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Promoter Structure Which Affects on the Expression of Yeast *MGMT* Gene

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Abstract : The present study was performed to analyze the molecular mechanism which dictates the transcription regulation of the O^6-methylguanine-DNA methyltransferase (*MGMT*) gene in *Saccharomyces cerevisiae*. Previously we identified one possible upstream repressing sequence (URS) in *MGMT* promoter by promoter deletion and competition analysis. In this paper we report another regulatory element (UAS: upstream activating sequence, -213 to -136) which affects the transcription activity of *MGMT* promoter. Gel mobility shift assay and Southwestern blot analysis using UAS probe showed several specific proteins which were able to bind to this sequence.

Key words : *MGMT* promoter, UAS (upstream activating sequence), yeast.

O^6-methylguanine-DNA methyltransferase (MGMT: DNA-O^6-methylguanine: protein-L-cysteine S-methyltransferase, EC 2.1.1.63) catalyzes the transfer of alkyl groups from O^6-methylguanine and other methylated moieties of DNA to the protein itself (Koike *et al.*, 1990). The methyltransferase seems to be widely distributed in a variety of organisms. This enzyme activity has been found in mammals, fish, insects, yeast and several species of bacteria. The methyltransferase activity, however, is dependent on the type of tissue and varies with species and developmental stage (Pegg, 1990; Fritz *et al.*, 1991). This strongly suggests that a number of factors, including tissue, cell type, age and cell cycle, appear to be involved in the regulation of expression level. However, it had been believed that *Saccharomyces cerevisiae* did not have an O^6-methylguanine-DNA methyltransferase because of its high recombination repair activity until Sassanfar and Samson (1990) identified and characterized biochemically this enzyme activity. The molecular weight of this enzyme is approximately 25 kD and it accepts a methyl group from O^6-methylguanine to form S-methylcysteine. A cloned yeast *MGMT* cDNA was also able to suppress alkylation-induced killing and mutation in *E. coli ada ogt* mutants (Xiao *et al.*, 1991).

This work sets the goal of understanding transcription regulation of the yeast methyltransferase gene. As a first step to achieve this goal, the full length of yeast *MGMT* genomic DNA was isolated by plaque hybridization using the probe DNA produced by a polymerase chain reaction (PCR) (Joo *et al.*, 1995). Using Northern blotting we previously showed that the cellular level of *MGMT* gene transcripts is regulated under cell growth but not under the treatments of alkylating agents, such as methylmethanesulfonate (MMS) or N-methyl-N'-nitroso-N-nitosoguanidine (MNNG). In this report we analyzed the structure of the *MGMT* gene promoter with a series of the deletion mutants along the 5'-flanking region of the gene by newly developed *in vitro* yeast transcription system.

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Materials and Methods

Bacterial, yeast strains and plasmids

E. coli DH5α (*supE44, ΔlacU169 (φ80lacZ M15), hsdR17A, recA1, gyrA96 thi1 rel1*) was used for transformation and DNA manipulation. The plasmid pBluescript SK(−) was used for subcloning. The protease-deficient *S. cerevisiae* BJ2168 (*leu2, trp1, ura3-52, prb1-1122, pep4-3, prc1-407, MATa*) was used for the preparation of whole cell extract.

Chemicals and reagents

All culture supplies were purchased from Difco Laboratories. Radioactive materials were obtained from Amersham Life Science. All restriction enzymes and DNA modifying enzymes were purchase from Promega or Boehringer Mannheim Biochemicals and used as recommended by the manufacturers. All other chemicals

used in this study were purchased from Sigma Chemicals Co. or Aldrich Company Inc.

In vitro transcription reaction

Transcription reactions were performed in a final volume of 20 μl essentially as described (Schultz *et al.*, 1992). Fourteen μl of reaction mixture was made up of 10 μl of S-100 (77 μg of protein) and 25 mM KCl, 10 mM magnesium acetate, 50 mM potassium glutamate, 5 mM EGTA, 0.05 mM EDTA, 2.5 mM DTT, 10% glycerol, and 5 U of RNasin (Promega, Madison, WI, USA). After 5 min pre-incubation of reaction mixture with nonspecific DNA (20 μg/ml pGEM3EX), an additional 15 min incubation was carried out followed in the presence of specific template. Nucleotides were added and the transcription reaction was stopped after 40 min by adding 20 volumes of stop buffer (10 mM Tris-HCl, pH 7.5, 1% SDS, 1 mM EDTA). After extraction with phenol/chloroform and chloroform, the products were precipitated with radioactive labeled probe and 10 μg *E. coli* tRNA. The hybridization reaction was performed at 70℃.

S1 nuclease protection assay

The single strand S1 probe detecting transcripts from the yeast *MGMT* gene was prepared by labeling the *Eco*RI-*Hin*dIII fragment of *MGMT* gene (spanning from −222 to +145 of the *MGMT* gene and extra 46 base of polylinker sequence from pBluescript SK(−)) by separating in non-denaturing 6% acrylamide gel. About 50 μg total RNA was mixed with probe DNA (5×10^5 cpm) and hybridized in 1× hybridization buffer (80% formamide, 40 mM PIPES, pH 6.4, 1 mM EDTA, 0.4 M NaCl) at 42℃ for 16 h. After digestion of this mixture using 500 U/ml S1 nuclease at 37℃ for 30 min, the size of protected DNA was analyzed on 6% polyacrylamide gel containing 8 M urea. This procedure yielded 191 base fragment for correctly initiated transcripts.

Deletion of 5'-flanking region

To obtain a nested set of deletions in the 5'-flanking region of the *MGMT* gene, Exonuclease III was employed. The recombinant plasmid pF0.88 (10 μg) was digested with *Sac*I and *Sph*I. The digested DNA was dissolved in *Exo*III buffer (66 mM Tris-HCl, pH 8.0, 0.66 mM $MgCl_2$) and 500 U of *Exo*III (Promega) were added at 30℃. Each 5 μl sample was taken from the mixture at 15 sec intervals and put into a microcentrifuge tube containing 7.5 μl of S1 buffer (300 mM potassium acetate, pH 4.6, 250 mM NaCl, 60 U of S1 nuclease) on ice. After all samples were taken, they were incubated at 37℃ for 30 min and flooded with S1 stop buffer (300 mM Tris-base, 50 mM EDTA). After heating at 70℃ for 10 min, samples from each time point were analyzed on 1% agarose gel to determine the extent of digestion. Each sample was ligated and transformed into *E. coli* DH5α cells.

Gel mobility shift assay

The gel retardation assay was carried out with minor modification of the method of Staudt *et al.* (1988). Binding reactions were similar to those described above for the *in vitro* transcription reaction. All reactions contained 4 μg of S-100 extract, 2 μg of poly (dA-dT), and 1× binding buffer (25 mM KCl, 10 mM magnesium acetate, 50 mM potassium glutamate, 5 mM EGTA, 0.05 mM EDTA, 2.5 mM DTT, 10% glycerol). After 10 min preincubation with or without competitor DNA, the mixtures were further incubated for 30 min with 1 ng of labeled probe. S-100 extract was prepared from BJ2168 cells as described before. All reactions were performed at room temperature. Reaction products were electrophoresed on 4% non-denaturing polyacrylamide gel, and the gel was dried and autoradiographed.

Southwestern blot analysis

Southwestern blotting was performed following the procedure of Silva *et al.* (1987) with slight modification. Yeast S-100 extracts were fractionated on 10% SDS-PAGE with a 4% stacking gel. After electrophoresis, the gels were incubated for 3×1 h periods in renaturation buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 2 mM EDTA, 0.1 mM DTT and 4 M urea) with gentle agitation and the proteins were transferred onto nitrocellulose filters by electroblotting. To prevent non-specific binding, the filters were blocked for 2 h by a gentle shaking in blocking renaturation buffer (50 mM Hepes-KOH, pH 7.9, 65 mM KCl, 2 mM EDTA, 2 mM 2-mercaptoethanol, 0.1% Nonidet P-40, and 5% non-fat dry milk) and then incubated at room temperature with the binding solution used for the gel mobility shift assay containing non-specific competitor. The binding buffer was replaced with the same buffer containing 2×10^6 cpm of DNA probe per ml with or without 100-fold excess specific competitor. Binding was carried out for 3 h at room temperature in a sealed plastic bag with gentle agitation. The filters were briefly rinsed three times with binding buffer. DNA-protein complexes were visualized by autoradiography.

Results and Discussion

Most gene regulation is the result of specific protein-

DNA interactions. In addition, transcription initiation is certainly one of the major steps of gene regulation and for this reason much effort has been directed toward the elucidation of its molecular mechanisms. In this study, the sequences involved in the regulation of transcription initiation of *MGMT* gene was analyzed using artificially altered genes in an *in vitro* transcription system. pF0.88 plasmid, which had MGMT promoter sequences ranged from −742 (*Spe*I site) to +145 (*Hin*dIII site), was used as wild type template for *MGMT* promoter analysis. S1 nuclease protection assay utilized a single stranded RNA probe which was developed using the T7 promoter on pF0.88. This RNA contained extra 46-base linker DNA after the *Hin*dIII site of the yeast *MGMT* gene and this sequence allowed us to distinguish *in vitro* transcripts from endogenous transcripts of the *MGMT* gene (Fig. 1).

In vivo or transfection analysis is particularly well suited for studies concerning the *cis*-acting elements of transcription and can only provide marginal information about the *trans*-acting elements involved. But *in vitro* transcription extracts capable of supporting specific transcription initiation provides a powerful tool for the study both of the *cis*- and *trans*-acting elements. The main advantage of the method used in this study are its easiness and the fact that specific initiation activity can be reliably measured early in the procedure. In fact, in the case of the *MGMT* gene, the results of primer extension, RNase protection assay and S1 mapping studies showed that the yeast *MGMT* gene produced a major transcript initiating with a cytosine residue (Joo *et al.*, 1995). Thus, the *in vitro* transcription system in this study supported active and specific transcription initiation from *MGMT* promoter.

Regulation of gene expression is the result of specific protein-DNA interactions. In this study, cell-free extracts from yeast were used for analyzing the structure of methyltransferase gene promoter. To examine the functional significance of the upstream sequence of the methyltransferase gene, nested deletion constructs were made using Exonuclease III. The nucleotide sequences of the 5'-end points of each deletion mutant were determined. Transcription activity analyzed by S1 mapping showed that the deletion to −213 dramatically enhanced the transcription activity to about five fold more than wild type promoter and that the further deletion to −136 reduced the enhanced activity to a level a little bit lower than wild type (Joo *et al.*, unpublished results). A putative GCR1 binding motif (Baker, 1991; Sinclair *et al.*, 1994) resides at −166 to −162. The GCR1 binds CT/ATCC sequences found in many glycolytic genes, and has little UAS (upstream activating sequence) activity by itself. However, the RAP1-GCR1 interaction can boost RAP1 (repressor/activator protein 1)-mediated expression almost 10-fold (Buchman *et al.*, 1988). Deletion to −136 resulted in decreased *MGMT* expression, and this suggested that the region between −213 to −136 possibly contains a UAS.

In order to find out whether any binding protein affects the above sequence, competition analysis was employed. The plasmid D-213, which has a deletion to −213 in the 5' flanking region of *MGMT* promoter, was used for an *in vitro* transcription reaction in the

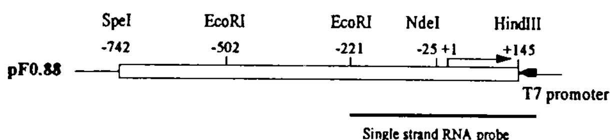

Fig. 1. Diagram of single stranded RNA probe which was used for S1 nuclease protection assay on pF0.88. This probe RNA was made using T7 promoter on pF0.88 plasmid which was cut by *Eco*RI. This RNA contained extra 46-base linker DNA after the *Hin*dIII site of yeast *MGMT* gene and this sequence allowed to distinguish in vitro transcripts from endogenous transcripts of *MGMT* gene.

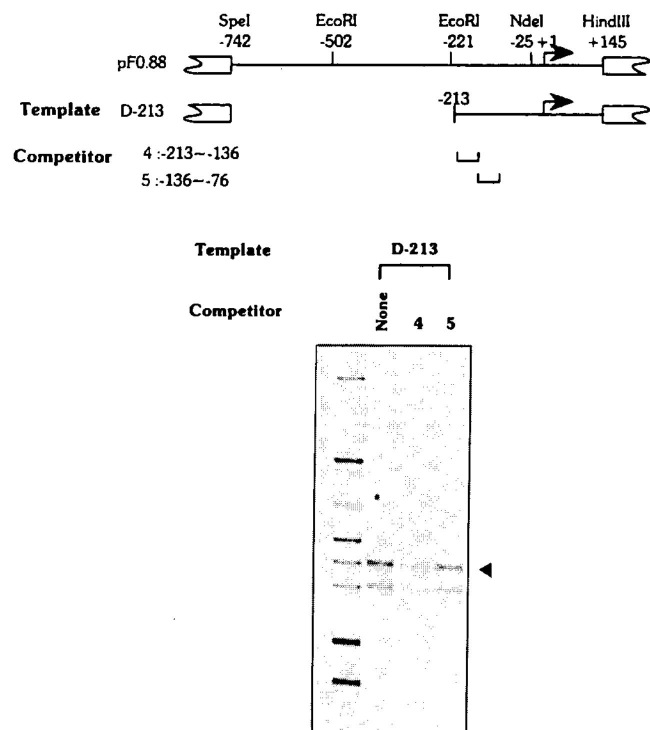

Fig. 2. *In vitro* transcription activity assay using D-213 template in the presence of competitor DNAs. The template DNA was *in vitro* transcribed and analyzed by S1 mapping. Competitors were synthesized by PCR and preincubated with S-100 extracts. The template and competitor DNAs for *in vitro* transcription were diagrammed at the top of the figure.

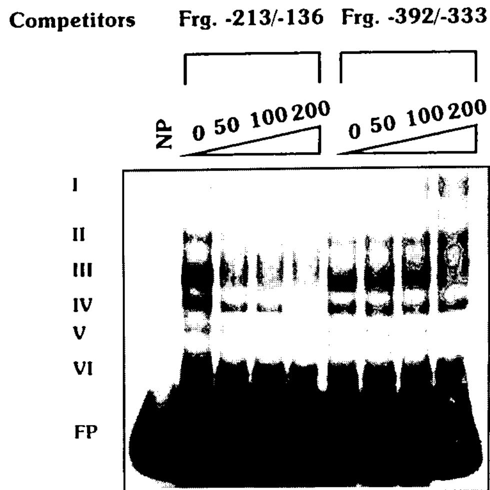

Fig. 3. Gel mobility shift assay using UAS probe. Each probe and competitors was obtained by PCR. Each assay contained 2 μg of poly (dA-dT). 4 μg of crude yeast cell extracts (S-100). and 1 ng of labeled UAS probe in 1x buffer. The UAS probe was made by integrating [α-^{32}P]-dCTP into the DNA fragments by PCR. Fold excess of competitor DNA used in this assay is indicated above the figure. FP : free probe; I. II. III. IV. V and VI : protein-DNA complexes.

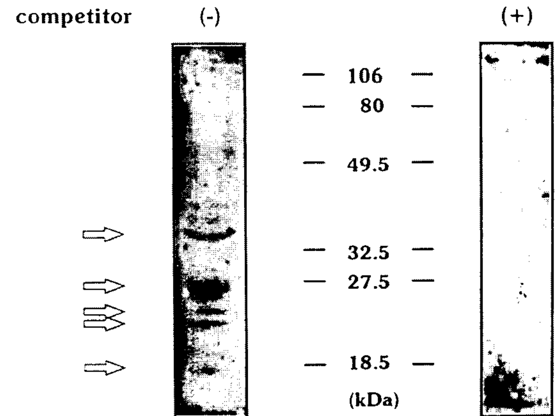

Fig. 4. Southwestern blot analysis with UAS probe. Yeast S-100 extracts were separated by SDS-polyacrylamide (12%) gel electrophoresis and transferred to a nitrocelluloce membrane. The membrane-bound proteins were renatured and incubated with the UAS probe in the presence or absence of specific competitor.

presence competitor DNAs (Fig. 2). Each competitor DNA was prepared by a polymerase chain reaction (PCR) and was added to the *in vitro* transcription reaction mixture to a 100-fold molar excess over the template DNA. When the fragment spanning from −213 to −136. in which a UAS element seemed to be located. was used as competitor. the transcription activity was significantly reduced. The fragment of −136/−73. however. did not show any competition against D-213 template for transcription. This suggested that a UAS element may be located in the region spanning from −213 to −136. and also that this sequence can bind to some specific proteins.

In an attempt to identify a specific DNA-binding protein for the UAS region. a double-strand DNA fragment containing the −213/−136 sequence of *MGMT* promoter was labeled. mixed with yeast whole-cell extracts, and subjected to gel electrophoresis. Six major bands were identified through this band shift assay (Fig. 3). Three of them (complex I. II. and V) significantly faded out with the addition of 50~200-fold molar excess of itself. The specificity of the formation of these protein-DNA complexes formation was corroborated by addition of the same molar concentration of unrelated DNA fragment (−392/−333). These results strongly suggested that these three bands are specific for UAS binding. After separating the S-100 extract on SDS-PAGE and transferring it to a nitrocellulose membrane, radio-labeled −213/−136 probe DNA was spread onto the membrane in the presence or absence of a specific competitor DNA. This Southwestern blotting experiment allowed us to identify five proteins which can bind to the −213/−136 fragment (Fig. 4). At this time we do not know how many proteins participated in the activation of the *MGMT* gene by the UAS element. and how these proteins worked together in order to act as activator. The only thing which could be suggested from our results is that at least five proteins were employed in the transcription activation. The real mechanism for the activation of the *MGMT* gene remains to be further studied.

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