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mel-18, a *Polycomb* group-related mammalian gene, encodes a transcriptional negative regulator with tumor suppressive activity

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The mammalian *mel-18/bmi-1* gene products share an amino acid sequence and a secondary structure, including a RING-finger motif, with the *Drosophila Polycomb* group (*PcG*) gene products Psc and Su(z)2, implying that they represent a gene family with related functions. As *Drosophila PcG* gene products are thought to function as transcriptional repressors by modifying chromatin structure, Mel-18/Bmi-1 might be expected to have similar activities. Here we have analyzed the function of *mel-18* and found that Mel-18 acts as a transcriptional repressor via its target DNA sequence, 5′-GACTNGACT-3′. Interestingly, this binding sequence is found within regulatory or non-coding regions of various genes, including the c-*myc*, *bcl-2* and *Hox* genes, suggesting diverse functions of *mel-18* as the mammalian homolog of the *PcG* gene. We also demonstrate that *mel-18* has tumor suppressor activity, in contrast to *bmi-1*, which has been defined as a proto-oncogene.

Keywords: *mel-18/bmi-1/Polycomb* group/transcriptional negative regulator/tumor suppressor

Introduction

The *Polycomb* group (*PcG*) genes of *Drosophila melanogaster* are required for maintaining the anterior restriction on expression of the homeotic selector genes of *BX-C* and *Ant-C* and hence for maintenance of segmental determination during embryonic development (reviewed by Epstein, 1992). The mechanisms of action of these *PcG* genes are thought to involve repression of homeotic gene expression. Recently mammalian homologs related to the *PcG* genes were discovered, which include *mel-18*, *bmi-1* and *M33* (Tagawa *et al.*, 1990; Brunk *et al.*, 1991; Pearce *et al.*, 1992). The *mel-18* and *bmi-1* gene products share amino acid sequence homology with proteins encoded by the *Drosophila PcG* gene *posterior sex comb* (*Psc*) and the neighboring *PcG* gene *suppressor two of zeste* [*Su(z)2*] (Brunk *et al.*, 1991; van Lohuizen *et al.*, 1991a; Ishida *et al.*, 1993).

The mouse *bmi-1* gene collaborates with the c-*myc* oncogene to cause B lymphomas and thus is considered to be a proto-oncogene (Haupt *et al.*, 1991; van Lohuizen *et al.*, 1991b). *bmi-1* null mutant mice demonstrate phenotypic alterations: (i) neurological abnormalities manifested by ataxic gait and sporadic seizures; (ii) posterior transformation, in most cases along the complete anteroposterior axis of the skeleton (van der Lugt *et al.*, 1994). These observations indicate that *bmi-1* plays an important role in morphogenesis during embryonic development and thus provide evidence of functional conservation of mammalian *PcG* homologs.

The Mel-18 protein is 342 amino acids long and the N-terminal 102 amino acid region, including the RING-finger motif, is 93% homologous to the Bmi-1 protein (Freemont *et al.*, 1991). In addition, the overall identity is 58%. Moreover, the deduced secondary structure shows high homology between the Mel-18 and Bmi-1 proteins. The *Drosophila Psc* and *Su(z)2* gene products also show similarities in their secondary structures (Ishida *et al.*, 1993). The strong conservation of amino acid sequence and predicted protein structure between the mammalian Mel-18/Bmi-1 and *Drosophila PcG* gene products reflects functional similarities and hence suggests involvement of the *mel-18* gene in embryogenesis and tumorigenesis.

To provide biological insights into these mammalian *PcG*-related gene products we carried out a systematic study of Mel-18 DNA binding, transcriptional activity and biological functions, since little is known about mammalian *PcG*-related gene products. Our results show that the Mel-18 protein is a sequence-specific DNA binding protein and is a transcriptional negative regulator via its cognate binding sequence, 5′-GACTNGACT-3′. Furthermore, we demonstrate that *mel-18* has tumor suppressor activity, in contrast to *bmi-1*, which has been described as a proto-oncogene.

Results

Mel-18 protein can bind both single strand and double strand DNA

To study Mel-18–DNA interactions we semi-purified recombinant Mel-18 protein (Figure 1A). The semi-purified recombinant Mel-18 was applied to columns of native (ds-DNA) or denatured (ss-DNA) calf thymus DNA (Figure 1B). The proteins bound to the columns were eluted with buffer containing 1.0 M KCl. The flow-through and 1.0 M KCl fractions were subjected to 12% SDS–PAGE and Mel-18 was detected by Western blot with an antibody against synthetic Mel-18 peptide. As shown in Figure 1C, Mel-18 protein could be detected in the eluted fractions from both columns, indicating that recombinant Mel-18 protein can bind to both ds- and ss-DNA.

Target sequence determination by the SAAB method

Since our previous studies have demonstrated DNA binding activity of Mel-18, we tried to identify the specific

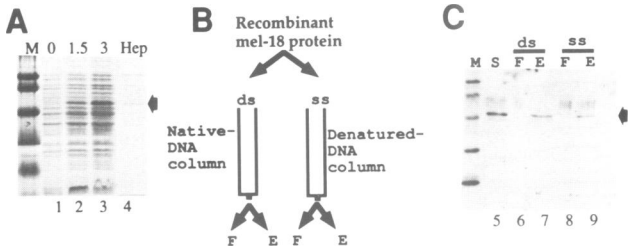


Fig. 1. Recombinant Mel-18 protein can bind to both ds- and ss-DNA columns. (**A**) Purification of bacterially expressed Mel-18 proteins. Mel-18 was expressed by the pET11 vector, induced for 1.5 or 3 h by addition of IPTG (indicated as 0, 1.5 and 3 at the top) and purified. Coomassie Blue stained SDS–PAGE gels of total protein from cells carrying the expression vector without induction (lane 1), proteins from IPTG-induced cells (lanes 2 and 3) and peak fractions from the second heparin column chromatography (lane 4) are shown. Molecular markers are indicated (M: 97.4, 66, 46, 30 and 21.5 kDa; Amersham). (**B**) Experimental scheme for the DNA binding assay. Semi-purified recombinant Mel-18 protein was applied to a native or a denatured calf thymus DNA column (Pharmacia). (**C**) The flow-through fraction (F), the 1.0 M KCl fraction (E) and unfractionated samples (S) were subjected to 12% SDS–PAGE and the Mel-18 protein was detected by Western blotting using rabbit anti-Mel-18 peptide antibody (Tagawa *et al.*, 1990). The arrow indicates the position of the Mel-18 protein on SDS–PAGE.

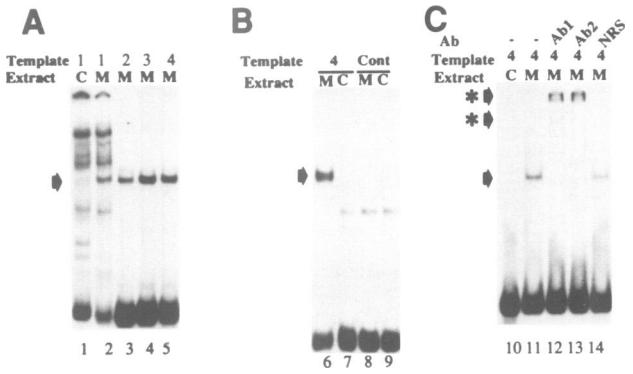


Fig. 2. Specific gel-shift of oligonucleotides containing Mel-18 binding sites from a pool of random oligonucleotides. (**A**) Progressive enrichment for Mel-18 binding sites using the SAAB method. Gel-shift assay was performed with materials obtained from each cycle of SAAB in the presence of recombinant Mel-18 (M) or the heparin column fraction from control bacteria (C). Template number (indicated as 1–4 at the top) corresponds to the number of cycles of SAAB enrichment (first to fourth cycles). (**B**) Specific gel-shifted band after four cycles of enrichment. A selected oligonucleotide template (template 4, lanes 6 and 7) and control template (Cont, lanes 8 and 9) were incubated with semi-purified recombinant Mel-18 (M, lanes 6 and 8) or the corresponding fraction (C) from a control lysate of *Escherichia coli* harboring the pET11 vector (lanes 7 and 9). (**C**) Specific supershift band formation with antisera against Mel-18 protein. The addition of anti-Mel-18 antiserum (Ab1 and Ab2, lanes 12 and 13), but not of pre-immune rabbit serum (NRS, lane 14), resulted in supershift band formation. Arrows indicate the positions of the Mel-18–DNA complex and the arrows with asterisks indicate the *mel-18*–DNA–antibody supershifted complex.

binding sequence. To determine the target binding sequence of Mel-18 we employed a random oligonucleotide polymerase chain reaction (PCR) selection technique, the SAAB method, with synthetic oligonucleotides containing 13 contiguous randomized bases as a template for gel-shift assay (Blackwell and Weintraub, 1990).

Figure 2A demonstrates the progressive enrichment for Mel-18 binding sites. In the first cycle we obtained several bands (Figure 2A, lane 2), but identified only one specific band by comparison with the control reaction (indicated by an arrow, Figure 2A). This band became a major fraction in the second to fourth cycles. The intensity of the band appeared to saturate after the third cycle. Figure 2B shows specific binding of the Mel-18 protein to the DNA template after four cycles of enrichment. The control template, which has the same 20 base flanking regions and a randomly selected but defined 13 base central region (see Materials and methods), showed only non-specific binding (Figure 2B, lane 8), indicating that the central 13 nucleotides of the enriched DNA template pool (template 4) contains the Mel-18 binding sites.

For further verification of specific binding we applied a 'supershift' assay to demonstrate whether this protein–DNA complex is really composed of Mel-18 protein, as the semi-purified Mel-18 protein fraction may still contain a certain amount of bacterial proteins. We performed the gel-shift reaction by adding rabbit anti-Mel-18 antiserum. We obtained supershift of the Mel-18–DNA complex only when the antiserum was added (Figure 2C, lanes 12 and 13), while addition of pre-immune rabbit serum had no effect (Figure 2C, lane 14). This clearly indicates that the protein–DNA complex we observed is really composed of Mel-18 protein, and not residual bacterial protein, with the enriched template. Moreover, we observed the same results with purified histidine-tagged Mel-18 protein expressed by the pET16 vector system (data not shown). Thus it is likely that the gel-shift band detected is solely Mel-18–DNA complex.

To identify the enriched sequences the pooled DNA after the fourth selection cycle was cloned and sequenced. Figure 3A shows the alignment of all selected sites. A consensus emerging from the 55 selected sites was 5′-GACTNGACT-3′ (numbers of appearances in the 55 sites for each conserved nucleotide are shown in Figure 3B by the bar graph), because most of the cloned DNA fragments contained a sequence with a GACT motif (55/60) and 48 contained a GACT direct repeat sequence with one nucleotide spacing. It was notable that 45 clones showed an identical sequence, 5′-TTACGACTTGACT-3′. Using this cloned DNA fragment with the central sequence 5′-TTACGACTTGACT-3′ as a template we obtained the same specific binding of Mel-18 (see below, Figure 4). Since this consensus sequence contains three elements, two directly repeated GACT sequences and a 1 bp spacer, we carried out further investigations to identify the essential binding motif using a series of mutants (M1–WT3, Figure 4). Surprisingly, none of the mutants was capable of binding the Mel-18 protein except for weak binding by the M4 mutant template, which has an intact direct repeat sequence with a mutated flanking region. It is thus demonstrated that the direct repeat sequence is necessary and sufficient for binding, whereas the flanking sequence, 5′-TTAC-3′, is important to increase the affinity of binding. It is also demonstrated that one GACT copy is not enough for binding (Figure 4, M1 and M3) and that the orientation of the repeat (WTi) and the spacing between the two GACT repeats (WT2 and WT3) are crucial for

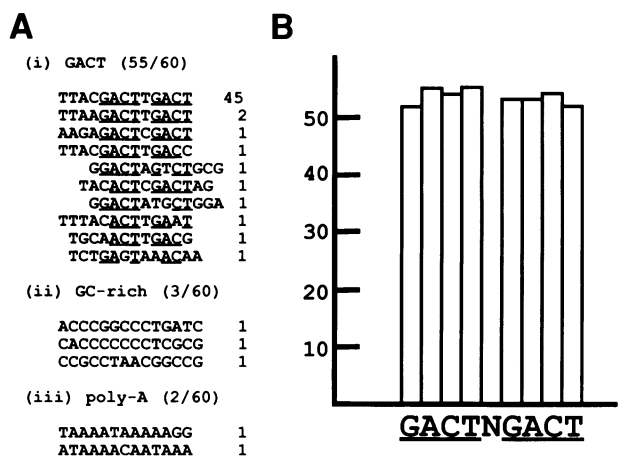


Fig. 3. Alignment of all selected Mel-18 binding sites. Sixty binding sequences for Mel-18 obtained by four rounds of selection are shown. These sequences are categorized into three groups, those containing: (i) GACT direct repeat core binding sequences; (ii) GC-rich sequences; (iii) poly(A) sequences. The consensus sequence is shown and the frequency of appearance of each conserved nucleotide among the 55 selected sites is shown in the bar graph (**B**). The underlined nucleotides at each site are those that occur in the consensus sequence (**A**).

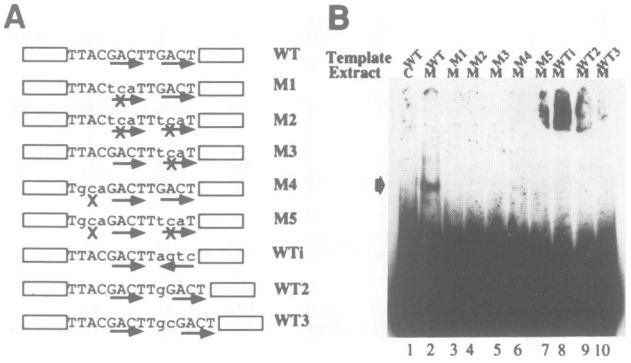


Fig. 4. Effects of mutations on the most enriched template DNA sequence 5′-TTACGACTTGACT-3′. (**A**) Nucleotide sequence of the enriched Mel-18 binding site (WT) and a series of related mutants (M1–WT3). Arrows indicate the direct repeat sequence and three base mutated regions are indicated as X. (**B**) Gel-shift assay on the series of mutated templates with bacterially expressed Mel-18 protein (M) and the control protein fraction (C). It should be noted that only the M4 template gave a shifted band at the same position as the WT template

Mel-18 binding. The data clearly demonstrate that Mel-18 is a sequence-specific DNA binding protein. Moreover, this can explain why the 45 isolated clones have an identical sequence, 5′-TTACGACTTGACT-3′, since the flanking 5′-TTAC-3′ sequence contributes to a higher affinity for Mel-18 binding. Although the *PcG* gene products and most RING-finger proteins have not been shown to possess sequence-specific DNA binding, we have detected sequence-specific binding of the *mel-18* gene product.

The function of Mel-18 in transcription

To investigate the transcriptional function of Mel-18 we first asked whether Mel-18 acts as a transcriptional activator, because the C-terminal part of the Mel-18 protein possesses a proline-rich region that is found in some transcriptional activators (Gerber *et al.*, 1994). On co-transfection of a pCAT-promoter plasmid (identical to the pCAT-control plasmid except for an SV40 enhancer; Figure 5A) carrying a tetramer of the wild-type (WT) or mutant (M2) oligonucleotide for Mel-18 binding (see Materials and methods) with a *mel-18* expression vector (pMel-18) we found no significant transcriptional activation in L or Cos cells (data not shown). We then tested the possibility that Mel-18 acts as a transcriptional repressor. The tetramer WT or M2 oligonucleotide was inserted upstream or downstream of a pCAT-control plasmid containing a SV40 promoter, a CAT (chloramphenicol acetyltransferase) reporter gene and a SV40 enhancer, resulting in p(WT)4-CAT, p(M2)4-CAT, pCAT-(WT)4 and pCAT-(M2)4 reporter plasmids (Figure 5A). Co-transfection with pMel-18 of p(WT)4-CAT or pCAT-(WT)4 caused repression of CAT activity in both orientations of the WT oligonucleotide, whereas co-transfection of p(M2)4-CAT or pCAT-(M2)4 produced no effect (Figure 5B and C). It must be stressed that repression by a tetramer of the WT oligonucleotide was observed regardless of the positions and orientations of the oligonucleotide. Thus the possibility that hindrance by the bound Mel-18 protein might interfere with the accessibility of other transcription factors and/or the basic transcriptional machinery to the promoter region can be ruled out. This repressive activity of Mel-18 is consistent with a suppressive function of *Drosophila* PcG protein on the *bithorax* complex (Orlando and Paro, 1993).

Tumorigenicity of antisense mel-18-transfected NIH 3T3 cells

Since the c-*myc* and *bcl-2* genes, which have emerged as possible target genes of Mel-18 (see Discussion), are known to act synergistically on cell proliferation and apoptosis, we tested the possibility that Mel-18 controls cell growth. We generated NIH 3T3 cell clones that overexpress sense or antisense *mel-18* RNA to block endogenous *mel-18* transcripts. The plasmids pSV-Mel18 (sense) and pSV-Mel18 (antisense) were transfected into NIH 3T3 cells. We obtained cloned cell lines S-627 and S-3G2 as sense *mel-18* transfectants and A-3G11 and A-8G7 as antisense *mel-18* transfectants respectively (Figure 6A).

To investigate tumorigenicity we injected transfectants subcutaneously into the backs of nude mice. Transfectants were scored as tumorigenic if a visible nodule appeared at the site of injection and subsequently increased in size during 90 days of observation. Mice that did not develop tumors were also observed for 90 days. The cells overexpressing antisense *mel-18* RNA displayed marked tumorigenicity, although they did not exhibit obvious morphological changes *in vitro*. When a relatively large number of cells (1×10^6 and 1×10^5) from the A-3G11 and A-8G7 clones were injected, tumors developed within 2–4 weeks and continued to grow unrestrictedly. No tumors developed during the same 90 day period in nude mice injected with cells from either the S-627 or S-3G2 clones, which overexpress sense *mel-18* RNA, or from clones transfected with control vector (Figure 6B and C). The antisense *mel-18* transfectants revealed undetectable levels of Mel-18 protein by Western blot analysis (Figure

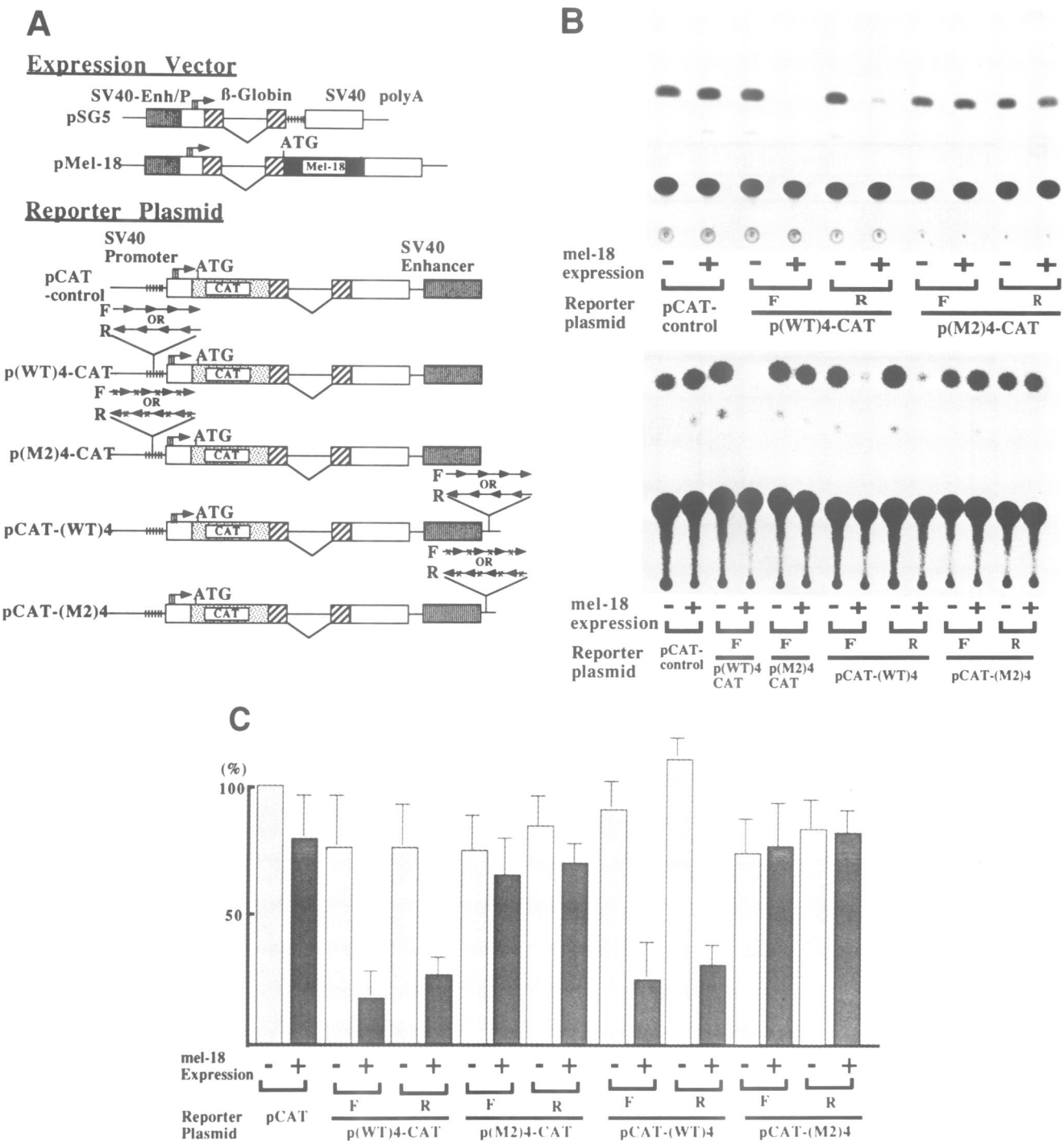


Fig. 5. Mel-18 binding to its target site results in transcriptional repression. (**A**) Schematic representation of the expression vectors pSG5 and pMel-18, as well as the reporter plasmids pCAT-control, p(WT)4-CAT, p(M2)4-CAT, pCAT-(WT)4 and pCAT-(M2)4. (**B**) Representative CAT analysis of enzyme activity synthesized from pCAT recombinant plasmids containing an oligomerized Mel-18 binding motif in the Ltk cell line. (**C**) Graph showing repression of CAT activity by co-transfection of the *mel-18* expression vector. The percentage shows the relative activity for each reporter construct in the presence (+) or absence (–) of the *mel-18* expression vector, where the pCAT-control reporter plasmid containing the SV40 enhancer with pSG5 is taken as 100%. The same results were obtained with the HSV-tk minimal promoter in place of the SV40 promoter (data not shown).

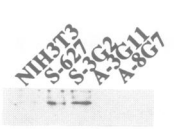
6A), indicating that acquisition of tumorigenicity is due to a decreased level of Mel-18 expression. These results demonstrate that *mel-18* has a tumor suppressive activity, in contrast to *bmi-1*, which has been identified as a collaborator with the c-*myc* oncogene in Eμ-*myc* transgenic mice by a retrovirus tagging method.

Discussion

DNA binding activity and transcriptional repression

In this study we have shown that a mammalian *PcG*-related gene, *mel-18*, encodes a protein with sequence-

A



B

Cell	tumorigenicity			latency
	$1x10^6$	$1x10^5$	$1x10^4$	(week)
NIH3T3	0/5	0/5	0/5	
Vector	0/5	0/5	0/5	
S-627	0/5*	0/5	0/5	
S-3G2	0/5*	0/5	0/5	
A-3G11	5/5	4/5	0/5	2-3
A-8G7	5/5	2/5	0/5	3-4

C

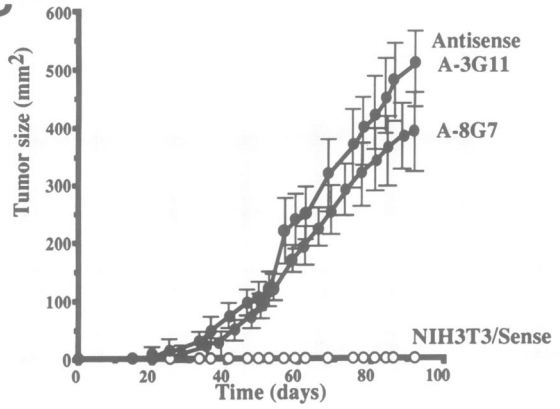


Fig. 6. Tumorigenicity of NIH 3T3 cells overexpressing antisense *mel-18*. (**A**) Western blot analysis of Mel-18 expression by anti-Mel-18 peptide antibody indicating overexpression in sense *mel-18* (S-627 and S-3G2) or undetectable levels of expression in antisense *mel-18* (A-3G11 and A-8G7) transformants. (**B**) NIH 3T3 transfectants overexpressing *mel-18* (S-627 and S-3G2) or antisense *mel-18* (A-3G11 and A-8G7), NIH 3T3 cells transfected with vector alone (Vector) and untransfected NIH 3T3 cells (NIH 3T3) were used for *in vivo* tumorigenicity tests involving five mice in each group. This assay was repeated at least three times. The asterisk indicates that the tumorigenicity frequency is 1/5 in one experiment. (**C**) Tumor growth curve. Tumor size in nude mice injected with the antisense *mel-18* overexpressing clones A-3G11 and A-8G7 (1×10^6 cells) was measured and plotted as the average size with standard deviation for five mice per experimental group. This experiment was repeated three times. Data for the sense *mel-18* overexpressing clones (S-627 and S-3G2) and mice injected with control NIH 3T3 cells are shown (no tumorigenicity).

specific DNA binding *in vitro*. Mel-18 provides the first evidence that a PcG-related protein has sequence-specific DNA binding activity and further confirms data presented previously (Tagawa *et al.*, 1990). While several investigators have identified RING-finger proteins localized in vacuoles and peroxisomes (Robinson *et al.*, 1991; Tan *et al.*, 1995) and act as a signal transducer in the cytoplasm (Hu *et al.*, 1994; Rothe *et al.*, 1994; Sato *et al.*, 1995), Mel-18 might be the first evidence of sequence-specific DNA binding activity. We have also demonstrated that Mel-18 protein functions as a potent repressor via its cognate DNA binding sequence in mammalian cells. The results are consistent with evidence that PcG proteins repress gene expression by formation of heterochromatin (Jones and Gelbart, 1993). We have not yet examined the effects of Mel-18 protein on chromatin structure. While we favor the simple interpretation of a direct interaction between Mel-18 protein and the transcriptional machinery via the cognate binding sequence 5′-GACTNGACT-3′, it is equally plausible that tethering of Mel-18 protein to transient reporter DNA alters the local chromatin structure and thereby represses reporter expression (Jeong *et al.*, 1991; Lauderdale and Stein, 1993).

Recently it has been demonstrated that the fusion proteins LexA–*Drosophila* PcG and LexA–murine Bmi-1 show repressor functions (Bunker and Kingston, 1994). The results support our present results on the repressor activity of Mel-18. Therefore, these negative regulatory functions might be phylogenetically conserved between *Drosophila* and mammals.

Possible target genes and function

Because a consensus binding sequence for Mel-18 was defined *in vitro*, we carried out a database search for the 5′-GACTNGACT-3′ sequence in the regulatory or non-coding region of known genes. As a real target often does not have the highest binding affinity we used the sequence 5′-GACTNGACT-3′ instead of the high affinity 5′-TTA-CGACTNGACT-3′ sequence. We obtained many putative binding sites from the database. It is not surprising that the Mel-18 protein may interact at many sites on the genome, because PcG gene products are known immunohistochemically to detect >80 sites on the polytene chromosome of *Drosophila* (Zink and Paro, 1989).

Among the putative binding sites the following two categories are most interesting. First, the Mel-18 binding motif was found in the regulatory region of the c-*myc* oncogene and the intron of the *bcl-2* gene. Indeed, c-*myc* expression level is elevated in NIH 3T3 cells transfected with antisense *mel-18* and is decreased in sense *mel-18* transfectants in a dose-dependent fashion (M.Hasegawa *et al.*, manuscript in preparation). The tumorigenicity of NIH 3T3 cells seen in this study might be explained by simultaneous overexpression of c-*myc* and *bcl-2*, greatly increasing the possibility of unregulated cell growth by stimulating the proliferation pathway and blocking the apoptosis pathway (Bissonnette *et al.*, 1992; Fanidi *et al.*, 1992; White, 1993). However, we do not know whether tumorigenicity is due solely to the direct effects of c-*myc*. It is conceivable that overexpression of some other genes with c-*myc* up-regulation might take over normal growth control, leading to unregulated growth, in a synergistic fashion.

In our previous report we described the expression pattern of Mel-18, which is mainly expressed in tumor cells of various origin (Tagawa *et al.*, 1990). This would argue against a tumor suppressive activity of Mel-18. However, there are many reports that the *p53* tumor suppressor gene is also overexpressed in tumors (Sarkis *et al.*, 1993). Moreover, clues to the further explanation of tumorigenicity might reside in the resemblance between the *mel-18* and *WT-1* genes (Kreidberg *et al.*, 1993), both of which possess zinc/RING-finger motifs with repressor activity. The *WT-1* gene product is reported to be a dual function regulator that switches activity by recruiting auxiliary factors to the promoter, either by protein–protein or protein–DNA interactions, which then act as co-activators or co-repressors. Indeed, the *WT-1* gene product

is an activator in the absence of *p53*, but a repressor in its presence (Maheswaran *et al.*, 1993). In this sense Mel-18 could also be a dual function regulator, depending on the context of the promoter architecture or the cellular microenvironment.

The second binding type category is present in the mouse *Hoxc-6* gene in the 3′-untranslated region. This strong phylogenetic conservation of the relationship between regulator and target genes implies the biological importance of the *mel-18* gene not only for the development of vertebrae, especially specification of the anteroposterior axis of the axial skeleton, but also for control of cell growth, since the *Hox* and *Pax* genes were reported to have oncogenic activity (Maulbecker and Gruss, 1993; Rabbits, 1994). Indeed, mice lacking the *bmi-1* gene and *mel-18* null mice recently developed in our laboratory demonstrate that *bmi-1/mel-18* act as regulators of anteroposterior axis formation (van der Lugt *et al*, 1994; Akasaka *et al.*, manuscript in preparation).

The categories mentioned above are the most interesting in which to test for possible target genes for Mel-18. In this sense mammalian *PcG*-related gene products (namely Mel-18 and Bmi-1) might control at least two different biological events, regulation of *Hox* code and control of cell growth/death.

Materials and methods

Purification of recombinant Mel-18 protein and column experiments

Recombinant Mel-18 protein was produced using the pET11 vector system (Rosenberg *et al.*, 1987). The fraction obtained by chromatography on DEAE–Sepharose (FT) and twice on heparin–Sepharose to ~60–80% purity was applied to 1 ml native or denatured calf thymus DNA columns. After washing with five bed volumes of 0.1 M KCl–TEMGN buffer (25 mM Tris–HCl, pH 7.5, 12.5 mM $MgCl_2$, 0.1 mM EDTA, 20% glycerol, 0.1% NP-40) the bound proteins were eluted with 1.0 M KCl–TEMGN buffer and concentrated to the same volume as the initial application. A 10 μl aliquot of each fraction was subjected to 12% SDS–PAGE with detection by the ECL Western blotting system (Amersham) with anti-Mel-18 peptide antibody.

Binding site selection by the SAAB method

The 53 bp random oligonucleotide containing a 13 base radomized internal region (5′-CTGGATCCTAGATATCCCTGNNNNNNNNNNN-NNAGGCTCAAAGCTGAATTCCT-3′) was subjected to the SAAB method as described (Blackwell and Weintraub, 1990). The oligonucleotide 5′-CTGGATCCTAGATATCCCTGTTACGACGATTGAAGGCTC-AAAGCTGAATTCCT-3′, designated as the control template, contains the same 20 base flanking regions and a randomly selected, but defined, 13 base central region. For gel-shift assay ~3–7×10^6 c.p.m. DNA (0.1 pmol) were incubated with 50 ng partially purified recombinant Mel-18 protein (dialyzed against TMGN buffer, identical to TEMGN buffer except without EDTA), 0.5 μg poly(dI–dC):poly(dI–dC) (Pharmacia) and 0.5 μg bovine serum albumin in 10 mM HEPES, pH 7.8, 50 mM KCl, 1 mM dithiothreitol and 20% glycerol in a 10 μl reaction volume. The reactions were incubated at room temperature for 30 min. The samples were analyzed on 7% polyacrylamide gels (79:1, acrylamide:bisacrylamide) in 1× TNE buffer (6.6 mM Tris–HCl, pH 7.5, 3.3 mM sodium acetate, 1 mM EDTA) for 2.5–3 h at 10 V/cm. The PCR mixtures were incubated for 1 min at 94°C, 1 min at 50°C and 1 min at 72°C for 15 cycles in a DNA thermal cycler (Perkin Elmer-Cetus or Hybaid/OmniGene).

Transient expression system and CAT assay for transcriptional activity

A pMel-18 expression vector was constructed by insertion of full-length *mel-18* cDNA into the *Eco*RI site of pSG5 (Green *et al.*, 1988). The reporter plasmids p(WT)4-CAT, p(M2)4-CAT, pCAT-(WT)4 and pCAT-(M2)4 were constructed as follows. Tetramerized WT oligonucleotide (5′-TCGGTTACGACTTGACTCGC-3′ and 5′-CCGAGCGAGTCAA-GTCGTAA-3′) or M2 oligonucleotide (5′-TCGGGTTACTCATTTCAT-CGC-3′ and 5′-CCGAGCGATGAAATGAGTAAC-3′) were introduced into the pCAT-control vector (Promega). For CAT assays 1 μg reporter plasmid and 1 μg *mel-18* expression vector were transfected into Ltk cells together with 1 μg pCH110 β-galactosidase expression vector (Pharmacia), used as an internal standard. The CAT assay was carried out as described peviously (Gorman *et al.*, 1982).

Tumorigenicity assay

NIH 3T3 cells (clone 5611, supplied by Dr S.A.Aaronson) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum. To obtain clones that express elevated amounts of sense *mel-18* or antisense *mel-18* RNA we transfected 10 μg plasmid pSV-Mel18, a derivative of pSV2gpt carrying 1.6 kb mouse *mel-18* cDNA under the control of RSV-LTR with the *Eco-gpt* resistance gene, into NIH 3T3 cells by electroporation (Hatakeyama *et al.*, 1985). After isolation of drug resistant clones the amounts of *mel-18* RNA and protein were quantitated by RNase protection assay and by Western blotting (Tagawa *et al.*, 1990). Expression of *mel-18* mRNA in the sense *mel-18*-transfected clones (S-627 and S-3G2) was >10 times the basal expression observed in vector-transfected cells. Expression of Mel-18 protein was also elevated in these clones, but was undetectable in the antisense *mel-18* transfectants (A-3G11 and A-8G7). Five- to six-week-old nude mice (Balb/c nu/nu; SLC Japan Inc.) were injected subcutaneously on both flanks with 1×10^4, 1×10^5 or 1×10^6 cells, as indicated, resuspended in Dulbecco's modified phosphate-buffered saline (100 μl). Cells were scored as tumorigenic if a visible nodule appeared at the site of injection and subsequently increased in size. Mice that did not develop tumors were also observed for 90 days. Cells from additional clones overexpressing antisense *mel-18* RNA were also found to be tumorigenic in this assay (Hasegawa *et al.*, manuscript in preparation).

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