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The nuclear targeting and nuclear retention properties of a human DNA repair protein O^6-methylguanine-DNA methyltransferase are both required for its nuclear localization: the possible implications

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Human O^6-methylguanine-DNA methyltransferase (MGMT) protects human cells from the mutagenic effects of alkylating agents by repairing the O^6-alkylguanine residues formed by these agents in the nuclear DNA. We report here a study showing a possible two-step model for the nuclear localization of the 21 kDa human protein. The first step is the translocation of the protein from the cytosol to the nucleus. This appears to require the nuclear targeting property associated with the holoprotein in combination with a cellular factor(s) to effect the nuclear translocation of MGMT. The second step involves the nuclear retention of MGMT (to prevent its export from the nucleus). This requires a basic region (PKAAR, codons 124–128) that can bind to the non-diffusible DNA elements in the nucleus. Supporting data for such mechanisms are: (i) the holoprotein can target the cytosolic 110 kDa β-galactosidase into the nucleus; (ii) purified recombinant MGMT requires a cellular factor for transport across the nuclear membrane; (iii) nuclear MGMT can be removed selectively by DNase I; (iv) the repair-positive K125L mutant, which alters the PKAAR motif, remains in the cytosol and fails to bind DNA *in vitro*; and (v) polypeptide containing the PKAAR motif has no nuclear targeting property. Interestingly, mutants in another basic region, KLLKVVK (codons 101–107) are DNA binding and repair deficient but entirely nuclear. As these substitutions affect the functional properties of human MGMT, they are potential targets for genetic screening of individuals for risk assessment to alkylating agents.

Keywords: nuclear retention/nuclear targeting/ O_6-methylguanine-DNA methyltransferase

Introduction

A ubiquitous repair process predominantly neutralizes the hazard of O^6-alkylguanine (6RG) lesions in cellular DNA (Loveless, 1969; Gerchman and Ludlum, 1973; Zarbl *et al.*, 1985; Mitra *et al.*, 1989) entailing a direct transfer of the alkyl moiety. This activity is mediated by O^6-methylguanine-DNA methyltransferase (MGMT) which transfers the alkyl group from 6RG to a cysteine residue (PCHRV) of the active site (Yarosh, 1985; Lindahl *et al.*, 1988; Pegg, 1990). Prokaryotic and mammalian MGMTs share strong homology around the active site region but differ significantly in others. This could be related to an evolutionary divergence of MGMTs towards their functions in the eukaryotic cell nucleus.

The importance of mammalian MGMTs in cellular defence against alkylating agents is highlighted by the observed protection of transgenic mice against *N*-nitrosourea-induced thymic lymphomas (Dumenco *et al.*, 1993). Interestingly, comparatively lower protection was observed when bacterial MGMTs were expressed in mammalian cells (Pegg, 1990). This is possibly due to the cytosolic distribution of the expressed bacterial MGMTs in mammalian cells (Dumenco *et al.*, 1989). Consequently, they are inadequate to repair the DNAs which are compartmentalized in the mammalian cell nucleus. This observation suggests that, to perform their functions, eukaryotic MGMTs must have their destinies in the cell nucleus. This agrees with most of the immunostaining data, including human biopsy tissues, showing that human MGMT is a nuclear protein (Ayi *et al.*, 1992; Lee *et al.*, 1992; Brent *et al.*, 1993). However, there are reports on its cytosolic appearance (Ishibashi *et al.*, 1994a,b). This could be due to the cell lines used, which might be defective in the mechanism of nuclear localization of human MGMT, antibody specificity or experimental conditions. Therefore, understanding the mechanisms behind the nuclear localization of the protein may provide information for the above unusual observations and, importantly, an insight into how the protein functions *in vivo*.

The present view on the nuclear localization of proteins concerns the balance between two processes. First is the import of proteins into the nucleus, i.e. nuclear translocation which requires a nuclear targeting signal (Silver, 1991) and cellular transport factors (Gorlich *et al.*, 1994). Second is the export of proteins from the nucleus (Schmidt-Zachmann *et al.*, 1993; Gerace, 1995). It appears that the imported protein will not be found accumulated in the cell nucleus (e.g. export from the nucleus), unless it can stabilize itself there (e.g. by its nuclear retention property). This may prevent proteins from accumulating in the nucleus unnecessarily and can also serve as a regulatory mechanism, i.e. regulating its nuclear localization property can control the *in vivo* activity of a transcription factor (Beg *et al.*, 1992; Henkel *et al.*, 1992).

We report here a study on the possible mechanisms of the nuclear localization of the 21 kDa human MGMT using defined molecular and biochemical techniques. The experiments involved are as follow: (i) comparing the nuclear targeting potential of the holoprotein and its individual regions (N- and C-terminal deletion analysis) with the common SV40 large T antigen-like nuclear localization signal (NLS, PKKKRKV, Kalderon *et al.*, 1984), when they are fused to the 110 kDa cytosolic β-galactosidase (β-gal) in transient transfection assays;

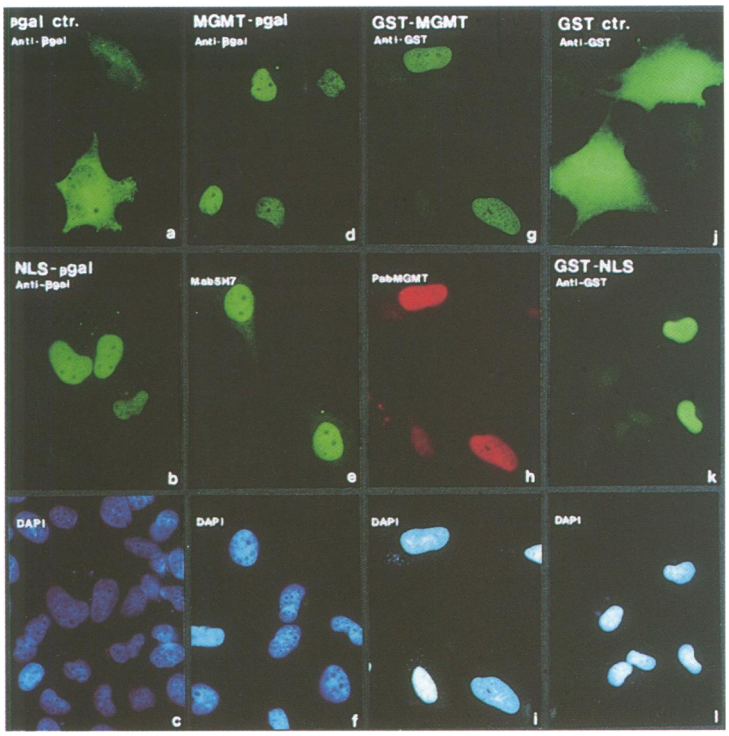

Fig. 1. Nuclear targeting property of human MGMT. Immunostaining of MRC5.SV40 cells 48 h after transfection with mammalian vectors containing the cDNAs for β-galactosidase (β-gal) in (**a**) (β-gal staining by 5 μg/ml of Mab.β-gal), for NLS-β-gal in (**b**) (β-gal) and (**c**) [nuclear DNA of (b) by co-staining with 1.5 μg/ml of DAPI], for MGMT–β-gal in (**d**) (β-gal), (**e**) (MGMT staining by 10 μg/ml Mab.5H7) and (**f**) [DNA of (e)], for GST–MGMT in (**g**) (GST staining by 5 μg/ml of Mab.GST), (**h**) [MGMT staining of (g) by co-staining with 1.5 μg/ml of Pab.MGMT] and (**i**) [DNA of (g) and (h)], for GST in (**j**) (GST) and for GST–NLS in (**k**) (GST) and (**l**) [DNA of (k)]. NLS is the SV40 oligopeptide **PKKKRK**V.

(ii) point mutation studies on the two basic regions, -**KLLKVVK** (codons 101–107) and -**PKAAR** (codons 124–128), of the protein; (iii) *in vitro* nuclear penetration assay using permeabilized cells; and (iv) probing the DNA binding activities of the protein *in vivo* by partial DNase I digestion of paraformaldehyde-fixed cells and *in vitro* by DNA gel mobility assay using the recombinant proteins. These experiments address the two possible steps involved; first, how the protein is translocated from the cytosol to the nucleus, i.e. its nuclear targeting property and the possible associated factor(s), and second, how the small protein (Dingwall and Laskey, 1986) can prevent itself from possible nuclear export after its import into the nucleus, i.e. its nuclear retention property.

Results

Nuclear targeting property of human MGMT: the 130 kDa MGMT–β-gal fusion protein is localized in human cell nuclei

To confirm that human MGMT is a nuclear protein and that it has a nuclear targeting property, we studied exogenous MGMTs expressed as fusion proteins in MRC5.SV40 *mer*$^-$ cells by transfection with mammalian vectors containing the human cDNA fused to the cDNAs of two cytosolic proteins β-gal (110 kDa) and glutathione-*S*-transferase (GST, 25 kDa). As the tag proteins are from other species and the parental cell line contains low levels of MGMT (Ayi *et al.*, 1992), the expressed fusion proteins can be monitored easily by staining with antibodies towards the tag proteins and MGMT. Immunostaining data in Figure 1 show that: (i) cells transfected with MGMT–β-gal and GST–MGMT constructs were stained positively in the nuclei by both antibodies [in MGMT–β-gal cDNA-transfected cells see Figure 1d for β-gal (by Mab.β-gal), 1e for MGMT (by Mab.5H7) and 1f for the DNA staining of 1e (which marks the nucleus) by DAPI; in GST–MGMT see Figure 1g for GST (by Mab.GST), 1h for MGMT (by MGMT.Pab) and 1i for DNA of 1h]; and (ii) in the control experiments, both the expressed tag proteins alone were either diffuse (GST) or localized in the cytoplasm (β-gal) [see β-gal in Figure 1a and GST in 1j]. These results suggest that exogenous (by transfection) MGMT in the form of larger size fusion proteins is also nuclear, similarly to endogenous MGMT in *mer*$^+$ cells (Ayi *et al.*, 1992). As the expressed 130 kDa MGMT–β-gal fusion protein is too large for the passive diffusion into the nucleus, its presence in the nucleus suggests that MGMT can target the cytoplasmic β-gal (or GST) protein into the nucleus. This is similar to the SV40 NLS oligopeptide (**PKKKRKV**) [see the nuclear staining for NLS–β-gal in Figure 1b (β-gal staining) and the nucleus (by DNA staining of 1b) in 1c, and in Figure 1k (GST) and 1l (DNA of 1k) for GST–NLS].

Nuclear localization properties of N- and C-terminal deletion mutants, as β-gal fusion proteins, of human MGMT

To map out the regions of MGMT involved, we transfected MRC5.SV40 (*mer*$^-$) cells with pCHL vector constructs containing β-gal cDNA fused to human MGMT cDNAs containing progressive 90 bp deletions from the 5′ ends

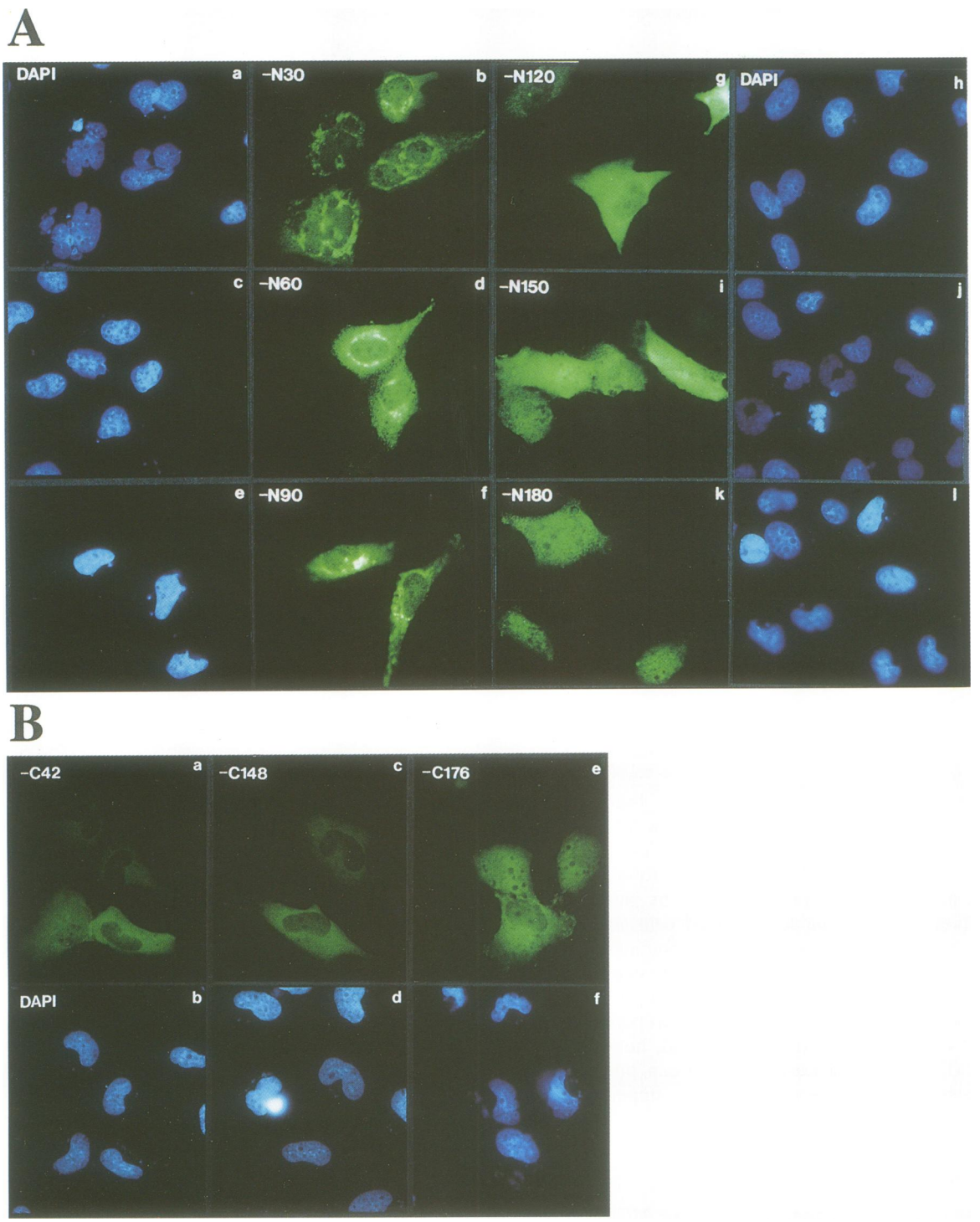

Fig. 2. Nuclear targeting properties of β-gal-fused N- and C-terminal deletion mutants of human MGMT. Immunostaining of MRC5.SV40 cells 48 h after transfection with mammalian vectors containing various 5′ and 3′ deletions of human MGMT cDNAs fused to β-gal. (**A**) 5′ Deletions (N-terminus) for codons 1–30 (–N30) in (a) (DNA staining by 1.5 μg/ml of DAPI) and (b) [β-gal staining of (a) by co-staining with 5 μg/ml of Mab.β-gal], similarly for codons 1–60 (–N60) in (c) (DNA) and (d) (β-gal), for codons 1–90 (–N90) in (e) (DNA) and (f) (β-gal), for codons 1–120 (–N120) in (g) (β-gal) and (h) (DNA), for codons 1–150 (–N150) in (i) (β-gal) and (j) (DNA) and for codons 1–180 (–N180) in (k) (β-gal) and (l) (DNA). (**B**) 3′ Deletions (C-terminus) for codons 165–207 (–C42) in (a) (β-gal) and (b) (DNA), for codons 59–207 (–C148) in (c) (β-gal) and (d) (DNA) and for codons 31–207 (–C176) in (e) (β-gal) and (f) (DNA).

and selective deletions from the 3′ ends. Figure 2A (N-terminal deletion) and B (C-terminal deletion) shows the expressed fusion proteins by immunostaining using Mab.β-gal. Surprisingly, no prominent nuclear staining can be observed in cells expressing these β-gal fusion proteins, except the appearances of uniform cytosolic and nuclear staining in the deletion mutants; –N150 (Figure 2A, i), –N180 (Figure 2A, k) and –C176 (some cells in Figure 2B, e). These results possibly suggest that the N- (–C176) and C- (–N150 and –N180) termini of MGMT

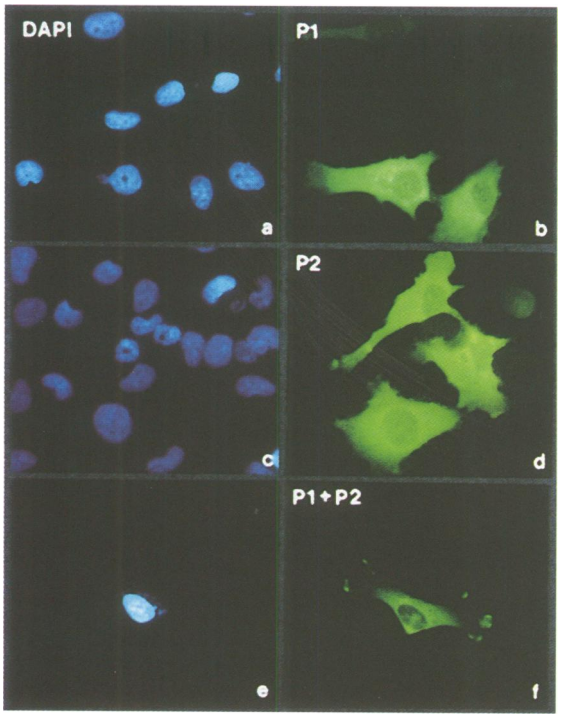

Fig. 3. Properties of GST fusion proteins containing the basic regions of human MGMT. Immunostaining of MRC5.SV40 cells 48 h after transfection with mammalian vectors containing cDNAs of the two basic regions of MGMT fused to GST cDNA; for P1 (codons 91–120, containing the **K**LL**K**VV**K**) in (**a**) (DNA staining by 1.5 μg/ml of DAPI) and (**b**) [GST staining of (a) by co-staining with 5 μg/ml of Mab.GST], similarly for P2 (codons 121–150, containing the P**K**AA**R** region) in (**c**) (DNA) and (**d**) (GST) and P1+P2 (codons 91–150, containing **K**LL**K**VV**K** and the P**K**AA**R** regions) in (**e**) (DNA) and (**f**) (GST).

were insufficient for the complete nuclear localization of proteins as compared with NLS–β-gal (Figure 1b) or MGMT–β-gal (Figure 1d and e). It appears that proper nuclear localization requires an additional domain(s) in the holoprotein.

The basic regions, KLLKVVK and PKAAR, of MGMT are not nuclear targeting

To confirm these results further, we tested again whether the two potentially NLS-like basic regions of MGMT (alone or collectively) could play the role of the NLS, in view of the specific arrangement of their basic amino acid residues (Kalderon *et al.*, 1984; Lanford *et al.*, 1988) and the strong nuclear staining observed for NLS– and MGMT–β-gal fusion proteins in Figure 1. In this experiment, we used the smaller reporter protein GST (25 kDa) rather than the larger β-gal (110 kDa). Three MGMT cDNAs were constructed: P1 [containing the **K**LL**K**VV**K** (codons 101–107) region]; P2 [containing the P**K**AA**R** (codons 124–128) region]; and P1+P2, and ligated to the 3′ end of GST cDNA. These constructs were inserted into the vector pXJ41neo and transfected into MRC5.SV40 cells. The expressed proteins were followed by immunostaining with Mab.GST.

Figure 3 shows that the GST–P1 (a and b), GST–P2 (c and d) and GST–(P1+P2) (e and f) proteins were all present in the cytosol. This is in contrast to the diffuse staining pattern of the GST protein alone (see Figure 1j), and the predominantly nuclear stainings found in the GST–MGMT and GST–NLS proteins (see Figure 1g, h and k respectively). These results, however, show that these basic regions differ significantly from the nuclear localization properties of the SV40 NLS (P**KKKRK**V) and the holoprotein.

Characterization of human MGMTs carrying point mutations in its basic regions: mutants in the PKAAR region are cytosolic but repair-normal

As multiple regions of MGMT may be required for its nuclear localization, we speculated that the central region, where the basic regions are located, might function in conjunction with the N- and C-termini. To avoid the introduction of drastic changes in the holoprotein, we constructed cDNAs containing systematic point mutations; **K**101**L**, **K**104**L**, **K**107**L**, **K**125**L** and **R**128**L**, around these basic regions to investigate the role of each basic residue in the nuclear localization of MGMT. After transfection of these cDNAs (cloned in pXJ41neo vector) into MRC5.SV40 cells, the expressed proteins were stained by MGMT.Pab and Mab.5H7 (Ayi *et al.*, 1992). Figure 4A shows that mutations at the lysine residues (K) of the **K**LL**K**VV**K** region did not affect the nuclear staining of MGMT (Figure 4A, c and d for **K**101**L**, e and f for **K**104**L**, g and h for **K**107**L**). In contrast, the **K**125**L** mutant protein was predominantly in the cytosol (see i, j1 and j2) whereas diffuse staining was observed for the **R**128**L** mutant (see k, l1 and l2).

Although mutations in the P**K**AA**R** region appear to perturb the nuclear localization of MGMT, this may be due to a gross structural alteration in the mutant proteins. However, if one assumes that structural integrity is essential for function, assaying the repair activities of these mutant proteins may provide such information. Therefore, we attempted to establish permanent cell lines of these mutants by multiple rounds of selection with Geneticin. Cell extracts from these stable cell lines were subjected to Western analysis and activity measurements. With the exception of the **K**107**L** transfectant, which had far fewer MGMT-positive cells, we obtained stable cell lines for **K**101**L**, **K**104**L**, **K**125**L** and **R**128**L** which expressed high levels of MGMT (see Western analysis in Figure 4B).

After quantification of their MGMT levels, these cell extracts were assayed for O^6-methylguanine repair activities by incubation with a known concentration of ^{32}P-labelled CGC6MGCG substrate (Liem *et al.*, 1993). Table I shows that the repair activities were significantly impaired in the **K**101**L** and **K**104L mutants. The **K**125**L** and **R**128**L** mutant cell extracts were repair-positive (albeit that the R128L mutant showed a 25% decrease in activity, Kanugula *et al.*, 1995), suggesting that they are not structurally altered and, therefore, their cytosolic appearances show a positive role for the P**K**AA**R** region in the nuclear localization of MGMT.

Deletion of the KLLKVVK region causes the misfolding of MGMT

The observation that the mutants (**K**101**L** and **K**104**L**) in the **K**LL**K**VV**K** region were repair-deficient (Table I) and nuclear (Figure 4), which is entirely opposite to the repair-normal and cytosolic behaviours observed from the mutants (**K**125**L** and **R**128**L**) in the P**K**AA**R** regions, led

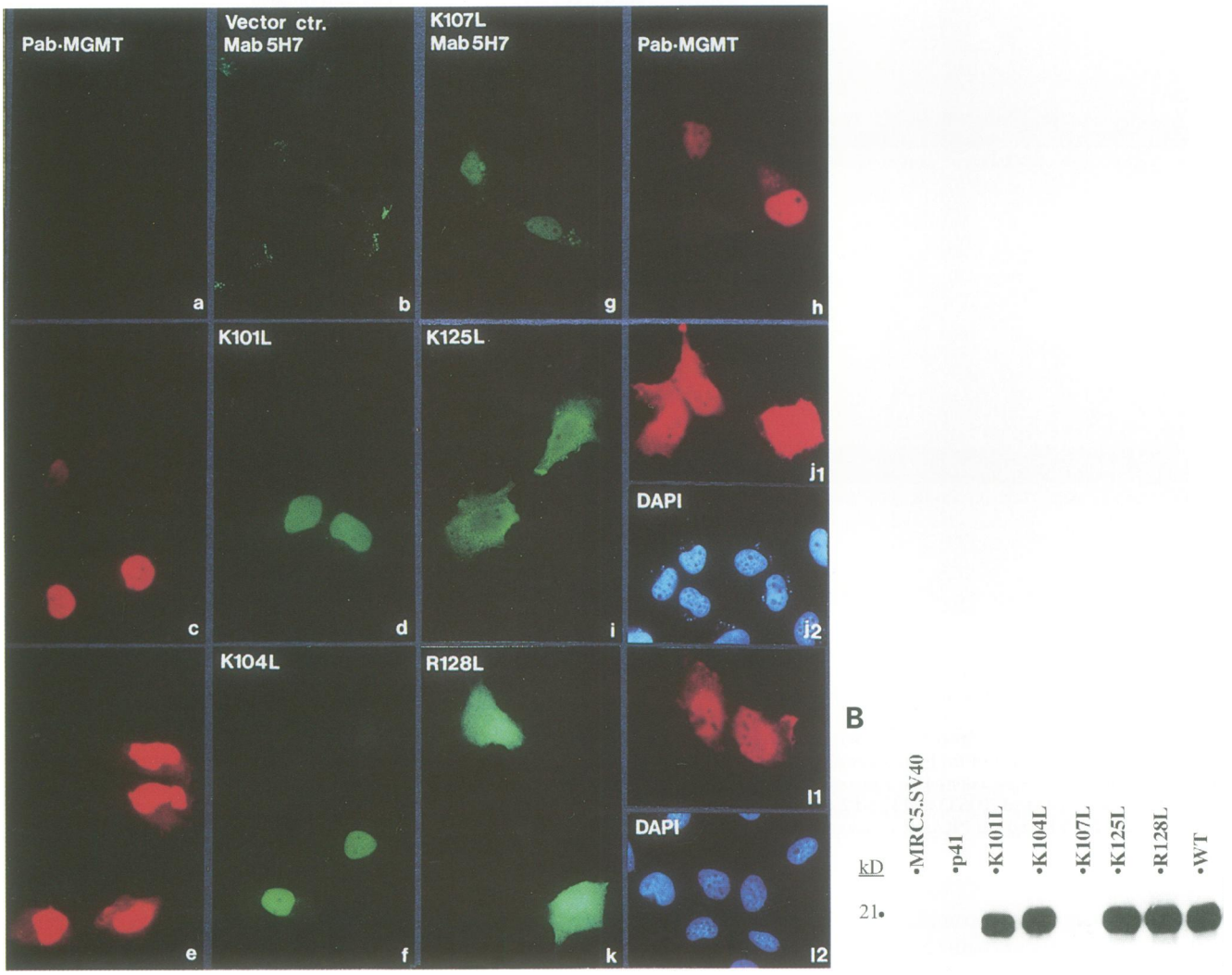

Fig. 4. Nuclear localization properties and Western blotting analysis of point mutation mutants in the basic regions of human MGMT.
(**A**) Immunostaining of MRC5.SV40 cells 48 h after transfection with mammalian vectors containing point mutations in the basic regions of MGMT: for control vector in (a) (MGMT staining by 1.5 μg/ml of Pab.MGMT) and (b) (MGMT staining by 10 μg/ml of Mab.5H7), for **K**101**L** [replacement of codon 101 lysine (K) residue by leucine (L)] in (c) (Pab.MGMT) and (d) (Mab.5H7), for **K**104**L** in (e) (Pab.MGMT) and (f) (Mab.5H7), for **K**107**L** in (g) (Mab.5H7) and (h) (Pab.MGMT), for **K**125**L** in (i) (Mab.5H7), (j1) (Pab.MGMT) and (j2) [DNA staining of (j1) by co-staining with 1.5 μg/ml of DAPI] and for **R**128**L** in (k) (Mab.5H7), (l1) (Pab.MGMT) and (l2) [DNA staining of (l1)]. (**B**) Western analysis of MGMT in cell extracts of stable cell lines transfected with MGMT cDNA carrying point mutations in the basic regions; 25 μg of total cell extracts/lane were resolved on a 13.5% SDS–PAGE, Western blot using Mab.2G1 and ECL; the time is 2 min. Labelled lanes are: MRC5.SV40, original cell line used for transfection; p41, established cell line transfected with mammalian expression vector pXJ41neo only; **K**101**L**, established cell line carrying MGMT cDNA, with the lysine residue (K) at codon 101 substituted by a leucine (L), in pXJ41neo and similarly for **K**104**L**, **K**107**L**, **K**125**L** and **R**128**L**; WT, wild-type MGMT cDNA. The negligible **K**107**L** MGMT detected is due to the lower number of MGMT-expressing cells.

us to study its function in MGMT. We deleted the entire **K**LL**K**VV**K** region from the holoprotein. Figure 5 shows that, unlike the N- and C-terminal deletion mutants (Figure 2), this mutant protein was retained preferentially in the periphery of the nucleus as distinctive granules (see Pab staining in Figure 5a, Mab.5H7 in b and their DNA stainings in c and d), which had been observed in misfolded or incompletely folded proteins (Hammond and Helenius, 1994). This result shows that the **K**LL**K**VV**K** region might be essential for structural integrity of the protein. Therefore, the repair-deficient **K**101**L** and **K**104**L** mutants (Table I) might have subtle structural alterations, which appear to affect only the repair activity but not the nuclear localization (Figure 4).

Nuclear translocation of human MGMT requires a cellular factor(s) and evidence for the nuclear retention property of MGMT: in vitro nuclear transport assay

As the P**K**AA**R** region has no nuclear targeting property (Figure 3), but mutations in this region perturb the nuclear localization of MGMT (Figure 4), what could be its role in this process? We addressed the possible presence of co-factors involved, first, in the initial translocation of MGMT from cytosol to the nucleus and, second, in the stabilization of MGMT in the nucleus.

The documented *in vitro* nuclear transport assay, which was used to identify cellular factors that aid the nuclear translocation of proteins (Adam *et al.*, 1990), was used to

Table I. Relative repair capacities of cell extracts obtained from established MRC5.SV40 cell lines expressing wild-type and mutant MGMTs

MGMT status	Cell lines	[^{32}P]CGCGCG (repaired) (c.p.m.)	[^{32}P]CGC6MGCG (unrepaired) (c.p.m.)	Repair (%)	Efficiency (%)
Wild-type	HeLa. CCL2B	5061 (±148)	211 844 (±6215)	1.93	–
–	MRC5.SV40	ND	215 345 (±8183)	–	–
–	p41	ND	211 296 (±7395)	–	–
Wild-type	HM37	174 394 (±6975)	35 394 (±1415)	83.1	100
K101L	HM101L	33 076 (±1389)	187 513 (±7815)	15.0	18.0
K104L	HM104L	859 (±32)	215 459 (±7972)	0.39	0.47
K107L	HM107L	45 (±3)	218 707 (±6998)	0.04	0.05
K125L	HM125L	173 942 (±7653)	41 382 (±1812)	80.8	97.2
R128L	HM128L	132 660 (±4245)	77 827 (±2490)	63.0	75.8

Duplicated samples of total cell extracts [adjusted to a similar amount of MGMT based on the intensity of MGMT bands quantified by a densitometer from a lower ECL exposure (30 s) negative in Figure 4B]. Two hundred μg for HeLa.CCL2B (containing ~10 ng of MGMT as positive control), MRC5.SV40 (control cells), p41 (MRC5.SV40 cells transfected with pXJ41neo only), wild-type and **K107L** [substitution of lysine (K) by leucine (L) at codon 107], 260 μg for **K101L**, 230 μg for **K104L**, 184 μg for **K125L** and 176 μg for **R128L** were incubated with the 5′-^{32}P-labelled substrate CGC6MGCG (221 540 c.p.m./22 pM) in assay buffer (100 μl, 50 mM Tris pH 8, 1 mM EDTA and 1 mM DDT) at 37°C for 1 h. The reaction mixtures were then analysed for the product [^{32}P]CGCGCG and the substrate [^{32}P]CGC6MGCG. Four HPLC runs (two injections per sample in duplicate) were carried out for each cell line. The average c.p.m. of the repaired product [^{32}P]CGCGCG and the unrepaired residual substrate [^{32}P]CGC6MGCG are summarized in columns 3 and 4 respectively, and ND represents 'not detectable'. Column 5 is the percentage of repair calculated from the ratio of product c.p.m. to the sum of product and residual substrate c.p.m. detected. Column 6 is the percentage efficiency of repair with respect to the wild-type cell extract. The negligible repair activity of HM107L is attributed to the lower number of MGMT-expressing cells, see Figure 4B.

study the first possibility of whether MGMT requires a cellular factor for its nuclear translocation. In this experiment, viable MRC5.SV40 cells that contained porous plasma but intact nuclear membranes were obtained by controlled streptolysin-O treatment (see Materials and methods). In these cells, the diffusible cytoplasmic proteins were removed during the procedure but not the non-diffusible cytoskeleton proteins, such as tubulin. These viable cells were then tested by immunostaining directly with antibodies (~200 kDa) towards tubulin (cytoskeleton) and histone (nuclear) before fixation with paraformaldehyde. The permeabilization conditions were then optimized until positive tubulin (due to the free entry of the antibodies through the punctured plasma membrane) but negative histone (restricted entry of the antibodies by the intact nuclear membrane) stainings were obtained. These cells were then used to test whether the wild-type and mutant MGMTs in the above established cell extracts and the recombinant MGMT (rMGMT) can be transported into the nucleus.

First, we tested the purified rMGMT. Figure 6 shows that, under these assay conditions, immunostaining of rMGMT appeared in the nucleus only when the assay mixture (rMGMT in transport buffer TB alone) was supplemented with MRC5.SV40 (*mer*$^-$) cell extracts [see the rMGMT staining by Pab.MGMT in Figure 6g (cell extract + rMGMT in TB) and the DNA staining in h and the negative stainings by Pab.MGMT in the control experiments (MRC5.SV40 cells only in Figure 6a, MRC5.SV40 extract in c, the rMGMT in TB only in e and their DNA staining in b, d and f respectively)]. Therefore, these results suggest that, even within the nuclear pore size exclusion limit, rMGMT could not diffuse passively across the nuclear membrane (Dingwall and Laskey, 1986). It required a cellular factor(s). Preliminarily observations suggest that this cellular factor may be negatively charged because passage of the MRC5.SV40 (*mer*$^-$) extract through a MonoQ column completely abolished its effect on rMGMT (A.Lim and B.Li, unpublished observation). Further experiments with the mutant cell

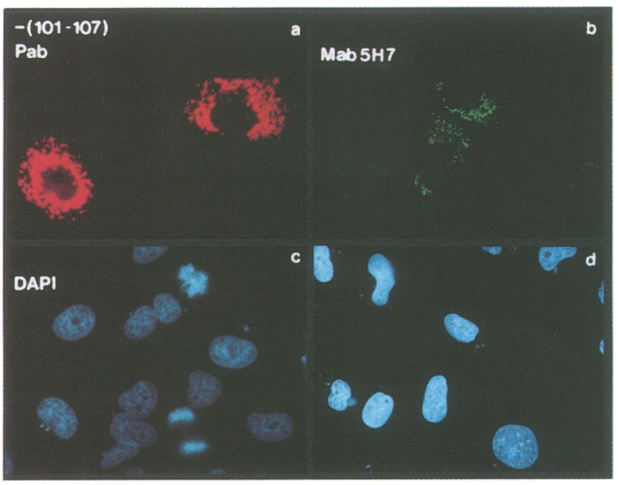

Fig. 5. Properties of MGMT carrying the **KLLKVVK** deletion. Immunostaining of MRC5.SV40 cells 48 h after transfection with mammalian vector containing MGMT cDNA with a deleted DNA sequence for the **KLLKVVK** region [codons 101–107, –(101–107)]. MGMT staining by Pab (1.5μg/ml) in (**a**) with the corresponding DNA staining by DAPI (1.5 μg/ml) in (**c**) and MGMT staining by Mab.5H7 (10 μg/ml) in (**b**) with the corresponding DNA staining in (**d**).

extracts alone showed that the **K101L** (see Figure 6k and l) and **K104L** (see m and n) mutants were similar to the wild-type protein (see i and j) which were found in the nuclei of the permeabilized cells, but not the **K125L** mutant (see o and p). This result, together with the transient transfection assay data in Figure 4, shows consistently that the **K125L** mutant could not accumulate in the nucleus. These observations strongly suggest that the **PKAAR** region may be involved in either the interaction with the cellular transport factor or the retention of the translocated MGMT in the nucleus. However, the first possibility can be ruled out by the exclusively cytosolic appearance of the fusion protein carrying the **PKAAR** polypeptide (see basic region analysis in Figure 3). The **PKAAR** region has no nuclear targeting, or NLS, property. As cellular factors that facilitate the nuclear translocation of proteins

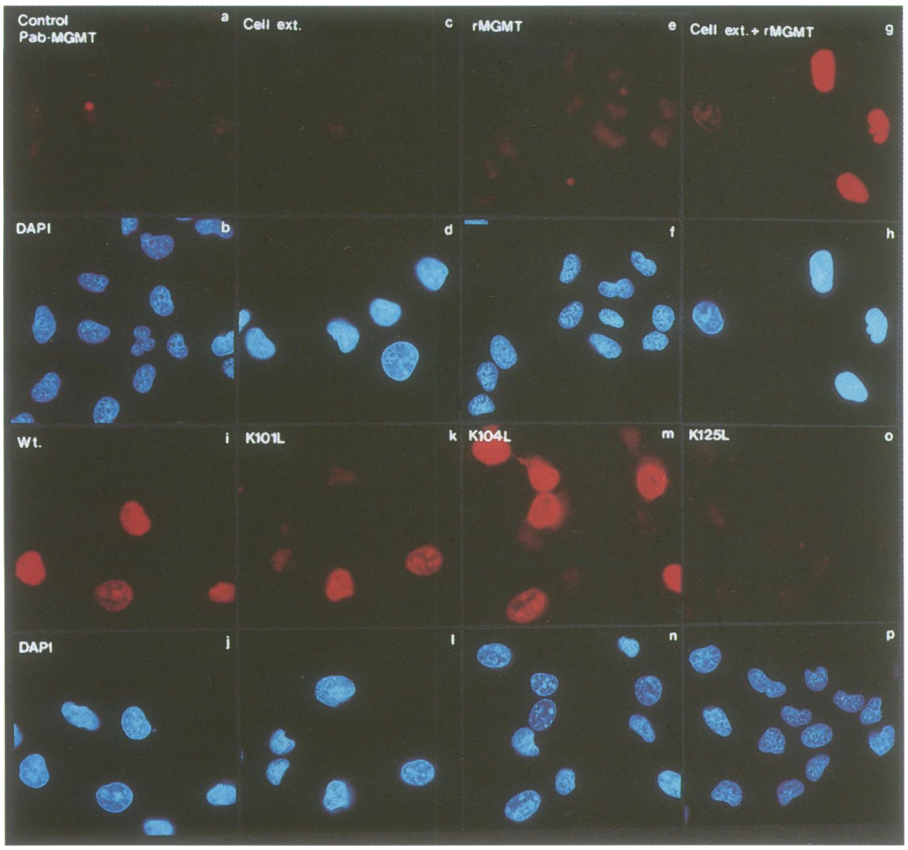

Fig. 6. *In vitro* nuclear transport assay of wild-type (Wt), recombinant and mutant MGMT proteins. Immunostaining of streptolysin-O-permeabilized MRC5.SV40 cells treated with various sources of MGMT: for control MRC5.SV40 cells in (**a**) (MGMT staining by 1.5 μg/ml of Pab.MGMT) and (**b**) [DNA staining of (a) by co-staining with 1.5 μg/ml of DAPI], for control MRC5.SV40 cell extracts (500 μl containing 200 μg of cellular proteins in transport buffer, TB) in (**c**) (MGMT) and (**d**) (DNA), for recombinant MGMT (rMGMT, 1 μg in 500 μl of TB) in (**e**) (MGMT) and (**f**) (DNA), for rMGMT in TB supplemented with MRC5.SV40 cell extract (200 μg) in (**g**) (MGMT) and (**h**) (DNA), wild-type cell extracts (200 μg of protein in 500 μl of TB) obtained from stable MRC5.SV40 cells (HM37) transfected with human Wt MGMT cDNA in (**i**) (MGMT) and (**j**) (DNA), and similarly for the **K**101**L** mutant cell extract in (**k**) (MGMT) and (**l**) (DNA), for **K**104**L** in (**m**) (MGMT) and (**n**) (DNA), and for **K**125**L** in (**o**) (MGMT) and (**p**) (DNA).

are potentially NLS binding proteins (Silver, 1991; Gorlich *et al.*, 1994), it is unlikely that the P**K**AA**R** region can be involved in the interaction with the cellular factor. Therefore, the P**K**AA**R** region is probably responsible for the nuclear retention of MGMT after its translocation from the cytosol to the nucleus.

Human MGMT binds to DNA in vivo and in vitro: the role of the PKAAR region in the nuclear retention of MGMT

To understand how the P**K**AA**R** region could retain the holoprotein in the nucleus, we investigated the possible factor(s) that stabilizes MGMT in the nucleus (the second possibility). As the human rMGMT binds to single- and double-stranded DNA *in vitro* (Ayi *et al.*, 1992; Kanugula *et al.*, 1995), it is possible that similar binding of MGMT to the non-diffusible nucleic acid components *in vivo* could enable its retention in the nucleus. Therefore, we used DNase I to probe the sensitivity of MGMT in the nuclei of HM37 cells (a stable MRC5.SV40 cell line expressing the wild-type MGMT). Established procedures, which show the sensitivities of RNA-related splicesome factors (e.g. SC35) towards RNase but not DNase I (Spector *et al.*, 1991), were used. Paraformaldehyde-fixed HM37 cells were partially digested with DNase I before immunostaining with MGMT antibodies and the DNA dye DAPI. Figure 7A shows that there was a concurrent loss of MGMT and DNA staining in these DNase I-digested cells (note that the nuclei are intact as revealed by the residual DNA staining). Upon extended DNase I digestion (5 h), no residual MGMT staining by Pab could be observed. This result shows that MGMT in the nucleus is either directly or indirectly associated with DNA.

Consequently, the cytosolic appearance of the **K**125**L** mutant (see Figure 4A i, j1 and j2) might be due to its defect in DNA binding. Therefore, we investigated the DNA binding properties of the mutant proteins. Gel mobility assays were carried out using a ^{32}P-labelled 71mer oligonucleotide duplex, with a degenerate $-N_{13}-$ (N = A, G, C and T) core (Blackwell and Weintraub, 1990; Pollock and Treisman, 1990), and partially purified recombinant GST–MGMT mutant proteins (Figure 7Ba). These fusion proteins were purified by glutathione–Sepharose, as compared with the common MonoS and ssDNA chromatography used for MGMT purification, to avoid concentrating DNA binding proteins in the bacterial cell extracts. GST–MGMT is repair-normal and nuclear (Figure 1). The diversified oligonucleotide probe was chosen to prevent the possible sequence-specific DNA binding exhibited by these mutant proteins. Figure 7Bb

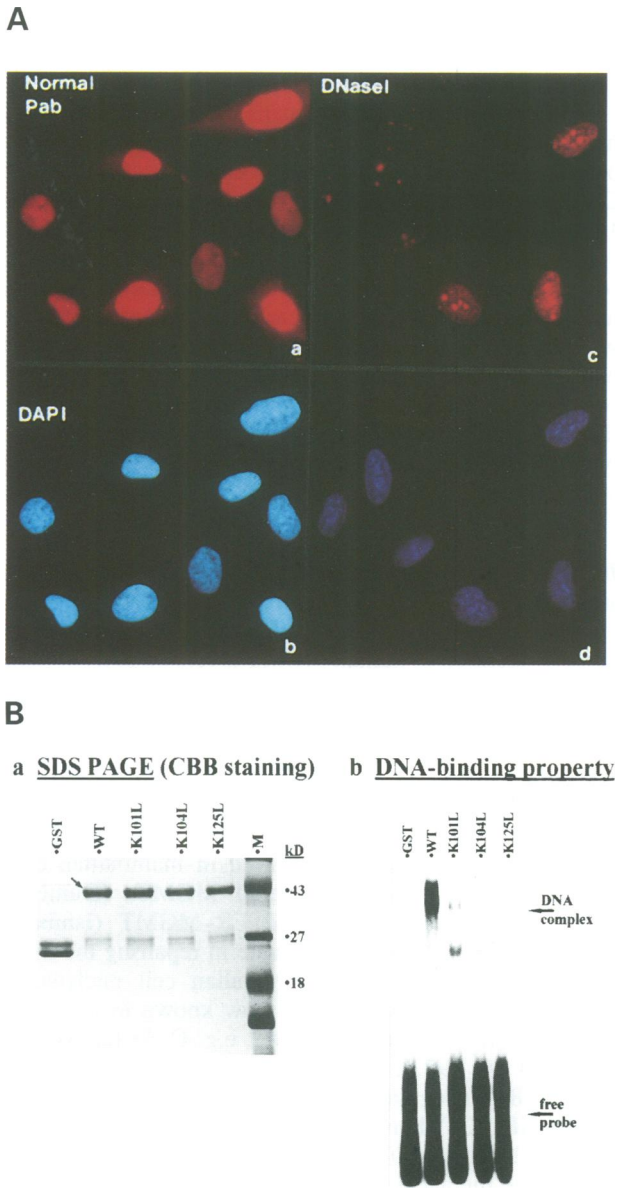

Fig. 7. Affinity of MGMT towards DNA *in vivo* and *in vitro*. (**A**) Immunostaining of paraformaldehyde-fixed HM37 cells (a stable MRC5.SV40 cell line transfected with wild-type MGMT cDNA) after partial digestion with DNase I (25 μg/ml for 2 h); control untreated in (a) (MGMT staining by 1.5 μg/ml of Pab) and (b) [the DNA staining of (a) by co-staining with 1.5 μg/ml of DAPI] and DNase I treated in (c) (MGMT) and (d) (DNA). (**B**) Gel mobility assays. (a) Coomassie blue staining of a 13.5% SDS–PAGE used in the analysis of expressed and glutathione–Sepharose-purified proteins loaded 2 μg/lane. Labelled lanes are: GST, 25 kDa band as control; WT, 43 kDa wild-type GST–MGMT; **K**101**L**, GST–MGMT with the lysine (K) residue at codon 101 replaced by leucine (L); and similarly for **K**104**L** and **K**125**L**; M, pre-stained protein molecular weight marker. (b) Autoradiograph (overnight at –80°C) of a 5% non-denaturing polyacrylamide gel used in the analysis of DNA complexes formed between proteins in (a) and the ^{32}P-labelled 71mer duplex containing a $-(N)_{13}-$ core (see Materials and methods); each lane contains 200 ng of protein and 100 000 c.p.m. of DNA probe.

shows that there was no observable DNA–protein complex formed by the **K**125**L** (lane 5) mutant as compared with the wild-type MGMT (lane 2). Surprisingly, the two mutants in the **KLLKVVK** region, **K101L** (lane 3) and **K**104**L** (lane 4), also showed the formation of fewer DNA–protein complexes.

These data are unexpected because they showed that both basic regions were required for DNA binding. However, the role of the **KLLKVVK** region is unlikely to be direct because we had shown in Figure 5 that deletion of this region from MGMT caused severe misfolding of the mutant protein. This indicates that its role could be structural, and appears to agree with the properties of the mutants (**K**101**L** and **K**104**L**) observed in this region, which were both repair and DNA binding deficient (see Table I and Figure 7B). Additional support for this came from the observation that the repair and DNA binding properties of the holoprotein might not be entirely (or mutually) dependent upon each other. This is because the free base O^6-benzylguanine can be repaired by human MGMT (Kanugula *et al.*, 1995) and MGMT can be inactivated through the direct alkylation of its active site *in vivo* and *in vitro* by agents that are ineffective in producing 6RG in DNA (Brent, 1986; Dolan *et al.*, 1990). With their corresponding peptide motifs (PCHRV from codons 144–148 for repair and P**K**AA**R** from codons 124–128 for the putative DNA binding) located in two different regions of the protein, these two properties are unlikely to be perturbed simultaneously by a single point mutation in another region of MGMT (**KLLKVVK** at codons 101–107), unless this region of the protein can play a structural role in maintaining MGMT in a repair-active and DNA binding conformation.

Based on these observations, it is possible to suggest that the P**K**AA**R** region is directly involved in the DNA binding property of MGMT. This agrees with the properties of the mutants (**K**125**L** and **R**128**L**) observed, which were defective in DNA binding (Figure 7B) but normal in repair (Table I). Consequently, the decreased, but not abolished, DNA binding observed in the **K**101**L** and **K**104**L** mutants (Figure 7B) could be interpreted as the weaker binding of the P**K**AA**R** region to DNA due to an unfavourable structure exhibited by the mutant proteins. These observations agree with the immunostaining data which show that the **K**125**L** mutant was cytosolic because it could not bind to the nuclear DNA due to the altered P**K**AA**R** motif. Therefore, it failed to retain itself in the nucleus, whereas the **K**101**L** and **K**104**L** mutants in the **KLLKVVK** region were nuclear because the intact P**K**AA**R** motif could maintain the DNA binding property, albeit weakly. These observations show the P**K**AA**R** region can play a role in the nuclear retention of MGMT after its translocation to the nucleus.

Discussion

Conformation of human MGMT in a possible two-step, 'factor-mediated nuclear transport and binding to DNA', model for its nuclear targeting and retention

In summary, these data show that the nuclear localization of the 21 kDa human MGMT involves two steps. The first step is the translocation of the protein from the cytosol to the nucleus. This appears to require the nuclear targeting property associated with the holoprotein in combination with a cellular factor(s) to effect the nuclear translocation of MGMT. The supporting data are: (i) the holoprotein

can effectively target the cytosolic 110 kDa β-gal into the nucleus (Figure 1); and (ii) a negatively charged cellular factor is required for the transport of the rMGMT across the nuclear membrane, as shown by the *in vitro* nuclear penetration experiment in Figure 6. The second step involves the nuclear retention of MGMT (preventing its export from the nucleus). This requires a basic region (**PKAAR**) that can bind to the non-diffusible DNA elements in the nucleus. Supporting data are: (i) MGMT in human cell nuclei can be removed by DNase I treatment (Figure 7A), inferring its direct or indirect association with DNA *in vivo*; (ii) failure of the **K125L** mutant (in the **PKAAR** region) in maintaining itself in the nucleus (Figure 4A, i, j1 and j2) coincides with its lack of DNA binding activity observed *in vitro* (Figure 7Bb); (iii) this mutant protein is repair-positive (Table I), therefore its lack of DNA binding is not due to gross structural alterations; and (iv) the **PKAAR** basic region has no nuclear targeting property (Figures 2 and 3), implying that it does not interact with the cellular transport factor.

Although the model for nuclear retention of MGMT seems direct, which involves the binding of DNA by the **PKAAR** region, a model for the nuclear translocation of MGMT has yet to be defined from the following: (i) the holoprotein (see Figure 1), which behaves similarly to the SV40 NLS oligopeptide, is required for its effective nuclear localization; (ii) a cellular factor is required for its nuclear translocation (Figure 6); and (iii) the **PKAAR** region, which is required for nuclear retention of MGMT (see Figure 4), retains proteins in the cytosol (see Figure 3). A simple interpretation of these observations would be the masking of the **PKAAR** (and, perhaps, the **KLLKVVK**) region of the holoprotein through its association with the cellular factor during its translocation from cytosol to the nucleus. This is similar to the nuclear translocation of the transcription factor NF-κB which is modulated by direct binding of its NLS to the cytosolic factor IκB (Beg *et al.*, 1992; Henkel *et al.*, 1992). This interpretation may also explain why the ER–MGMT fusion protein (unlike the MGMT–β-gal and GST–MGMT reported here) is cytosolic (Ishibashi *et al.*, 1994b) since the specific mechanism which regulates the cytosolic ER (estrogen receptor) may override the nuclear targeting potential of human MGMT. As the protein binds to nucleic acids independently (Figure 7Bb), subsequent release of the attached cellular factor (which leads to unmasking of the **PKAAR** region) after translocation into the nucleus would be sufficient for MGMT to be retained in the nucleus through the interaction between its **PKAAR** region and the non-diffusible nuclear DNA. However, MGMT must be able to adopt various dynamic conformations to accommodate the structural changes involved. Apparently, this is feasible because an altered conformation of MGMT had been detected when the recombinant human MGMT was treated with nucleic acids (Chan *et al.*, 1993), or when the protein was inactivated through the alkylation of its active site (Ayi *et al.*, 1994). Interestingly, the N- (**E**30) and C- (**E**172) termini of human MGMT are significantly flexible (Ayi *et al.*, 1994), which coincides with their weak nuclear targeting properties observed in the N- and C-terminal deletion experiments (see the diffused staining in Figure 2Ai and k; Figure 2Be). It could be possible that the active nuclear targeting conformation of MGMT may contain exposed N- and C-termini but a buried central region of the protein contains the basic regions.

In this respect, the nuclear localization of human MGMT may require the holoprotein, since the regions of the protein involved are distantly located; at the N- and C-termini for its nuclear targeting (Figure 2), at codons 101–107 (**KLLKVVK**) for its proper structure and at codons 124–128 (**PKAAR**) for its nuclear retention. The overall experimental data also suggest that neither the nuclear targeting nor the nuclear retention property alone would be sufficient for the proper nuclear localization of human MGMT. It is interesting that the SV40 NLS oligopeptide **PKKKRK**V, which is extremely effective in the nuclear localization of proteins (Figure 1), may have overlapping NLS and DNA binding properties (Fanning and Knippers, 1992; LaCasse and Lefebvre, 1995), i.e. nuclear targeting and retention.

Implications in carcinogenesis related to alkylating agents

The salient finding in this study is the demonstration that nuclear retention of human MGMT is related to its binding to the DNA in the nucleus. Being in the close vicinity of the DNA (its target substrate), the repair protein can provide an efficient mechanism to prevent the accumulation of the mutagenic 6RG lesions in the DNA and, therefore, protect cells from the mutagenic effects of alkylating agents. This agrees with the lack of protection towards alkylating agents observed in mammalian cells expressing the cytosolic bacterial MGMTs (Dumenco *et al.*, 1989; Pegg, 1990) and ER–MGMT (Ishibashi *et al.*, 1994b) which are inadequate in repairing the DNA compartmentalized in the mammalian cell nucleus. In addition, various mutations are now known to affect the repair activities of human MGMT, e.g. **C**145 (active site, Chueh *et al.*, 1992), **E**172 (Rafferty *et al.*, 1994), **Y**114 and **R**128 (Kanugula *et al.*, 1995) and **K**101 and **K**104 (in the **KLLKVVK** region). If an effective repair activity for 6RG lesions in DNA is crucial in the cellular defence against the mutagenic effects of environmental alkylating agents, such as *N*-nitroso compounds, screening of individuals for these mutations in the human MGMT gene may be a useful biomarker for the assessment of risk from alkylating agents.

Materials and methods

Oligonucleotides

Oligonucleotides used were (their exact sequences are available on request): (i) cloning of full-length human MGMT in pGEX-2T; pGXHM1 and pGXHM2; (ii) site-specific mutagenesis; HM101L, HM104L, HM107L, HM125L, HM128L; (iii) N-terminal deletion; PZ-HM, HJ22 for –N180, HJ23 for –N150, HJ24 for –N120, HJ25 for –N90, HJ26 for –N60 and HJ27 for –N30; (iv) C-terminal deletion; pGXHM1, PZ-170 for –C42, for PZ-60 for –C148 and PZ-30 for –C176; (v) basic region peptides; HJ25 and HJ19 for P1, HJ24 and HJ20 for P2 and HJ25 and HJ20 for P1+P2; (vi) as the GST fusion construct from pGEX-2T; GX-1 and GX-2; (vii) KLLKVVK deletion; pGXHM1 and HMΔ101, pGXHM2 and HMΔ101X; (viii) NLS; GST–NLS1 and GST–NLS2 for GST–NLS, β-gal–NLS1 and β-gal–NLS2 for NLS–β-gal; (ix) linkers; PZ-1 and PZ-2; and (x) sequencing primers; CW51 and CW52 for MGMT, pGX-F and pGX-R for GSTMGMT and M13F for SK.

Reagents and antibodies

Lowry reagent (all protein measurements), DAPI and streptolysin-O are from Sigma Co. (USA); ECL reagents and [γ-^{32}P]ATP from Amersham

(UK); sequencing kit from USB chemical (USA); PCR kits from Perkin Elmer (USA); lipofectamine and Opti-MEM I from GIBCO-BRL (USA); mouse Mab.β-gal from Promega (USA); mouse Mab.GST from Santa Cruz Biotechnology Inc. (USA). Secondary and human MGMT antibodies were described in Ayi *et al.* (1992).

Cell culture and transfections

MRC5.SV40 cells (a gift from Dr Peter Karran, ICRF, UK) were grown on coverslips in 6-well plates (Ayi *et al.*, 1992). Confluent cells (70–90%) were rinsed twice with Opti-MEM I before transfection with DNA [1 ml/well, prepared by pre-incubating DNA (3 μg), lipofectamine (2 μl) and Oopti-MEM I (100 μl) at room temperature for 45 min before dilution with Opti-MEM I to 1 ml]. After 6 h, the wells were washed and replaced with fresh medium. Transfected cells were analysed for the expressed proteins at 24 and 48 h by immunostaining. Stable cell lines were obtained by selection of the above transfected cells (after 24 h) with Geneticin (200 μg/ml) for several days until single-cell colonies were formed, which were isolated and re-screened by immunostaining.

Preparation of total cell extracts, immunochemistry and activity assay

Procedures for cell extracts preparation (except the use of aprotinin and leupeptin, 1 μg/ml each), SDS–PAGE, immunostaining (except DNA was stained by 1.5 μg/ml of DAPI during the final wash) and Western analyses were described previously (Ayi *et al.*, 1992, 1994). 5′-^{32}P-labelled CGC6MGCG substrate was used as described previously (Liem *et al.*, 1993). MGMT contents in total cell extracts (25 μg) were quantified by Western analysis using Mab.2G1 (Ayi *et al.*, 1994). Between 150 and 300 μg of total cell extracts were used for assay.

In vitro nuclear protein transport assays

The procedure for selective permeabilization of the plasma membrane of cultured cells by streptolysin-O was adapted from Adam *et al.* (1990) for digitonin (see text for criteria). MRC5.SV40 cells were seeded on poly-L-lysine-treated (30 min) coverslips. Cells were rinsed with pre-cooled transport buffer [TB, 20 mM HEPES pH 7.3, 110 mM KOAc, 5 mM NaOAc, 2 mM $Mg(OAc)_2$, 0.5 mM EGTA, 2 mM dithiothreitol (DTT), 2 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml each of aprotinin and leupeptin, 1 mM ATP, 0.2 mM GTP, 10 mM creatine phosphate and 100 μg/ml creatine kinase] and followed by incubation with TB at 4°C for 10 min. Streptolysin-O (10 U/ml from 20 000 U/ml in water) in TB (at 4°C) was added and the cells were left for 10 min (4°C). After washing twice with cold TB, fresh TB at room temperature was added to the coverslips which were left at 37°C for 10 min (puncturing of the plasma membranes by the bound streptolysin-O). These permeabilized cells were washed immediately with cold TB to remove the streptolysin-O and the diffusible cytosolic components. Various transport assay mixtures [TAM, 500 μl containing rMGMT (1 μg) or 200 μg of total cell extract proteins in TB] were added onto the coverslips followed by incubation at 37°C for 30 min in a CO_2 incubator. Each TAM was prepared by diluting the total cell lysates, obtained as above using TB containing 0.3 M NaCl but no energy-regenerating system, with TB. After incubation, the cells were rinsed three times with cold TB and processed for immunostaining.

Cloning

Mammalian vector containing MGMT–β-gal and GST–MGMT fusion constructs. A polylinker carrying *Hin*dIII, *Bgl*II, *Sma*I and *Kpn*I restriction sites (constructed by the PZ-1 and PZ-2 duplex) was used to replace the *Hin*dIII–*Kpn*I fragment, which contains the *Escherichia coli TrpS* and *GPT* genes fused to the 5′ end of the β-galactosidase gene, of the eukaryotic expression vector pCHL (Pharmacia, Sweden). The human MGMT insert was obtained by PCR using the primers PZHM and pGXHM1, and a human MGMT cDNA [identical to that used in Tano *et al.* (1990), as a *Bam*HI–*Eco*RI fragment in SK]. After *Bam*HI and *Kpn*I digestion, the PCR product was ligated into *Bgl*II and *Kpn*I sites of the modified pCHL vector which expresses the construct as an N-terminal fusion protein of β-galactosidase. For cloning GST–MGMT cDNA, the human cDNA in pGEX-2T was amplified with 5′-phosphorylated primers GX-1 and GX-2. The PCR product was ligated into the blunted *Eco*RI site of mammalian vector pXJ41neo. Ligated vectors were transformed into DH5αF, and positive clones were confirmed by DNA sequencing (Sanger *et al.*, 1977).

Control GST–NLS and NLS–β-gal constructs. The control SV40 NLS (P**KKKRK**V) was constructed by using two sets of linker oligonucleotide duplexes. (i) GST–NLS1 and GST–NLS2 duplex, for the GST–NLS construct, was ligated into the 3′ end of GST in pGEX-2T and cloned into pXJ41neo as described above. (ii) β-gal–NLS1 and β-gal–NLS2 duplex, for the NLS–β-gal construct, was cloned into the 5′ end of β-galactosidase cDNA enclosed in the modified pCHL vector as described above.

N- and C-termini deletion mutants of MGMT as β-galactosidase fusion proteins. N-terminal deletion mutants –N30, –N60, –N90, –N120, –N150 and N–180 were amplified from human GST–MGMT cDNA by PCR using HJ27, 26, 25, 24, 23 and 22 as forward primers, and PZ-HM as reverse primer. The C-terminal deletion mutants –C42, –C148 and –C176 were amplified using forward primer pGXMH1 (containing a *Bam*HI site) and reverse primers PZ170, 60 and 30 (containing a *Kpn*I site). The PCR products were digested with *Bam*HI and *Kpn*I and cloned into the *Bgl*II and *Kpn*I sites of the modified pCHL vector as described above.

GST fusion protein containing the basic region of MGMT. P1 [codons 91–120, containing the (99)-LW**K**LL**K**VV**K** region], P2 [codons 121–150, containing the (124)-P**K**AA**R**AV and active site P**C**HRV regions] and P1+P2 (codons 91–150) inserts were obtained by PCR using primers HJ19 and HJ25 for P1, HJ20 and HJ24 for P2 and HJ20 and HJ25 for P1+P2. The products and pGEX-2T vector were digested with *Bam*HI and *Eco*RI before ligation. The fusion inserts were obtained as described above and cloned into pXJ41neo.

Point mutations in the basic regions of MGMT. The 5′-phosphorylated oligonucleotides, HM101L for **K**101**L**, HM104L for **K**104**L**, HM107L for **K**107**L**, HM125L for **K**125**L** and HM128L for **R**128**L**, were annealed to a single-stranded M13 template containing the human MGMT cDNA (see above). An oligonucleotide-directed mutagenesis kit from Amersham (UK) was used for the subsequent steps. Mutant plasmids were transformed into JM109 and identified by DNA sequencing. The mutant cDNAs were released from SK either by *Bam*HI and *Eco*RI digestion for cloning into pGEX-2T and expressed as GST fusion proteins (and purified using glutathione–Sepharose), or by *Eco*RI followed by Klenow (blunt) and *Bam*HI for cloning into *Bgl*II and *Kpn*I (blunted) sites in pXJ41 neo as described above.

Codon 101–107 deletion mutant of full-length MGMT. This deletion mutant was created by PCR using two sets of primers: pGXHM1 and 5′-phosphorylated HMΔ101; and 5′-phosphorylated HMΔ101X and pGXHM2. After digestion with *Eco*RI (for the front fragment) and *Bam*HI (back), the two fragments were cloned simultaneously into SK. The deletion insert was identified by sequencing, and cloned into pXJ41neo as described above after digestion with *Bam*HI and *Kpn*I (blunt).

DNase I digestion and gel mobility assay of MGMT–DNA complex

Paraformaldehyde-fixed HM37 cells, a stable MRC5.SV40 cell line carrying the transfected wild-type human MGMT cDNA in pXJ41neo, were digested with 25 μg/ml of DNase I (Sigma, USA) in phosphate-buffered saline (PBS) containing 5 mM Mg^{2+} at room temperature for 2 h. After washing with PBS (3×5 min), the cells were stained with MGMT.Pab and DAPI for DNA (Ayi *et al.*, 1992). ^{32}P-Labelled duplex was obtained by priming the 71mer, 5′-GTAAAACGACGGCCAGT-GAATTCAGATCT–$(N)_{13}$–GGATCCCTCGAGGTCGTGACTGGGAA-AAC with antisense primer 5′-GTTTTCCCAGTCACGAC (kit from Amersham, UK). Reaction mixtures [containing annealed 0.01 OD at 260 nm of each oligonucleotide in 15 μl of water, 5 μg of bovine serum albumin (BSA), 10 μl of labelling buffer, 5 μl of [α-^{32}P]dCTP, 2 μl of Klenow] were left at 37°C for 30 min and chased with cold dCTP (1 μl of 0.1 M) for 30 min. After phenol–$CHCl_3$ extraction, the probe was desalted on an NAP5 column (using TE) and used directly. Glutathione–Sepharose-purified proteins, GST, wild-type GST–MGMT, GST–**K**101**L**, GST–**K**104**L** and GST–**K**125**L** (220 ng in 2 μl) were first incubated with 1 μl (0.1 μg) of poly(dI·dC) in Dignam buffer (16 μl containing 0.1% NP-40, 0.1 mg of BSA/ml, 1 mM DTT) at 4°C for 10 min. The probe (100 000 c.p.m./μl) in DNA-binding buffer (3 μl, containing 20 mM HEPES–KOH pH 7.5, 20% glycerol, 20 mM KCl, 2 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT) was added to the protein mixture. After incubation for 10 min (at 25°C), gel loading buffers (5 μl of 0.1% BPB and XB in 50% glycerol) were added to the mixtures which were analysed on a 5% non-denaturing PAGE (containing 2.5% glycerol) using TGE buffers (50 mM Tris, 0.38 M glycine, 2 mM EDTA, pH 8.0) at 120 V for 5 h at 4°C. The dried gels were autoradiographed.

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