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Cloning and expression of cDNA for rat O^6-methylguanine-DNA methyltransferase

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ABSTRACT

cDNA for O^6-methylguanine-DNA methyltransferase was isolated by screening rat liver cDNA libraries, using as a probe the human cDNA sequence for methyltransferase. The rat cDNA encodes a protein with 209 amino acid residues. The predicted amino acid sequence of the rat methyltransferase exhibits considerable homology with those of the human, yeast and bacterial enzymes, especially around putative methyl acceptor sites. When the cDNA was placed under control of the *lac* promoter and expressed in methyltransferase-deficient *Escherichia coli* (*ada*$^-$, *ogt*$^-$) cells, a characteristic methyltransferase protein was produced. The rat DNA methyltransferase thus expressed could complement the biological defects of the *E.coli* cell caused by lack of its own DNA methyltransferases; e.g. increased sensitivity to alkylating agents in terms of both cell death and mutation induction.

INTRODUCTION

O^6-Methylguanine can pair with thymine during DNA replication, leading to G·C to A·T transition mutation in the DNA (1–4). Such mutations are often present in DNA sequences of organisms exposed to relatively low doses of alkylating agents (5). Mammary tumors in rats, as induced by injection of MNU carry this type of mutation in the Ha-ras-1 gene (6). To counteract such effects, organisms possess mechanisms to repair modified bases (7). O^6-Methylguanine-DNA methyltransferase catalyzes transfer of methyl groups from O^6-methylguanine and other alkylated moieties of the DNA to its own molecule, thereby repairing the lesions in a single-step reaction (8). The structure and function of methyltransferase have been studied extensively in *Escherichia coli* (9–12). Disruption of the genes coding for methyltransferases leads to an increased susceptibility of cells to alkylating agents, with respect to both mutation induction and cell killing (13).

The methyltransferase activity is present in various mammalian tissues (10). Attempts have been made to clone genes for mammalian methyltransferase enzymes and, as a first step, cDNA for the human O^6-methylguanine-DNA methyltransferase was isolated (14–16). Taking advantage of the overproduction of the enzyme in HeLa cells harboring the cDNA, human methyltransferase was purified to physical homogeneity and its properties elucidated (17). The purified enzyme catalyzed transfer of methyl groups from O^6-methylguanine as well as from a minor methylated base, O^4-methylthymine, of methylated DNA to the enzyme molecule.

Cellular contents of methyltransferase vary with tissues, and its was pointed out that more tumors are formed in tissues with less methyltransferase activity, in alkylnitrosourea-administered animals (2, 18). Some human tumor-derived cell lines are hypersensitive to alkylating agents, and these Mer$^-$ cells have little or no methyltransferase activity (19, 20). It was suspected that this methyltransferase deficiency may be related to occurrence of tumors in certain cases, though it is uncertain whether this deficiency is the cause or the result of tumor formation. To resolve this problem, appropriate animal models with various levels of methyltransferase activity are required.

We describe here the isolation, expression and sequence of rat methyltransferase cDNA. Recently, the cDNA sequence for rat methyltransferase was presented also by Potter *et al.* (25). The amino acid sequence of the rat enzyme, as predicted from its nucleotide sequence, exhibits a wide homology with those of the other mammalian, yeast and bacterial enzymes.

MATERIALS AND METHODS

Chemicals, cell lines, bacteria and libraries

[^{3}H]MNU (17.7 Ci/mmol), [^{32}P]dCTP (3000 Ci/mmol) and ^{14}C-labeled protein molecular size markers were purchased from Amersham Japan. Taq DNA polymerase was from Promega. Oligonucleotides were synthesized using an Applied Biosystems model 381A DNA synthesizer. 25×blot wash is 0.3 M Na_2HPO_4-0.2 M NaH_2PO_4-0.034 M sodium pyrophosphate-1.25% SDS. 2×SSCB is a 1:1 mixture of 4×SSC and 2×blot wash. *E.coli* strain KT233 (*Δada Δogt*) was as described (13). Rat cell line RLB-N2 and Rat-2 are our laboratory stocks. Rat liver cDNA libraries were obtained from Clontech (Cat. No. RL1001b and RL1020a).

Cloning, sequencing and PCR methods

To obtain the cDNA for rat O^6-methylguanine-DNA methyltransferase, we have screened a λgt11 cDNA library made from rat liver (Clontech, RL1001b) using an *Eco*T14I-*Pma*CI fragment derived from pUC9MGMT (human MGMT cDNA) as a probe

(14). Six positive plaques were selected, and the phage DNAs prepared were digested with *Eco*RI and subcloned into pUC18 vector. They carried almost the same size (about 700 bp) of cDNA fragments. One of the plasmid clones, termed pRM2, was used for sequence determination. The nucleotide sequence was determined by GENESIS 2000 automatic DNA sequencer (Dupont).

Since nucleotide sequence analysis revealed that clone pRM2 apparently lacks the 5'-region, including an initiation codon, another library RL1020a was screened. Oligonucleotide P1, CTCTGTGG*CTGCAG*GTTCGT, corresponding to bases 297−316 within the coding region (for the transcribed strand) of rat methyltransferase cDNA, which contains a *Pst*I site (shown by italic letters), was used as a primer. Either GCTGGGTAGTCCCCACCTTT (gt10F) or CTTATGAGTATTTCTTCCAGGGTA (gt10R) primer, derived from vector (λgt10) sequence, was used as an opposite primer. Template DNA was prepared by boiling a mixture of 5 μl of phage library (corresponding to 3.2×10^8 p.f.u.) and 20 μl of water for 10 min. Based on the number of independent clones (1.4×10^6) of the library, it was estimated that the mixture contains about 230 copies each of phage clones.

PCR was performed in 50 μl of reaction mixture containing 10 mM Tris·Cl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100, 0.2 mM each of dATP, dGTP, dCTP and TTP, 0.25 μM of P1 primer, 0.25 μM of one of the opposite primers and template DNA. Each cycle was set for 1 min of denaturation step at 94°C, 30 sec of annealing step at 60°C and 1 min of elongation step at 75°C, and 30 cycles of reactions were carried out in a DNA thermal cycler (Takara Shuzo Co., Ltd.).

The PCR products were digested with *Pst*I and *Eco*RI and cloned into pUC18. Among fragments amplified, the longest one, which was expected to contain the upstream region was digested with *Pst*I and *Eco*RI and cloned into pUC18, yielding plasmid pRM71. It had 99 additional nucleotides upstream of the pRM2 sequence. By joining the *Pst*I/*Eco*RI fragment of pRM71 to the *Pst*I/*Eco*RI fragment of pRM2, a composite plasmid pRM712 which contains the complete coding sequence for rat methyltransferase was obtained.

Expression of cDNA in *E.coli*

E.coli strain KT233, which lacks both *ogt* and *ada* genes was transformed with plasmid pRM712. One ml of culture was added to 10 ml of LB broth containing 50 μg/ml of ampicillin and 1 mM IPTG, and the mixture was incubated at 37°C. After 3 h of incubation, the cells were harvested and used to prepare a crude extract by sonication or to determine the mutation frequency and survival. Protein concentrations were determined by Protein Assay (Bio-Rad), using bovine serum albumin as a standard. Methyltransferase activity was determined according to Nakabeppu *et al.* (12). Fluorography was performed using ENLIGHTNING (NEN). Mutation frequency and survival of cells were determined according to Takano *et al.* (13).

RESULTS

Nucleotide sequence of the cDNA for rat methyltransferase

Figure 1 shows the nucleotide sequence of the cDNA for rat methyltransferase. For more than 96% of the region of the cDNA fragment, sequences of both strands were determined using an automatic DNA sequencer. There was one open reading frame, corresponding to a protein composed of 209 amino acid residues. The molecular weight of the protein, calculated from the predicted amino acid sequence was 22,247, a value similar to one estimated from gel filtration of the enzyme (21). In the cDNA sequence, 73 nucleotides of the 5'-untranslated sequence and 99 nucleotides of the 3'-untranslated sequence, prior to polyA sequence, were also present. The amino acid sequence and 3'-untranslated sequence were very similar to those of human.

PolyA$^+$ RNAs were prepared from Rat-2, a cell line derived from rat fibroblasts, and RLB-N2, derived from rat liver, and subjected to Northern blot analysis using the rat methyltransferase cDNA as a probe. One major 1.0 kb RNA band was detected (data not shown), thus the cloned cDNA should contain most of the mature mRNA sequence. Southern blot analysis with Rat-2 genomic DNA showed four bands corresponding to sizes of 7.8, 6.2, 6.0 and 4.4 kbp by *Eco*RI digestion (data not shown). Because the rat methyltransferase cDNA sequence contains no *Eco*RI site, the gene for rat methyltransferase consists of at least 4 exons.

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  1 CTCAGTTCCAGAGCCTGTGTACCGTTCCAGGTTACGGAGTTTCTGCACAG
 51 TTTGCAAACTGGAACTTGGCAGA

 74 ATG GCT GAG ATC TGC AAA ATG AAA TAC ACG GTG TTG GAC
  1 Met Ala Glu Ile Cys Lys Met Lys Tyr Thr Val Leu Asp

113 AGC CCT TTG GGG AAG ATA GAG CTG TCC GGC TGT GAG CGA
 14 Ser Pro Leu Gly Lys Ile Glu Leu Ser Gly Cys Glu Arg

152 GGC CTG CAT GGG ATA CGA TTT CTC AGT GGG AAG ACC CCA
 27 Gly Leu His Gly Ile Arg Phe Leu Ser Gly Lys Thr Pro

191 AAC ACT GAC CCC ACA GAG GCT CCA GCC TGT CCT GAG GTG
 40 Asn Thr Asp Pro Thr Glu Ala Pro Ala Cys Pro Glu Val

230 CTC GGT GGG CCA GAG GGA GTG CCA GAG CCC CTG GTG CAG
 53 Leu Gly Gly Pro Glu Gly Val Pro Glu Pro Leu Val Gln

269 TGC ACA GCC TGG CTG GAA GCC TAT TTC CAC GAA CCT GCA
 66 Cys Thr Ala Trp Leu Glu Ala Tyr Phe His Glu Pro Ala

308 GCC ACA GAG GGG CTT CCC TTG CCT GCT CTC CAT CAC CCT
 79 Ala Thr Glu Gly Leu Pro Leu Pro Ala Leu His His Pro

347 GTG TTC CAG CAA GAT TCA TTC ACC AGA CAG GTG TTA TGG
 92 Val Phe Gln Gln Asp Ser Phe Thr Arg Gln Val Leu Trp

386 AAG CTG CTG AAG GTT GTG AAA TTC GGA GAA ATG GTT TCT
105 Lys Leu Leu Lys Val Val Lys Phe Gly Glu Met Val Ser

425 TAC CAG CAA TTA GCA GCC CTG GCA GGC AAC CCC AAA GCG
118 Tyr Gln Gln Leu Ala Ala Leu Ala Gly Asn Pro Lys Ala

464 GCT CGT GCA GTA GGA GGA GCG ATG AGG AGC AAT CCA GTC
131 Ala Arg Ala Val Gly Gly Ala Met Arg Ser Asn Pro Val

503 CCC ATC CTC ATC CCC TGC CAC AGG GTG ATT CGC AGT GAC
144 Pro Ile Leu Ile Pro Cys His Arg Val Ile Arg Ser Asp

542 GGT GCC ATT GGC AAT TAC TCT GGA GGA GGA CAG ACT GTG
157 Gly Ala Ile Gly Asn Tyr Ser Gly Gly Gly Gln Thr Val

581 AAA GAG TGG CTT CTG GCC CAT GAG GGC ATC CCA ACT GGA
170 Lys Glu Trp Leu Leu Ala His Glu Gly Ile Pro Thr Gly

620 CAG CCG GCC TCC AAA GGC TTG GGT CTG ATT GGG AGC TGG
183 Gln Pro Ala Ser Lys Gly Leu Gly Leu Ile Gly Ser Trp

659 CTC AAG CCA TCC TTC GAG TCT TCC AGC CCA AAG CCG TCT
196 Leu Lys Pro Ser Phe Glu Ser Ser Ser Pro Lys Pro Ser

698 GGC TGA
209 Gly END

704 AATTGAGTAACCGTTTGAATGACACATAGATGTAATGCGGTGTTGGAAGC
754 GGATGTGTGGTGGGTACCACTATATTAAAAGAGCTGCATGTGTCCTGGGA
804 AAAAAAAAA
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Figure 1. Nucleotide sequence of cDNA for rat O^6-methylguanine-DNA methyltransferase and the deduced amino acid sequence. First nucleotide of the cDNA is numbered 1. Putative polyadenylation signal sequence, ATTAAA, is underlined.

Expression of rat methyltransferase cDNA in *E.coli*

To confirm that the cloned cDNA encodes O^6-methylguanine-DNA methyltransferase, the cDNA was expressed in *E.coli* cells. The *lac* promoter was placed in front of the presumptive coding sequence, and the construct was introduced into methyltransferase-deficient *E.coli* strain KT233 (*Δada Δogt*). A significant level of methyltransferase activity was found in an extract prepared from cells possessing such a construct, pRM312, which carries 8 bases shorter cDNA than does pRM712 (Fig. 2A). When the crude extract prepared from cells carrying pRM712 was incubated with ^{3}H-labeled MNU-treated DNA and the reaction product was analyzed by SDS-PAGE following fluorography, a distinct band appeared (Fig. 2B). From patterns on the fluorogram, the size of the enzyme produced in *E.coli* was estimated to be about 25 kDa, the same value as that for the enzyme present in rat RLB-N2 cells.

Biological activities exerted by expression of the cloned cDNA

We determined the survival of KT233 (*Δada Δogt*) cells, with or without the rat cDNA after MNNG treatment. As shown in Fig. 3A, cells carrying the rat methyltransferase cDNA and cells with the *E.coli ada* gene were equally resistant to MNNG. Thus, the rat methyltransferase functions to repair lethal lesions on the *E.coli* chromosome as efficiently as does the *E.coli* Ada protein. It should be noted that in these experiments a mutant form of *ada* gene was used to produce Ada (Ala^{69}) protein that can repair O^6-methylguanine and O^4-methylthymime but lacks a transcriptional activator activity (22).

We next examined the effects of rat methyltransferase on the MNNG-induced mutagenesis. Bacterial strains, with or without cDNA, were exposed to relatively low levels of MNNG. The mutation frequency of KT233 cells with the rat cDNA was as low as that of cells producing the *E.coli* Ada protein (Fig. 3B).

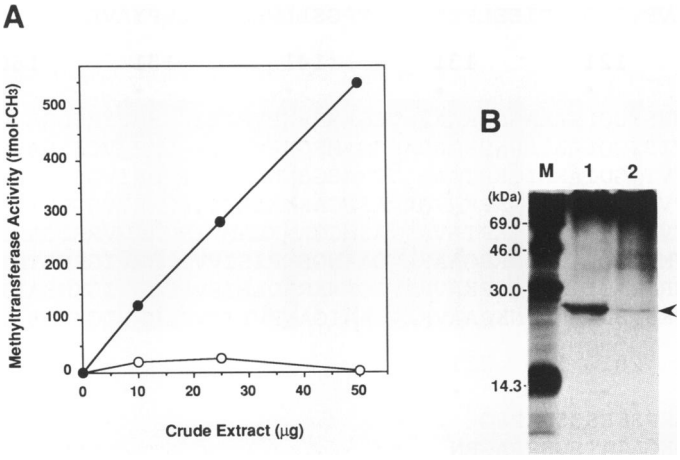

Figure 2. Expression of rat methyltransferase cDNA in *E. coli*. (A) Methyltransferase activities expressed in *E. coli*. Substrate DNA treated with ^{3}H-labeled MNU (17.7 Ci/mmol) was incubated with various amounts of crude extracts of *E. coli* KT233 cells carrying either one of the following plasmids at 37°C for 15 min and radioactivity transferred to the protein was counted. —●—, pRM312 which carries a rat methyltransferase cDNA; —○—, pUC18 (vector). (B) Fluorography of the methyl-accepted proteins. Crude extracts were incubated with DNA treated with ^{3}H-labeled MNU (17.7 Ci/mmol), and analyzed by 15% SDS-PAGE followed by fluorography. Position of the methyl-accepted methyltransferase protein is indicated by an arrow. Lane 1, 50 μg protein of extract of *E. coli* KT233 harboring pRM712; lane 2, 100 μg protein of extract of RLB-N2 cells; lane M, molecular size markers. Sizes of marker proteins are given on the left.

It is evident, therefore, that most of the DNA lesions responsible for induction of mutations can be efficiently repaired by the rat cDNA product.

DISCUSSION

Using a cDNA for human O^6-methylguanine-DNA methyltransferase as a probe (14), we isolated the cDNA for rat methyltransferase which contains an open reading frame encoding a protein composed of 209 amino acid residues. Sequence **CAGAATGG** around the putative translation initiation site matches sequence **CACCATGG** that is the Kozak-sequence (23) and is complementary to part of rat 18S ribosomal RNA (24). Nucleotide sequence analysis revealed that the predicted amino acid sequence has a high homology with the sequence for the characterized human enzyme (17). Within the coding region, there is a single 3-nucleotide deletion in the rat sequence as

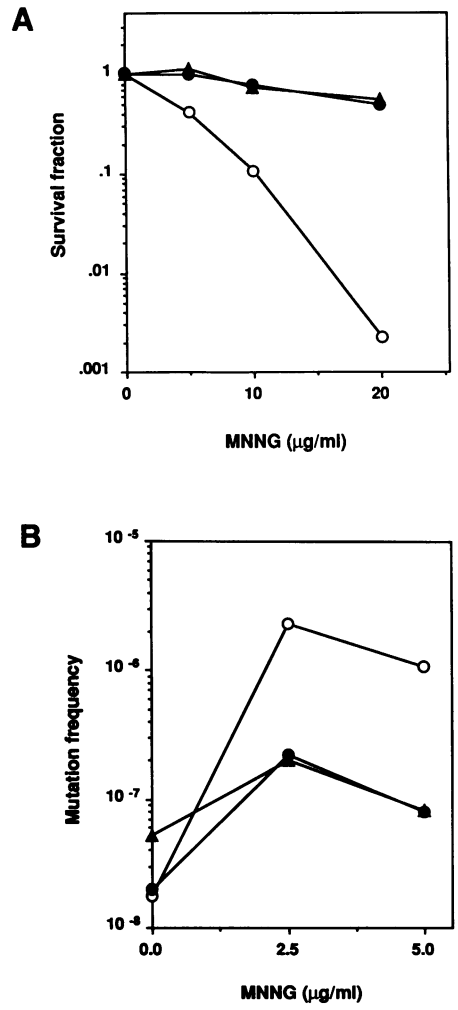

Figure 3. Complementation of biological defects of a methyltransferase-deficient *E. coli* mutant. *E. coli* KT233 (*ada*$^-$, *ogt*$^-$) cells carrying one of the following plasmids were treated with various concentrations of MNNG at 37°C for 10 min and the survivals and mutation frequencies were determined. —●—, pRM312; —○—, pUC18; —▲—, pKT101 which carries a mutant form of *ada* gene that produces Ala^{69} protein possessing only O^6-methylguanine-DNA methyltransferase activity (22). (A) Survivals of cells after treatment with MNNG. (B) Mutation frequency.

compared with the human one and, in reverse, a 12-nucleotide and a 3-nucleotide deletions are found in the human sequence. It was shown, moreover, that a protein carrying the corresponding methyltransferase activity was formed when the cDNA was expressed in methyltransferase-deficient *E.coli* cells. Potter *et al.* (25) cloned cDNA for methyltransferase from a rat cDNA library. Their reported sequence was essentially the same as ours, except for a single base change in the coding region. This alteration locates at the third position of the codon for the 190th amino acid, Gly, thus the actual amino acid sequences coded by the two clones are the same.

In addition to the factor of size, high homology can be observed with nucleotide sequences of the coding regions for the rat and human enzymes. Proportion of identical bases in the coding regions between rat and human is 72.3%. This homology can be extended to the 3'-flanking region and 59.8% of the bases are identical. The putative polyadenylation signal ATTAAA was found at almost the same position (data not shown). As the consequence of the high homology in nucleotide sequence, the amino acid sequences of the enzymes are well conserved, and the proportion of identical amino acids is 67.9%.

When the amino acid sequences of the mammalian, yeast and bacterial methyltransferases are aligned, a distinct feature emerges (Fig. 4). There are highly conserved sequences among the 8 species of methyltransferase proteins; in the C-terminal half of proteins, 22 completely conserved amino acids are distributed within the 87-amino acid stretch. Among them, most notable is the five consecutive sequence, **PCHRV**, which locates near the C-terminus. With the *E.coli* Ada methyltransferase, the cysteine residue within the sequence is the methyl acceptor site (8, 11), a finding concordant with that in the human enzyme (26, 27). The role of the conserved amino acid sequence has been examined with regard to the systematic changes of amino acids by site-directed mutagenesis of the cloned human cDNA (Chueh *et al.*, in preparation). They show that each of the 5 amino acids contributes to exert the enzyme activity and to stabilize the protein. There is another conserved sequence, **RAV**, located at position 132−134 for the rat sequence. Sequences around **RAV** are also conserved and the **RAV** sequence may be regarded as a core of sequence composed of 10 amino acids, **PXA(A/V) RAV(G/A)XA**. These conserved sequences may possibly constitute the active site of methyltransferase enzyme. Within the highly conserved sequences presented here, only yeast enzyme possesses substituted amino acids somewhere. It is known that the yeast methyltransferase is more thermolabile than are the others (28), and these amino acid substitutions might account for it.

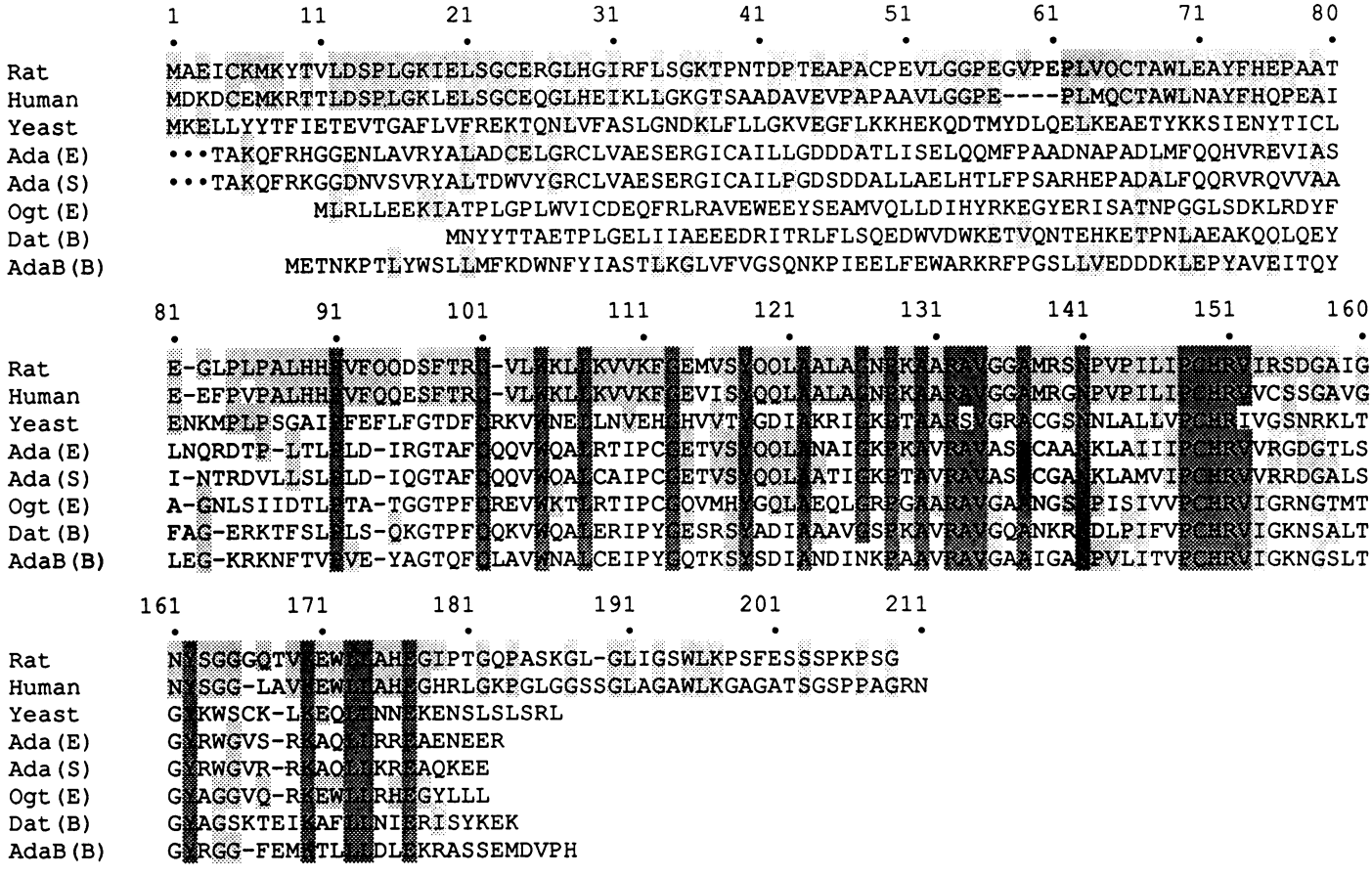

Figure 4. Comparison of the amino acid sequences for O^6-methylguanine-DNA methyltransferases from various sources. Amino acids are numbered according to rat methyltransferase. Ada(E) and Ada(S) show amino acid sequences of the C-terminal halves of the proteins. Amino acids the same as the rat methyltransferase are lightly shadowed. The dark shadowed areas mean that more than 7 of 8 methyltransferases have the same amino acids. Spaces, shown by −, were introduced to obtain maximum matches. E, *Escherichia coli*; S, *Salmonella typhimurium*; B, *Bacillus subtilis*. Data were from: rat (this paper), human (14), yeast (32), Ada(E) (12), Ada(S) (33), Ogt(E) (34), Dat(B) (35), AdaB(B) (36).

Rat methyltransferase activity increased following treatment with alkylating agents (29). In contrast to the case of adaptive response in bacteria, which is specifically induced by alkylating agents (30), the increase was also caused by UV, γ-ray and 2-acetylaminofluorene (25, 31, Fukuhara *et al.*, in preparation). It is likely that the mechanism functioning in mammalian cells may differ from that found in bacterial cells.

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Note added

Rahden-Staron,I. and Laval,F. also reported cDNA cloning of the rat O^6-methylguanine-DNA methyltransferase (*Biochem. Biophys. Res. Commun.*, 1991, **177**, 597–602).