity in the nuclear levels of GAPDH (Fig. 4B). Basal intranuclear levels of GAPDH differed between cell lines, as evidenced by Western blot analysis (Fig. 4B). Confocal microscopy experiments also revealed that the human ALL cells studied have different ratios of nuclear to cytosolic GAPDH (Fig. 4C). Moreover, constitutive levels of nuclear GAPDH in these human leukemia cells before MP treatment as measured by Western blot analysis, significantly correlated with their sensitivity to 48 h ($p = 0.002$) or 72 h ($p = 0.001$, Fig. 5) treatment with 10 μ M mercaptopurine. 697 Cells were used in this study as a negative control because of their very low level (more than 10-fold decreased) of thioguanosine incorporation into DNA, because of low hypoxanthine phosphoribosyltransferase activity (Krynetskaia et al., 1999) and correspondingly low sensitivity to MP. Our data indicate that basal GAPDH intranuclear localization per se is not sufficient to trigger cell death but that a high level of nuclear GAPDH is associated with cytotoxicity when accompanied by sufficient DNA modification.

The newly identified protein complex containing GAPDH binds with similar affinity to DNA duplex containing either a G^ST or the more prevalent G^SC mismatch, in contrast to MutS α , which binds primarily to DNA with a G^ST mismatch. Earlier reports linking GAPDH to apoptosis (Sheline and Choi, 1998), together with the present finding that GAPDH is part of a protein complex that interacts with dG^S-DNA, points to an important role for this protein in cellular responses to DNA modifications. We hypothesize that the complex containing GAPDH serves as a sensor of structural alterations in DNA and links relatively small perturbations in DNA structure to the cellular apoptotic machinery.

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Send reprint requests to: Dr. William E. Evans, St. Jude Children's Research Hospital, 332 N. Lauderdale, Memphis, TN 38163. E-mail: william.evans@stjude.org
