



Title	Gelsolin gene silencing involving unusual hypersensitivities to dimethylsulfate and KMnO <sub>4</sub> in vivo footprinting on its promoter region
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GELSOLIN GENE SILENCING INVOLVING UNUSUAL HYPERSENSITIVITIES TO  
DMS AND  $\text{KMnO}_4$  IN VIVO FOOTPRINTING ON ITS PROMOTER REGION

short title: gene silencing of gelsolin in bladder cancer

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*Key words: gelsolin, gene silencing, dimethylsulfate hypersensitivity, bladder cancer,  
in vivo footprinting*

*Abbreviations* : HDAC, histone deacetylase; TSA, trichostatin A; CHIP, chromatin immunoprecipitation; DIG, digoxigenin; DMS, dimethylsulfate; LM-PCR, ligation mediated polymerase chain reaction

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We previously reported that gelsolin gene expression is reduced in various tumors. In an effort to gain further insights into the mechanism of gelsolin downregulation in tumors, we examined the *in vivo* properties of the gelsolin promoter in urinary bladder cancer cell lines. Neither mutation nor hypermethylation were responsible for gene silencing at the promoter. After exposure to trichostatin A (TSA), a histone deacetylase inhibitor, gelsolin promoter activity was markedly enhanced in the cancer cells not in cells derived from normal tissue. Chromatin immunoprecipitation (ChIP) assays revealed that both histones H3 and H4 were hypoacetylated in the promoter region of the cancer cells, and the accumulation of acetylated histones were detected by TSA treatment. *In vivo* footprinting analysis revealed the presence of dimethylsulfate (DMS) hypersensitive site in the untranslated region around nucleotide -35 only in the cancer cells but not in cells derived from normal tissue, and analysis of  $\text{KMnO}_4$  reactive nucleotides showed that the stem loop structure could be formed *in vivo* of the cancer cells. This novel stem loop structure may play a part in regulating the transcription of the gelsolin gene in the cancer cells. These results suggest that nucleosome accessibility through histone deacetylation and structural changes (DMS hypersensitivity and stem loop structure) in the promoter region form the basis of the mechanism leading to the silencing of gelsolin gene in human bladder cancer.

## (INTRODUCTION)

Cancers occur due to the accumulation of abnormalities in gene function. While many of them are genetic (mutation and deletion), epigenetically mediated changes in gene expression have been increasingly investigated. Hypermethylation of CpG islands, that are GC-rich regions usually located at the 5'-end of genes, results in alterations in the histone acetylation and methylation status around gene promoter regions. Aberrant cytosine methylation and concomitant histone modifications play important roles in the repression of over half of the tumor suppressor genes during malignant transformation<sup>1, 2</sup>.

Previously, we reported that the expression of gelsolin, an actin-regulatory protein, is frequently silenced in various cancers including stomach, colon, urinary bladder and lung, and that gelsolin functions as a tumor-suppressor<sup>3-7</sup>. These findings were also demonstrated in breast and prostate cancers<sup>8, 9</sup>. On the other hand, a subset of non-small cell lung cancers is characterized by high expression levels of gelsolin correlated with lymphatic invasion<sup>10</sup>, and there was a gradual increase in gelsolin with an increase in tumor grade and stage in uroepithelial carcinoma<sup>11</sup>. Gelsolin induced invasion of epithelial cells as well as colon adenocarcinoma cells<sup>12</sup>. It is therefore possible that a decrease in expression during a particular stage is followed by an increase during another stage. To understand roles of gelsolin in the whole process of tumorigenesis, mechanisms for both down- and up-regulations should be analyzed. In breast cancers, gelsolin genes were not mutated, however epigenetic changes in the chromatin structure were responsible for the deficiency<sup>13</sup>. Histone deacetylase (HDAC) inhibitors, such as trichostatin A (TSA) and apicidin, selectively induced the expression of gelsolin<sup>14, 15</sup>. Recent studies have suggested a new mechanism for cell-specific gene regulation linking nuclear architecture, chromatin

structure and functional organization of DNA sequences<sup>16</sup>.

In an effort to determine the molecular basis of gelsolin downregulation in urinary bladder cancer and to better understand the carcinogenic process, we investigated the gelsolin promoter through the use of methylation-specific sequencing, luciferase assays and chromatin immunoprecipitation (ChIP) assays. Furthermore, we employed in vivo footprinting using the ligation mediated polymerase chain reaction (LM-PCR) to examine the DNA elements in the cis-acting region of the gelsolin promoter in an attempt to speculate the chromatin structure in this region and to identify potential DNA-protein interactions in cancer cells.

## MATERIALS AND METHODS

### *Cell cultures and chemicals*

Three human bladder cancer cell lines: DAB-1 (established by Dr. T. Demura, Hokkaido University), KU-7 (provided by Dr. M. Tachibana, Keio University, Tokyo) and UMUC-2 (provided by Dr. B. Grossman, University of Michigan) were maintained in DMEM containing 10% FCS and 0.05% glutamine in a 37°C incubator. Cell lines derived from normal uroepithelial tissues (SV-HUC-1, HMKU-1) were cultured in F-12 medium and in Keratinocyte-SFM (Gibco-BRL, Gaithersburg, MD), respectively. SV-HUC-1 is an immortalizing cell line, which was obtained after infection of human uroepithelial cell (HUC) with simian virus 40 (SV40) (CRL-9520 ATCC Manassas, VA). HMKU-1 was isolated from a young patient with ectopic ureter by the method of Truschel et al<sup>17, 18</sup> and is mortal and very difficult to cultivate under some conditions such as TSA treatment. Exponentially growing cells were used in all experiments. For TSA treatment, cell lines were grown and plated into culture dishes, switched to medium containing TSA (Wako, Tokyo, Japan) at 0, 10, 100 and 500 ng/ml for 24h, or 500 ng/ml for 8, 16 and 24h.

### *Mutational analysis*

The gelsolin promoter from both normal (SV-HUC-1) and cancer (DAB-1) cells was amplified using PCR with the following primer pairs: 5'-TGCCCAAAGTTACAGGACT-3' (-599 to -581; S1), 5'-CTCCCCTACCTGCTGCTG-3' (+93 to +76; AS1). The PCR cycle consisted of a denaturation step at 94°C for 1 min, annealing at 57°C for 1 min, followed by elongation at 72°C for 1 min. The PCR products were sequenced using the following 4 primers: S1, 5'-GGCCCCAGCCCAGCTCAGGA-3' (-287 to -268; S2), AS1,

5'-CTGAAGGGGCCTAGGGTTCG-3' (-173 to -195; AS2).

### *Genomic Bisulfite Sequencing*

Genomic DNA isolated from SV-HUC-1, DAB-1 or UMUC-2 cells was subjected to sodium bisulfite treatment using a CpGenomic modification Kit (Oncor Inc., Gaithersburg, MD) according to the manufacturer's protocol. With this procedure, only methylated CG was conserved as CG while the other C was converted to T. The following specific primers were constructed:

5'-ATAGGATTAGTC/TGATGTTTGGG-3' (-588 to -567; MetS1),  
5'-GC/TGGTGTTAGTTGAGTATTG-3' (-336 to -317; MetS2),  
5'-CCAAACCCTCTACTTTCCCCTAA-3' (-173 to -195; MetAS1),  
5'-CCTCCCCTACCTACTAC-3' (+94 to +78; MetAS2). The modified DNA were amplified by nested PCR and sequenced.

### *Transient transfection and luciferase assay*

The cells were seeded and pBgl II gelsolin promoter luciferase reporter (Fujita H. unpublished data) was transfected with FuGene6 reagent (Roche Diagnostics, Mannheim, Germany) in triplicate. After incubation for 48h, luciferin substrate (Promega Corp., Madison, WI) was added, and the luminescence was measured using the Picagene kit (Toyooki, Tokyo, Japan) and a Berthold Lumat LB9501 luminometer. Cells were co-transfected with pSV beta as an internal control and the luciferase activity was normalized. In an effort to determine whether the acetylation of histones alters promoter activity, transfected cells were incubated with various doses of TSA as described above. Relative luciferase activities were standardized relative to an untreated control.

### *Western blot analysis*

Purified gelsolin protein was produced with human cytoplasmic gelsolin expression plasmid pET-11a (Novagen, San Diego, CA) in *E. coli* as described previously<sup>19</sup>. The extracts (5 micro g total proteins) of cells derived normal tissue (SV-HUC-1) and cancer (DAB-1, KU-7) cells were subjected to 10% SDS-PAGE with the purified gelsolin standards (100, 50, 25 ng). Next these cells were also treated with TSA and 20 micro g total proteins were extracted, subjected to SDS-PAGE, and then transferred to a nitrocellulose membrane. The blots were incubated with anti-gelsolin mouse monoclonal antibody and alkaline phosphatase-conjugated secondary antibody (Sigma Chemical Co., St. Louis, MO). The expression of gelsolin was detected by ECL chemiluminescence (Amersham Pharmacia Biotech, UK). The protein was quantified with LAS-1000 mini imager (FUJIFILM, Tokyo, Japan). Cell extracts from HMKU-1, which strongly express the gelsolin protein, were used as a positive control.

### *Chromatin Immunoprecipitation (ChIP) Assay*

We performed ChIP assays using SV-HUC-1, DAB-1, KU-7 and UMUC-2 cells treated with or without TSA using an anti-acetylated histone H3 (#06-599), H4 (#06-866) ChIP Assay Kit (Upstate Biotechnology, Upstate, NY) according to the manufacturer's protocol. All the cells were treated with formaldehyde to covalently cross-link histones to DNA, and chromatin was isolated and immunoprecipitated with anti-acetylated histone antibodies as described. Five-fold serial dilutions of DNA from cancer cells and cells derived normal tissue (with or without TSA) were blotted to Hybond N Nylon membrane (Amersham). The digoxigenin (DIG) moiety was

introduced into a nucleic acid probe using a gelsolin promoter sequence (nucleotides -229 to -5). The DIG-labeled probe was hybridized to the membrane and detected using a DIG-specific antibody conjugated to alkaline phosphatase. Following treatment with DIG / CDP-*Star* reagents (Roche Diagnostics, Mannheim, Germany), the chemiluminescent signals were detected by exposure of the blots to X-ray film and were quantified using NIH imaging. The ratios of values of cancer cells to those of SV-HUC-1 were calculated and were shown as mean and standard deviation (SD).

#### *In vivo dimethylsulfate (DMS) footprinting*

The LM-PCR for *in vivo* DMS footprinting was performed as described previously<sup>20, 21</sup>. Briefly, bladder cancer (DAB-1, KU-7) cells and cells derived from normal uroepithelium (SV-HUC-1, HMKU-1) were treated with DMS (Nacalai Tesque, Kyoto, Japan) (0.05, 0.1%) *in vivo*. Genomic DNA was extracted, purified and then cleaved with 1 M piperidine (Nacalai Tesque) at 90°C for 30 min<sup>22</sup>. Control naked genomic DNA from DAB-1 cells was treated with DMS *in vitro*. The nucleotide sequences of the gelsolin primers used to evaluate the overall promoter region between -235 and +42 from the transcriptional start site were as follows: for the sense strand: 5'-CCCATCTGAGCTCTTTGGCC-3' (-303 to -284; L1), GCCCTGGGTCTCCTCCCC (-264 to -247; L2), and GGGTCTCCTCCCCGACCCTAGGCC (-259 to -236; L3), for the antisense strand: 5'-CCTCCCCTACCTGCTGC-3' (+94 to +78; U1), CACTGGAGACAGCGGCTGCTGG (+72 to +51; U2), and GAGACAGCGGCTGCTGGGTCCAGCTGC (+67 to +41; U3). The nucleotide sequences of the primers used to evaluate the proximal promoter regions between -128 to +22 from the transcriptional start site were as follows: the gelsolin primers used to analyze the sense strand were: 5'-GGGAAAGCAGAGGGCTTGG-3' (-191 to

-173; L4; 5'-CCTTCCTGCGAGGTGAAGCGAGG (-165 to -143; L5), and 5'-CCTGCGAGGTGAAGCGAGGGGTCCCC (-161 to -136; L6). Primers L1 or L4 and U1 were used for first-strand synthesis, whereas primers L2 or L5 and U2 were used for PCR amplification. Primers L3 or L6 and U3 were labeled at their 5' ends with [<sup>32</sup>P] ATP and used for the detection of the ladder. Samples were analyzed on a 6% polyacrylamide sequencing gel. Nucleotide positions of cleavage signals derived from LM-PCR products were determined from the guanine ladders of control naked DNA and our revised sequence information (Fig. 1).

#### *In vivo KMnO<sub>4</sub> treatment*

Potassium permanganate (KMnO<sub>4</sub>) modification of whole cells was performed as described previously<sup>23</sup>. Naked genomic DNA from human epidermoid carcinoma KB cells was treated *in vitro* with 200 micro l of 10 mM KMnO<sub>4</sub> for 1 or 3 min, and the reaction was stopped by the addition of 25 micro l (final concentration of 1.5M) of beta-mercaptoethanol. The modified bases were subsequently cleaved with piperidine and assayed by LM-PCR which was performed as described above. Many different kinds of the stem and loop structures were proposed from computer analysis (Genetyx-Secst Software Development, Tokyo, Japan) depending on the regional selections and parameters used.

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## RESULTS

### *Mutation and methylation*

Some molecular mechanisms resulting in the silencing of the gelsolin gene in cancer cells can include gene mutation, DNA methylation and alteration of chromatin structure. We first carried out direct sequencing of the gelsolin promoter (from -580 to +75, relative to the transcription start site) using the PCR products derived from human bladder cancer cells (DAB-1, KU-7, and UMUC-2) and the cells derived from normal uroepithelium (SV-HUC-1). Although it was found that the gelsolin promoter of cancer and normal tissue-derived cells were identical in sequence, this sequence differed from that previously reported at two nucleotide positions: -153 G (from K which shows G or T in the original report<sup>24</sup>) and -170 T (our addition) from the transcription start site (Fig. 1).

The gelsolin gene promoter sequence is rich in G/C and contains many CpG sites. Forty CpG sites were found in the region 600 bp upstream from the transcription start site with 25 of these being clustered between -229 to -5. Bisulfite sequencing analysis using cancer (UMUC-2, DAB-1) cells and the cells derived from normal uroepithelium (SV-HUC-1) showed no difference in the methylation state of these cells, and all CpG sites in the proximal promoter region were unmethylated (data not shown).

### *Effect of trichostatin A on promoter activity and protein expression*

In an effort to determine the effects of TSA on the gelsolin promoter, bladder cancer cell lines (DAB-1 and KU-7) were transiently transfected with a reporter luciferase gene under the control of the core promoter construct (pBgl II). As shown in Fig. 2A, elevated promoter activities in cancer cells were observed in a dose- and

time-dependent manner. The production of gelsolin protein in the DAB-1 and KU-7 cancer cells under untreated conditions was much lower than that in SV-HUC-1 cells derived from normal epithelium, which was consistent with our previous findings<sup>4</sup>. The expression of gelsolin was 35.5 ng / 5 micro g lysate in SV-HUC-1, 3.8 ng in DAB-1 cells, and 4.0 ng in KU-7 cells (Fig. 2B). The positive effects of TSA were also detected in relation to the expression of gelsolin protein in DAB-1 and KU-7 cells, but not in SV-HUC-1 cells. The induction of gelsolin protein after treatment with TSA was observed in the both bladder cancer cell lines, and not in SV-HUC-1 cells, in a dose- and time-dependent manner (the time-dependency data are not shown).

#### *Histone deacetylation*

Since TSA seems to have many effects on cells besides inhibiting histone deacetylase, we analyzed the fluctuation in histone acetylation levels at the gelsolin promoter using the ChIP assay. DNA fragments were purified from immunoprecipitated material using antibodies against acetylated histones H3 and H4, respectively. These fragments were dot blotted and hybridized with the probe representing the gelsolin promoter (from -229 to -5). Without TSA treatment, a significantly lower level of hybridization signals was observed in the cancer cells than in cells derived from normal tissues. The relative ratios  $\pm$  standard deviation of acetylated histone H3 in bladder cancers DAB-1, KU-7 and UMUC-2 compared to SV-HUC-1 were  $0.17\pm 0.03$ ,  $0.11\pm 0.04$  and  $0.09\pm 0.06$ , respectively. Similar results were obtained when acetylated histone H4 was examined, where the relative ratios were  $0.15\pm 0.01$ ,  $0.28\pm 0.20$  and  $0.20\pm 0.05$ , respectively. TSA treatment resulted in a remarkable accumulation of acetylated histone H3 in cancer cells, with the increase relative to SV-HUC-1 cells derived from normal tissues being 8.9-fold (DAB-1),

3.8-fold (KU-7) and 6.6-fold (UMUC-2). Similar trends were observed with acetylated histone H4: 8.3-fold (DAB-1), 1.9-fold (KU-7) and 3.3-fold (UMUC-2) (Fig. 3). These results suggest that the increase in gelsolin promoter activity and consequent induction of gelsolin protein synthesis by TSA are mediated by a histone deacetylase inhibitor, and that the histones associated with the gelsolin promoter in bladder cancer cells are hypoacetylated.

#### *In vivo DMS footprinting*

In vivo DMS footprinting was performed in an effort to identify the presence of sequence-specific transcription factors bound to the gelsolin gene promoter, and to relate these findings to changes in the histone acetylation status. We have characterized the gelsolin promoter region from -235 to +1 using three primer sets (L1 - L3, L4 - L6 and U1 - U3). In the case of the distal region of the promoter, we conducted in vivo footprinting using the L1 - L3 primer set, as shown in Fig. 4A. We observed specific decreases in the intensity, reflecting protection from DMS methylation, in the guanines of the GC-box sequence (-109 to -104) on the sense strand in SV-HUC-1 cells derived from normal tissue, but not in cancer DAB-1 and KU-7 cells. Similarly, protection of guanines at -138 to -130 on the sense strand was detected in SV-HUC-1 cells but not in KU-7 cancer cells. Although signals around -135 are weak, especially 0.05 % DMS treated DAB-1 specimen, signal intensities of -130 guanine on the sense strand of both cancer cells are almost equal to that of “G ladder” derived from naked genomic DNA, whereas that of SV-HUC cells is clearly low (protected). Sometimes a hypersensitive signal is observed in adjacent position to the protected region<sup>25, 26</sup>, and we detected such a type of hypersensitive signal at -126 cytosine in SV-HUC cells but not in DAB-1 and KU-1 cells. Thus, a low signal

intensities (protection) in guanine cluster (-135 to -138), protection of -130 guanine and appearance of hypersensitive signal at -126 cytosine were observed in only SV-HUC-1 cells. From these three aspects, we suspected that some transcriptional factors bind to the region -130 to -138 in SV-HUC-1 cells but not in DAB-1 and KU-1 cells.

Reverse GC-boxes were clustered in the proximal region of the gelsolin gene promoter. To examine these reverse GC-boxes, *in vivo* footprinting using the L4 - L6 primer set was performed as shown in Fig. 4B. The guanine residue of the GC-box at -50 to -45 on the sense strand, especially the last guanine at -50, showed canonical hypersensitivity to DMS in DAB-1 and KU-7 cancer cells but not in SV-HUC-1 cells. The other guanines exhibited weak protection in KU-7 cancer cells. This protection of four guanines from the 5'-end of the GGGCGG consensus sequence and the hypersensitivity of the last guanine are representative features of a transcription factor Sp1 binding to the GC-box<sup>18</sup>. These findings suggest that Sp1 binds at -50 to -45 on the sense strand of gelsolin promoter region in human bladder cancer cells.

The most striking point of the *in vivo* footprinting in cancer cells was the appearance of a superhypersensitive guanine at -35. This guanine residue is located on a spacer portion between the GC-box and the reverse GC-box from -41 to -27. A similar superhypersensitive signal was also observed for the guanine at -36 on the antisense strand of cancer cells (data not shown). Besides the superhypersensitive signal in the palindromic sequence, hypersensitive regions were also detected from -113 to -108 in cancer cells, but only in the -113 and -112 region in cells derived from normal tissues. Similar hypersensitive sites were observed in regions from -25 to -21, -58, -79 and -85 to -86, especially in cancer cells. The novel superhypersensitive site and the conspicuous cluster of hypersensitive sites in the gelsolin promoter imply that

the proximal region in cancer cells might form an extraordinary structure such as a stem and loop structure.

#### *In vivo KMnO<sub>4</sub> analysis*

In an effort to obtain evidence supporting the presence of a stem and loop structure at the gelsolin gene promoter, we carried out LM-PCR analysis using KMnO<sub>4</sub>, which reacts with unpaired bases, especially thymine, and to a lesser extent cytosine *in vivo*. The relative intensity of piperidine cleavage signals was much higher in cancer cells, suggesting that cancer cells could contain this putative stem and loop structure (Fig. 5A). Cleavage signals (-39 to -36) near the -35 guanine of the DMS superhypersensitive site, and those nucleotides, consisting mainly of thymines and cytosines, at -131 to -127, -122, -121, -115, -114, -95 to -86, -61, -60, -56, -55, -42, -41 and -2 were detected. With the results of the DMS hypersensitive sites, we suggest that the core promoter region of the gelsolin gene tends to form a stem and loop structure, especially in cancer cells. Taken together, we proposed a model in gelsolin promoter region of human urinary bladder cancer cell lines as shown in Fig. 5B. In this model, KMnO<sub>4</sub> reactive nucleotides located in a loop portion or at a boundary between a stem and a loop. In addition, other cleavage signals (around -90 and around -120) observed in Fig. 5A implied that much more complex secondary structure might be formed in the gelsolin gene promoter, especially in human bladder cancer cells.

## DISCUSSION

Investigations over the past few years have revealed the importance of chromosomal infrastructure in gene control, determining both active and repressed states. DNA methylation and histone acetylation are major processes that can determine chromatin structure, which in turn are primary regulators of gene transcription<sup>27</sup>. Reduced expression of several tumor suppressor gene families, such as the cadherin family<sup>28</sup> and cyclin-dependent kinase inhibitors<sup>29</sup>, have been implicated in the development of human urinary bladder cancers. We previously reported that over 70% of human urinary bladder carcinomas reduced or lost the production of gelsolin protein<sup>4</sup>. Our present analysis revealed that the downregulation is not due to mutation or hypermethylation of the gelsolin gene, but instead due to histone deacetylation. We directly demonstrated the hypo-acetylated state of the gelsolin promoter in bladder cancer cell lines. Several models have been proposed that describe the relationship between histone acetylation and transcription. The morphology of the bladder carcinoma cell line T24 dramatically changed, and actin stress fibers reappeared during treatment with TSA<sup>14</sup>. It was shown that incubation with TSA resulted in a dramatic upregulation of gelsolin RNA and protein levels that preceded apoptotic death of breast cancer cell lines<sup>30</sup>.

Recently, Dong et al. demonstrated the presence of a cis-element defined as a 27-bp sequence located approximately -135 bp upstream of the transcription start site that mediated gelsolin gene silencing. Gel shift and supershift assays, together with Southwestern blotting analysis, indicated that activating transcription factor-1 (ATF-1) and a 400 kDa protein may possess cancer cell-specific DNA-binding activity to the 27-bp gelsolin cis-element<sup>31</sup>. However, in bladder cancer,

we did not observe any protection at position -134 to -129 of the TGGACG region of the sense strand. Instead, our present in vivo footprinting analysis identified a broad and strong hypersensitive region of guanine bases within the gelsolin promoter in urinary bladder cancer cells but not in immortalized uroepithelial cells at nucleotides -113 to -108, sense strand (Fig. 4A, 4B), suggesting structural alteration of gelsolin promoter DNA in bladder cancer cells. Breast cancer and bladder cancer show ATF-1 binding and hypersensitivity, respectively, and this fact suggests that these regions are common targets of carcinogenic process. In addition, we showed that the promoter region of the gelsolin gene in human urinary bladder cancer cells contained a superhypersensitive site on a guanine located at -35 of sense strand. We also observed cancer cells specific binding of Sp 1 on the GC-box at -45 to -50 (Fig. 4B). In our model of Fig. 5B showing superhypersensitive guanine at -35, Sp 1 is the most feasible candidate as a single strand binding transcription factor<sup>32</sup>.

The increase in DMS accessibility can result from alterations in the local DNA topology induced by protein binding or unknown mechanisms in bladder cancer cells. Some factors can bind to the gelsolin promoter in the vicinity of the upstream superhypersensitive guanine. The fact that analysis using  $\text{KMnO}_4$  revealed the presence in the gelsolin promoter of positive signals at -42 to -36 and other regions in cancer cells suggests the formation of a single strand DNA structure at a broader region including the superhypersensitive site at -35. The palindromic arrangement in bladder cancer cells may form a stem and concomitant loop structure. A stem loop is created when two complementary sequences within a single strand come together to form double-helical structures, and in some cases the structures include mismatched or unmatched (bulged) bases<sup>33</sup>. Such mismatches destabilize the local structure but

introduce deviations from the standard double-helical structure that can be important for higher-order folding and function.

Although some single-stranded DNA stabilizing proteins not only Sp 1 might participate to the maintenance of this large loop structure, Sp 1 binding and subsequent formation of the stem and loop structure seem to be a crucial step of the gelsolin gene repression. Sp1 is a DNA-binding protein that usually acts as a positive regulator of various cellular and viral gene expressions<sup>34</sup>. However, there are several reports that Sp1 serves the unusual role of repressing gene expression and acts as a silencer of gene expression in many cell lines<sup>35-37</sup>. Moreover, it was reported that a nuclear protein, p74, interacts directly with the transactivation domain of Sp1 protein, and negatively regulates the Sp1-mediated transactivation<sup>38</sup>. These findings suggest that in our present report Sp1 binds gelsolin promoter and directly or indirectly repress expression of gelsolin of human bladder cancer cells. Although we do not know at present the causality between the hypoacetylation of histones H3 and H4 and the formation of the stem and loop structure, these two events might function synergistically to repress the gelsolin gene transcription in human urinary bladder cancer.

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## FIGURE LEGENDS

FIGURE 1. Nucleotide sequence of the 5'-flanking region of the human gelsolin gene. The first nucleotide of the transcription start site (Kwiatkowski et al., 1988), indicated in lowercase, was designated as +1. The corrected sequence described in this study is shown in bold as **G** (-153) corrected from K (G or T) and **T** (-170) revised additionally. The positions of the primers used in LM-PCR are indicated by arrows under this sequence.

FIGURE 2. Gelsolin promoter activity and expression following TSA treatment. (A) The pBgl II plasmid containing basal gelsolin promoter was transiently transfected into human urinary bladder cancer DAB-1 and KU-7 cells, and their luciferase activities were analyzed after treatment with TSA at various concentrations (0, 10, 100, 500 ng/ml) for 24h (left) or 500 ng/ml for various times (0, 8, 18, 24h) (right). (B) Western blot analysis of gelsolin proteins in DAB-1 and KU-7 cancer and immortalized uroepithelial SV-HUC-1 cells were compared and quantified using purified gelsolin standards. These cells were treated with various concentrations of TSA (0, 10, 100, 500 ng/ml) for 24h using anti-gelsolin and anti-actin antibodies. Normal uroepithelial HMKU-1 cells, that fully express gelsolin, were also used as positive controls.

FIGURE 3. Acetylation status of the gelsolin promoter. The chromatin structures of human uroepithelial immortalized (SV-HUC-1) cells and cancer (DAB-1, KU-7 and UMUC-2) cells (with or without TSA treatment) were cross-linked and subjected to the ChIP assay. Chromosomal immunoprecipitations were performed with both anti-acetylated histone H3 and H4, and gelsolin basic promoter DNA was quantified

using DIG hybridization. The intensities of cancer cells were compared to that of normal cells, and TSA-treated cells were compared to an untreated control, respectively.

FIGURE 4. In vivo DMS footprint analysis on the promoter region of the gelsolin gene in human uroepithelial cells and bladder cancer cells. (A) corresponds to the core region (-230 to -20) of the gelsolin promoter on the sense strand (L1 - L3 primer set). The lane labeled G ladder represents naked DNA, purified from DAB-1 cells and treated with DMS *in vitro*. Lanes SV-HUC-1, DAB-1 and KU-7 represent DNA from in vivo DMS (0.05 or 0.1%)-treated nuclei from each cell line. (B) corresponds to the proximal region (-130 to +30) of the gelsolin promoter (L4 - L6 primer set). The lane labeled G ladder represents naked DNA, purified from DAB-1 cells and treated with DMS *in vitro*. Lanes HMKU-1, SV-HUC-1, DAB-1 and KU-7 represent DNA from in vivo DMS (0.05 or 0.1%)-treated nuclei from each cell line. Numbers beside the guanine ladder represent nucleotide positions relative to the transcription initiation site. Open arrowhead, a residue showing the super-hypersensitive site.

FIGURE 5. Assay of unpaired bases in the gelsolin promoter. (A) KB genomic DNA was treated with DMS *in vitro* and used as a guanine ladder (G Ladder). SV-HUC-1, DAB-1 and KU-7 cells were treated in vivo with  $\text{KMnO}_4$  for 0.5 or 1 min, and KB genomic DNA was also treated *in vitro* with  $\text{KMnO}_4$  for 1 or 3 min. Samples were cleaved with piperidine prior to LM-PCR treatment. (B) Experimentally deduced model of the stem and loop structure in the gelsolin gene promoter in human bladder cancer cell lines. Nucleotides in circles and squares denote residues reactive and hyper-reactive to  $\text{KMnO}_4$ , respectively. Large open arrow at guanine -35 indicates a

superhypersensitive site observed in DMS footprinting in Fig. 5. Thin closed arrow at guanine -50 denote a canonical hypersensitive site found on the G-rich strand of GC-box, in the case of Sp 1 binding. Dotted arrow at thymine +1 showed a transcriptional start site.



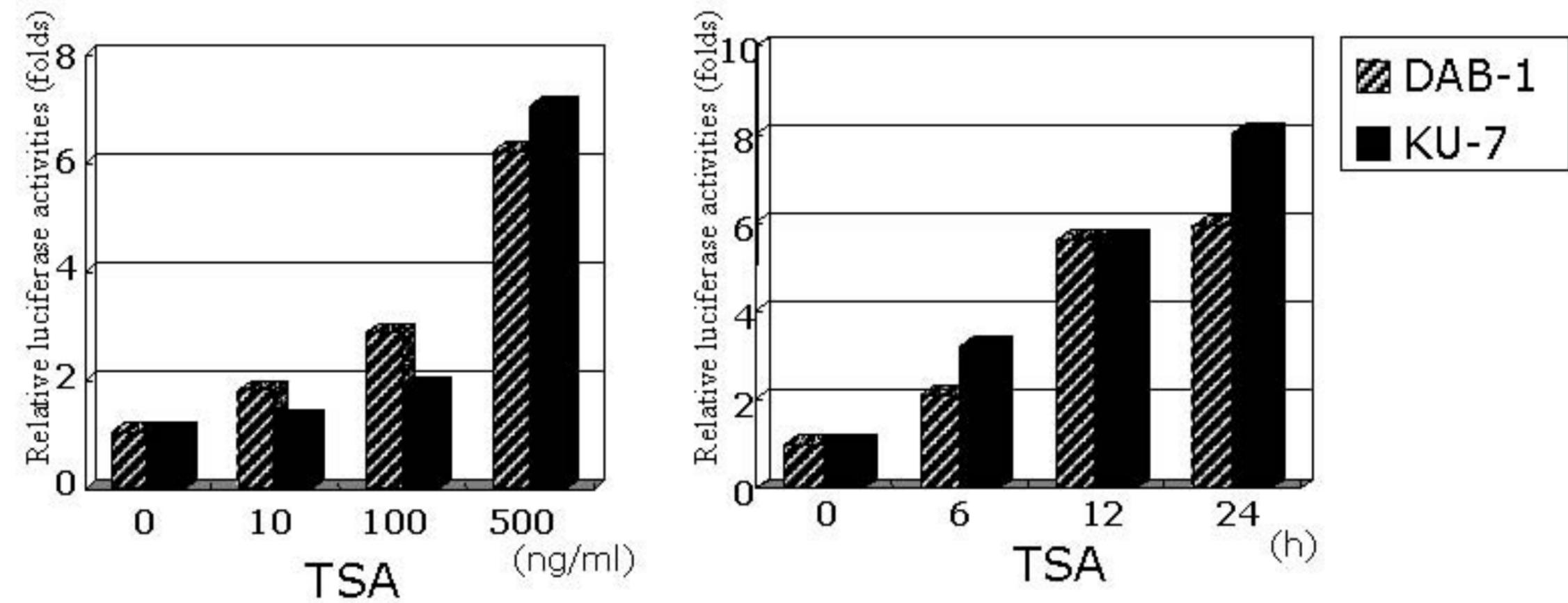
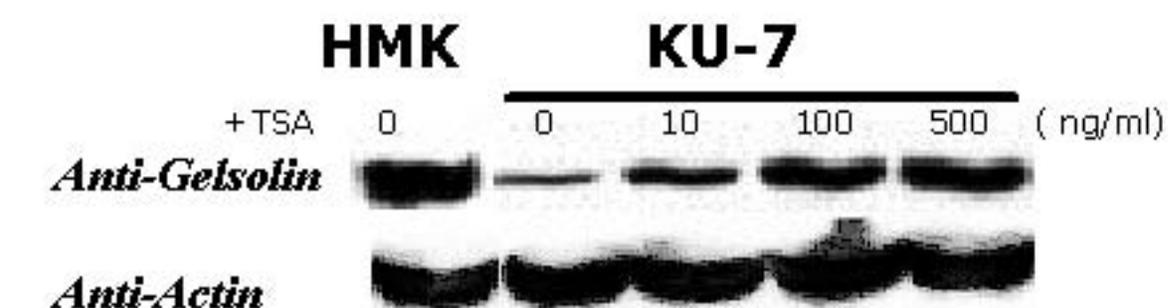
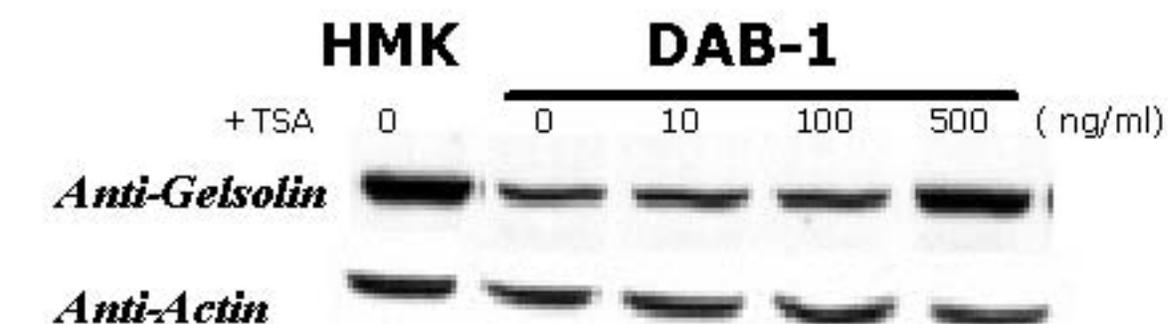
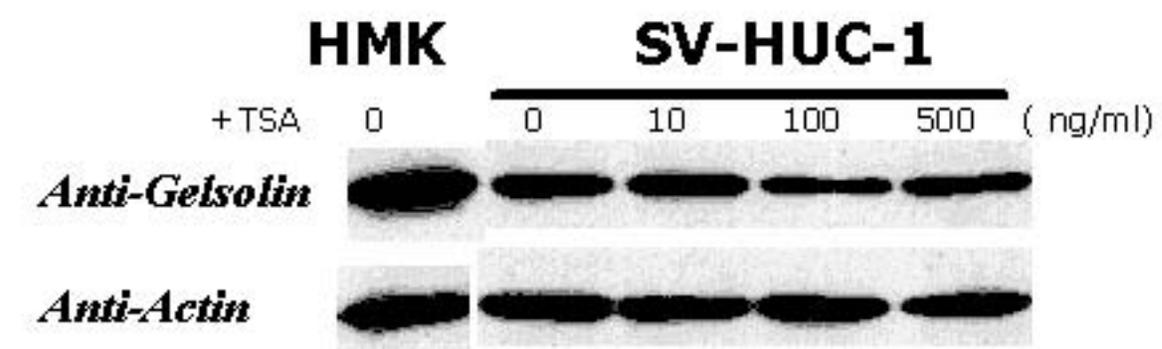
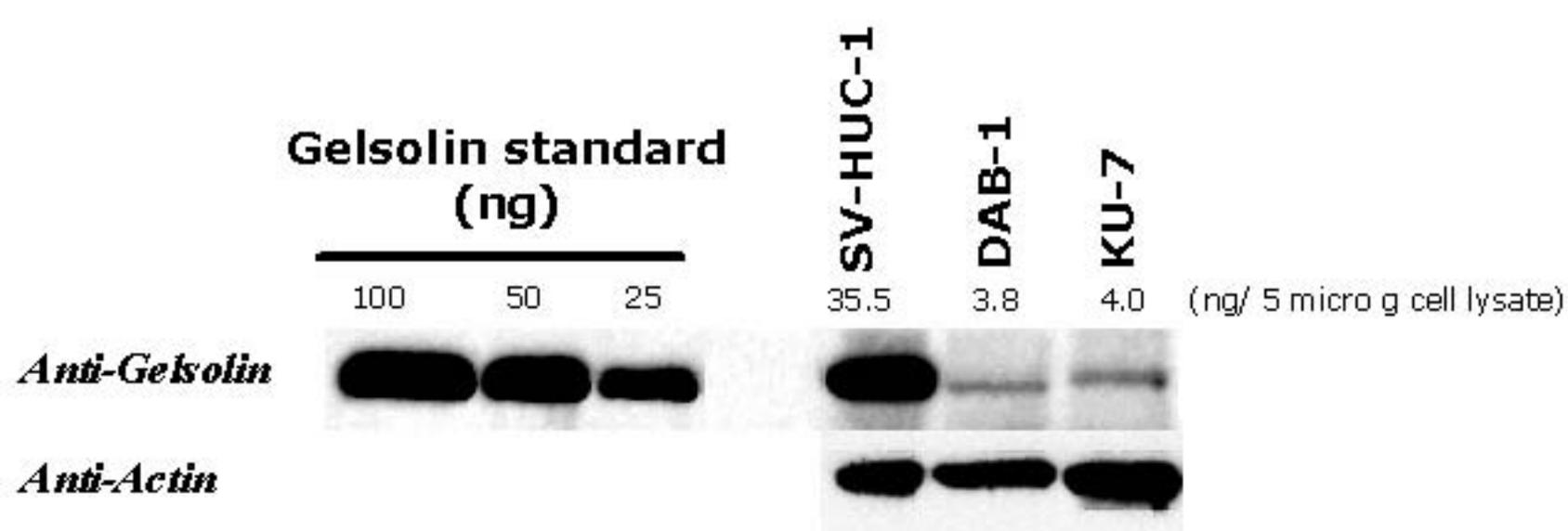


Fig. 2A



HMK:HMKU-1

Fig. 2B

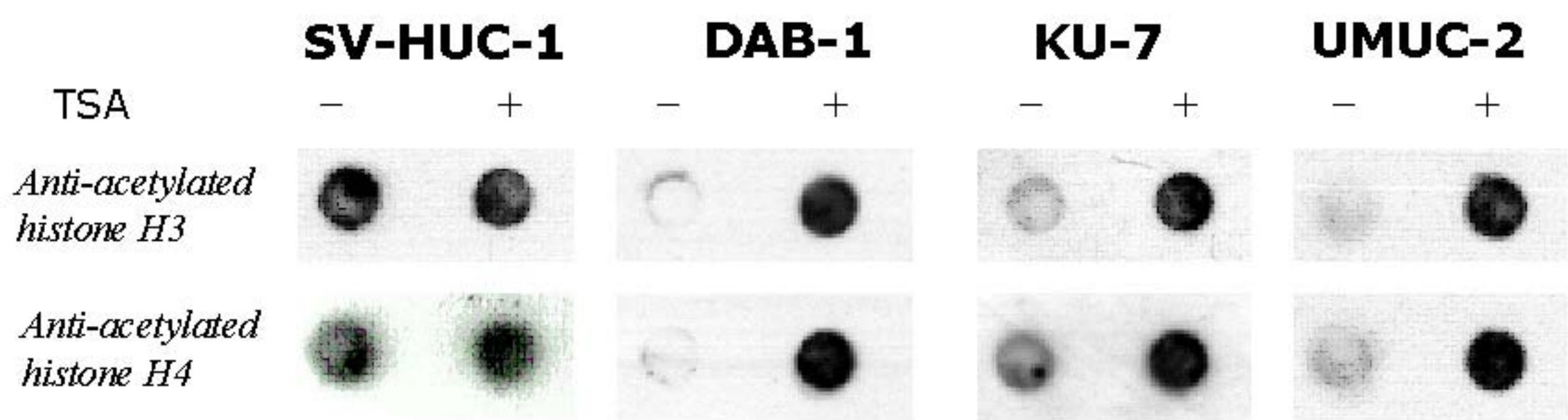


Fig. 3

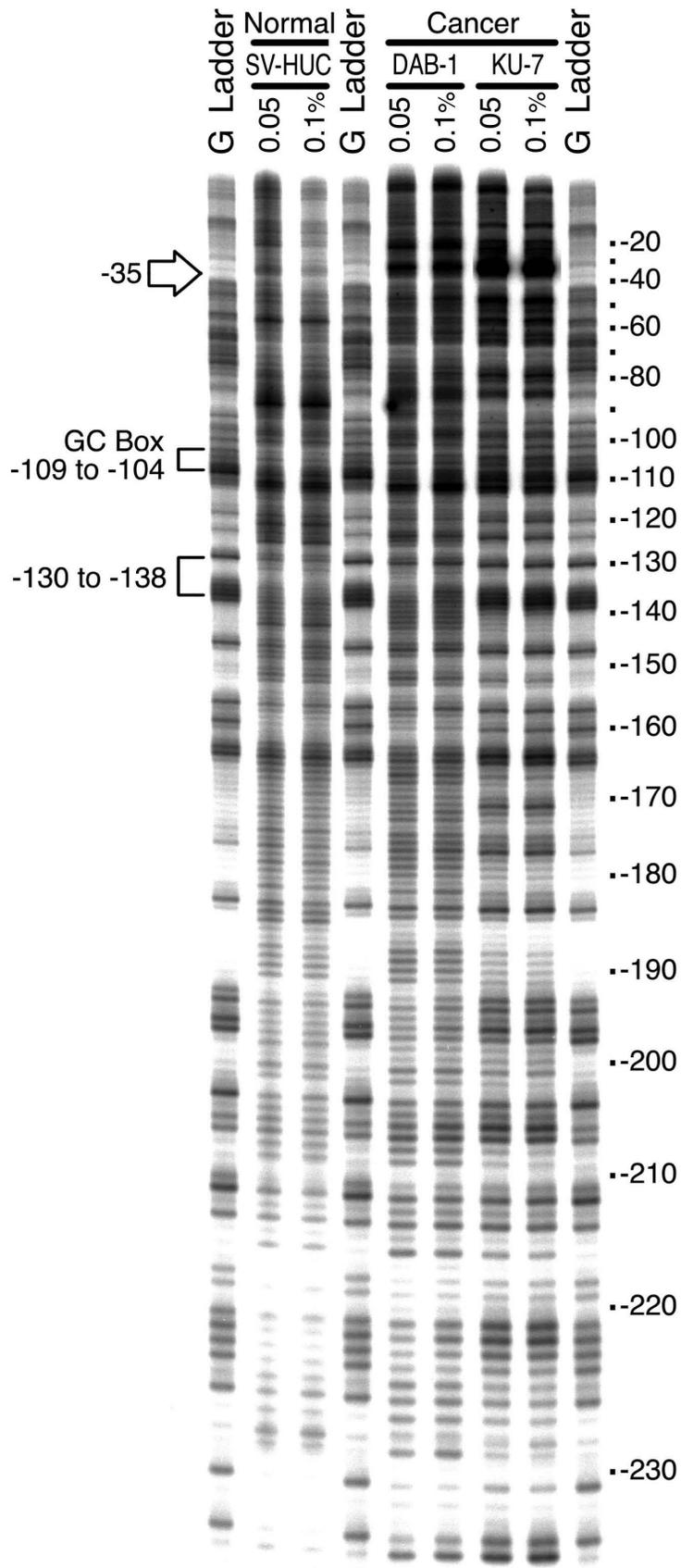


Fig.4A

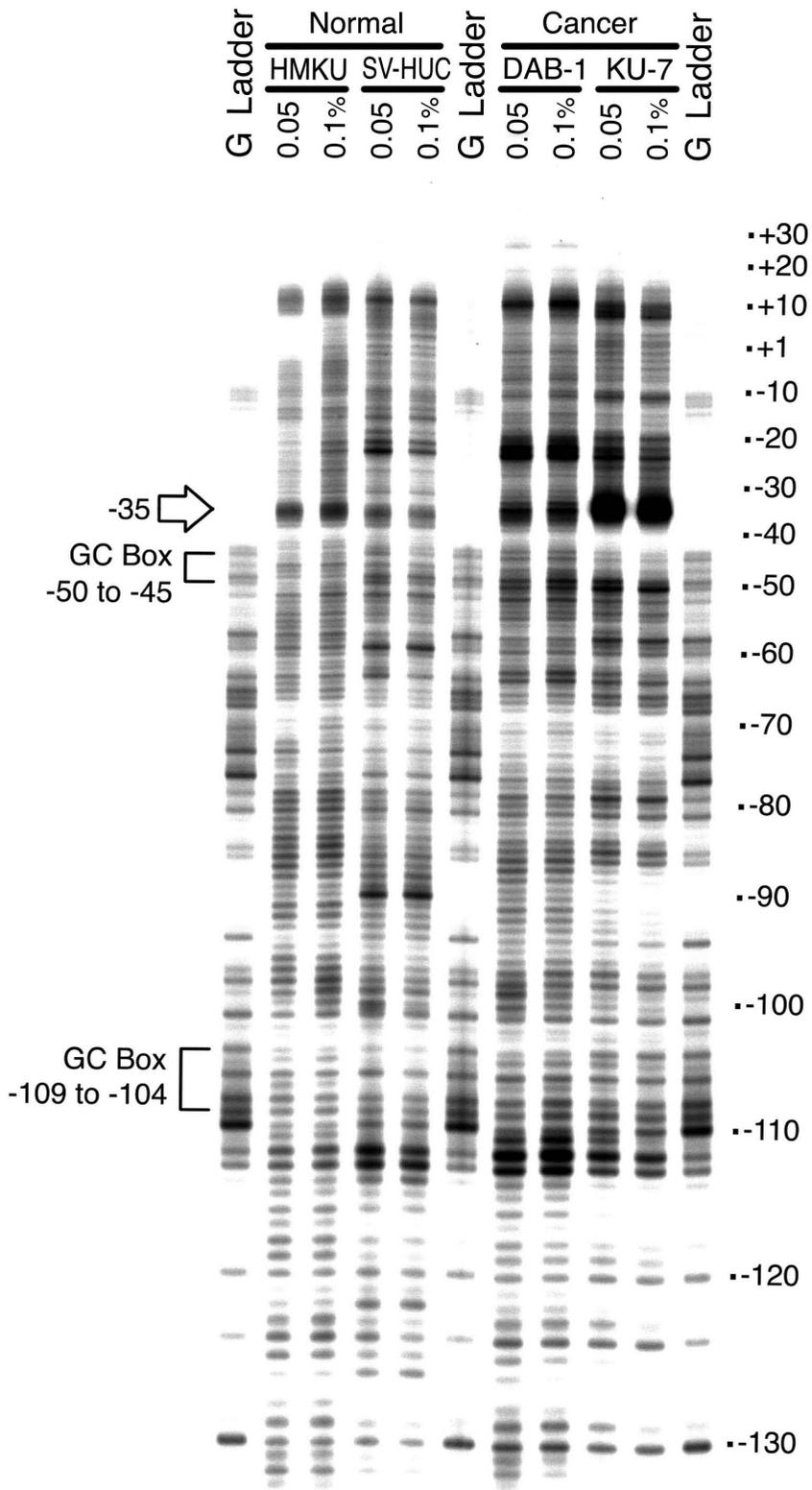
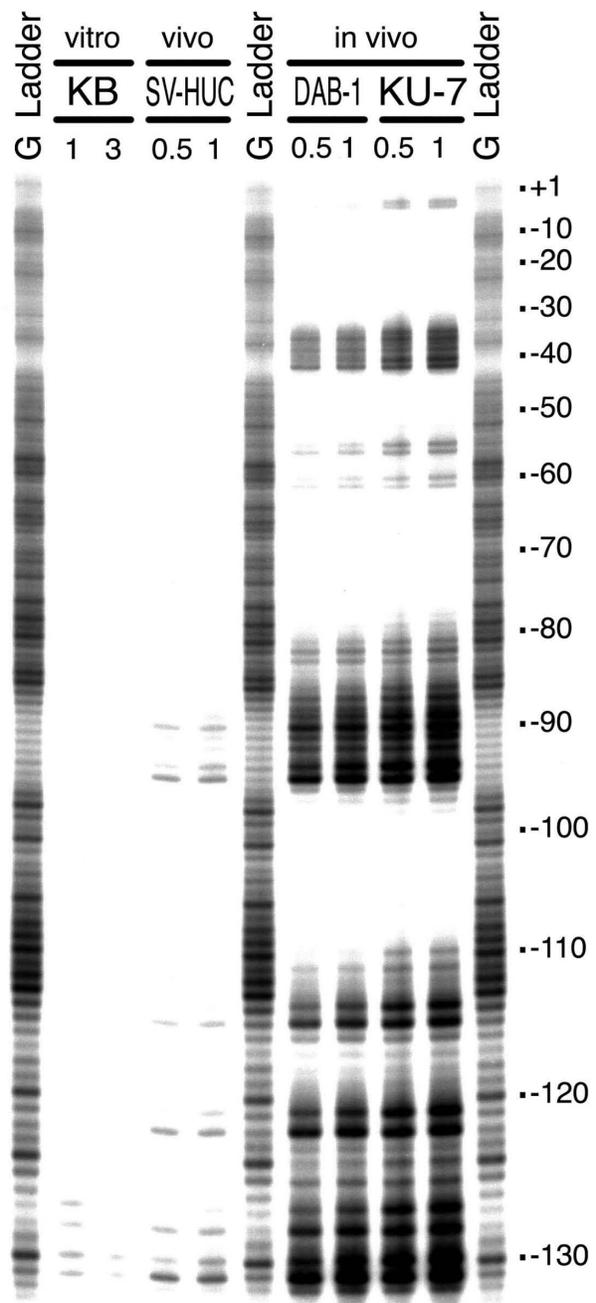
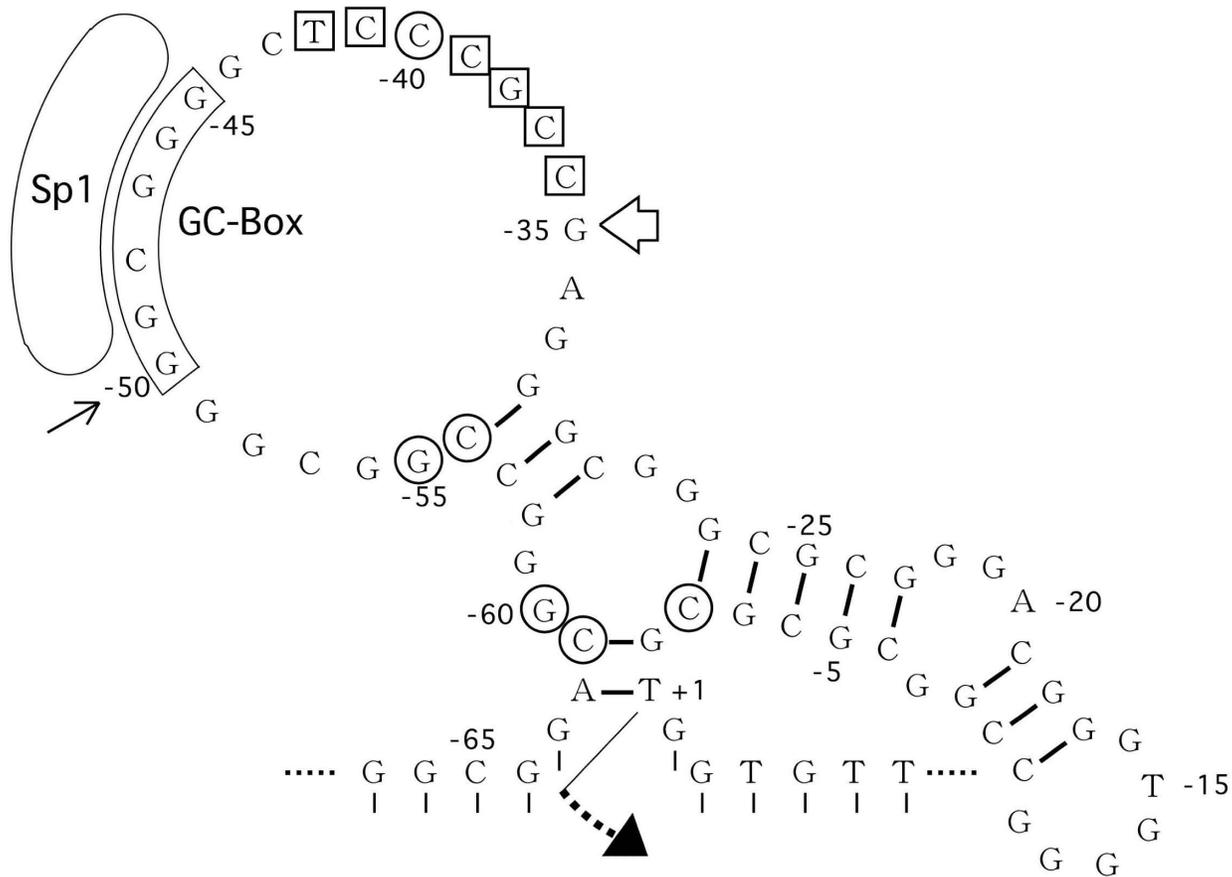


Fig.4B

**(A)****(B)****Fig. 5**