Regular paper

Maintenance of integrated proviral gene expression requires Brm, a catalytic subunit of SWI/SNF complex

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SUMMARY

We show here that MuLV-based retrovirus vector transgene expression is rapidly silenced in human tumor cell lines lacking expression of Brm, a catalytic subunit of the SWI/SNF chromatin remodeling complex, even though these vectors can successfully enter, integrate, and initiate transcription. We detected this gene silencing as a reduction in the ratio of cells expressing the exogenous gene rather than a reduction in the average expression levels, indicating that down-regulation occurs in an all-or-none manner. Retroviral gene expression was protected from silencing and maintained in Brm-deficient host cells by exogenous expression of Brm but not BRG1, an alternative ATPase subunit in the SWI/SNF complex. Introduction of exogenous Brm to these cells suppressed recruitment of protein complexes containing YY1 and histone deacetylase (HDAC) 1 and 2 to the 5’-LTR region of the integrated provirus, leading to the enhancement of acetylation of specific lysine residues in histone H4 located in this region. Consistent with these observations, treatment of Brm deficient cells with HDAC inhibitors but not DNA methylation inhibitors suppressed retroviral gene silencing. These results suggest that the Brm-containing SWI/SNF complex subfamily (trithorax-G) and a complex including YY1 and HDACs (Polycomb-G) counteract each other to maintain transcription of exogenously introduced genes.
INTRODUCTION

Retroviruses are known to integrate into host cell chromosomes as proviruses and to express viral genes even after host cell proliferation. One problem limiting development of retroviral vectors with long-term expression, however, is gene silencing (1). DNA methylation has been reported as the major epigenetic DNA modification that occurs in conjugation with provirus down-regulation (2-4) and serves as a signal for association with methyl-CpG-binding protein 2 (MeCP-2), which together form large protein complexes containing histone deacetylases (HDACs) (5). Therefore, silenced retroviral genes have been sometimes reactivated by treating cells with either the DNA methyltransferase inhibitors, 5-azacytidine (5-aza-C) and 5-azadeoxycytidine (5-azadC) or HDAC inhibitors, such as trichostatin A (TSA). In many cell types that exhibit gene silencing, however, it is not clear whether provirus methylation is the primary cause or simply reflects transcriptional repression. Gene silencing that is not mediated by DNA methylation has been suggested (6-9).

In multicellular organisms, epigenetic regulation of transcription supports distinct cell type-specific gene expression. For example in *Drosophila* and mammalian cells, the Polycomb-group (Pc-G) protein complex and trithorax-group (trx-G) protein complex
epigenetically regulate the expression of essential developmental genes such as *Hox* (10).

The proteins required to maintain a repressed state are Pc-G complex, whereas those required for persistence of expression are trx-G complex. Pc-G and trx-G protein complexes do not contribute to the initiation of specific target genes but instead counteract each other to repair previously established chromosomal domains of specific genes throughout development (10).

Insight into the role of the trx-G protein complex in transcriptional regulation has came from studies of the *Drosophila Brahma* gene and its mammalian homologues *Bgm* and *BRG1*. The products of these genes have DNA-dependent ATPase activity and are classified as SWI2/SNF2 family proteins. The mammalian SWI/SNF chromatin remodeling complex contains either Brm or BRG1 but not both (11). The differences in biochemical function between Brm and BRG1 are largely unknown, however we observed the Brm or BRG1 subunit has distinct target specificity to facilitate gene activation through AP-1 (12). These findings provide mechanistic links between epigenetic transcriptional regulation and chromatin remodeling.

We herein describe retroviral gene silencing, which occurs very rapidly in a discontinuous and stochastic manner, in certain human tumor cell lines, and we show that
this phenomenon caused by lack of Brm gene expression in the cell. In addition, we present biochemical evidence suggesting that a trx-G protein complex (Brm-containing SWI/SNF subfamily) and a Pc-G protein complex (containing YY1 and HDACs) counteract each other to maintain expression of retrovirally introduced exogenous genes as was reported for endogenous genes (10).

**EXPERIMENTAL PROCEDURES**

*Cell Lines*--Human tumor cell lines SW13 (vim-) (13, adrenocortical carcinoma), SW620 (adrenocortical carcinoma), Saos2 (osteosarcoma), C33A (cervical carcinoma), H1299 (non-small cell lung carcinoma), MDA-MB435 (breast ductal carcinoma), G401 (rhabdoid tumor), PtG-S2 (14, prepackaging cell line for retrovirus vector production), Hela S3 (epitheloid carcinoma) and rat fibroblast cell line 3Y1 were maintained in high glucose DMEM (Gibco BRL., Gaithersburg, MD) supplemented with 10% fetal calf serum and incubated at 37°C. CHAP31 (15) (kind gift from Japan Energy Corp., Saitama, Japan) at 4-8 nM and trichostatin A (TSA; Sigma Chemical Co., St. Louis, MO) at 60-200 nM were added to inhibit HDAC, and 5-azacytidine (5-aza-C; Sigma) at 0.5-2 μM and 5-azadeoxycytidine (5-azadC; Sigma) at 0.5 μM was added to inhibit DNA
methylation.

**Plasmid Construction**--pBabe-hBrm-IRESpuro was generated by inserting the 4.8-kb *DraI-EcoRI* fragment of pSVhSNF2α (16) (encoding full-length Brm) into the *SnaBI-EcoRI* site of pBabe-IRESpuro (17). The 5.2-kb *SalI-NotI* fragment of pSVhSNF2β (16) (encoding full-length BRG1) was inserted into the *SalI-NotI* site of pGEX-4T3 (Amersham) to generate pGEX-4T3-hBRG1. The 2.5-kb *BamHI* fragment and the 2.5-kb *BamHI-StuI* fragment were isolated from pGEX-4T3-hBRG1 and inserted into the *BamHI-SnaBI* site of pBabe-IRESpuro to generate pBabe-hBRG1-IRESpuro. The 0.6-kb *ClaI-HindIII* fragment encoding hBrm C-terminal region was excised from pSVhSNF2a and ligated into the unique *ClaI-HindIII* site of pBluescript SK+ to generate pBS-hBrm, which is used as the template of the *Brm* probe.

**Production and Transduction of Vectors**--All retrovirus vectors were VSV-G pseudotyped and were produced as described previously (14). Control vector was produced with pBabe-IRESpuro (17). MFGnlsLacZ (14), which encodes LacZ with a nuclear localization signal, was used as the LacZ virus. For titration of Brm or BRG1 virus, expression of viral RNA was detected by *in situ* hybridization with *gag* probe (17), and positive clones were counted 2.5 days after the virus transduction into the same cell
lines to be used. To obtain single colonies, transduced cultures were trypsinized 1 day after transduction and seeded into collagen-coated, 90-mm dishes at dilutions to yield less than 400 colonies/dish. Fixed colonies were stained for detection of LacZ for LacZ virus or processed for in situ hybridization with the gag probe or the Brm probe for the other vectors (17).

Western Blotting--Whole cell extracts (20 µg) were prepared under denaturing conditions, separated by electrophoresis on 8%-SDS polyacrylamide gels, transferred to PVDF membranes, immunostained with anti-BRG1 rabbit polyclonal antibody (Santa Cruz, Biotechnology, Santa Cruz, CA), anti-Brm monoclonal antibody (Transduction Laboratories, Lexington, KY) or anti-Brm goat polyclonal antibody (Santa Cruz) and detected with an ECL kit (Amersham) as described previously (12).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)--Total RNA was prepared from cells with ISOGEN RNA isolation reagent (Wako Pure Chemicals, Tokyo, Japan). RT-PCR was performed within the linear range with Superscript One-step RT-PCR with Platinum Taq Kit (Life Technologies, Inc. Rockville, MD). The primer sets were as follows: 5'-ctggcctccccctggagccatgct-3' and 5'-aggccgggtcctgttgcggacac-3' for BRG1, and 5'-ctgcaagagcgggaatacagacttcaggcccg-3' and
5'-ggctgcctgggcttgcttgtgctcccaaacc-3' for Brm, and 5'-tcattgacctcaactacatggtttac-3' and 5'-ggcatggactgtggtcatgagtc-3' for GAPDH. RNA was reverse-transcribed for 30 min at 50°C. Amplification conditions were, an initial denaturation of 94°C for 3 min followed by 30 cycles (for Brm and BRG1) of 94°C for 30 s, 67°C for 2 min, and 72°C for 1 min or 25 cycles (for GAPDH) at 94°C for 30 s, 56°C for 1 min, and 72°C for 1 min.

Chromatin Immunoprecipitation (ChIP) Assay--ChIP assay was performed according to the manufacturer’s protocol (Upstate Biotechnology, Inc., Lake Placid, NY) except that the sonication condition was changed to five times for 20 s each at 10% output (BRANSON Model 250). Specific antibodies for immunoprecipitations were anti-BAF60a (Transduction Laboratories), anti-YY1 (Santa Cruz), anti-HDAC1 (Upstate Biotechnology), anti-HDAC2 (Santa Cruz) and anti-histone H1 (clone AE4, Upstate Biotechnology) antibodies. The antibody to tetra-acetylated histone H4 (on residues 5, 8, 12 and 16) (pan-acetylated H4) or antibodies to H4 acetylated on individual residues (H4-K5, H4-K8 and H4-K12, respectively) were purchased from Upstate Biotechnology. After protein-DNA crosslinks in the immunoprecipitates were reversed, DNA was extracted for the PCR. Conditions for semi-quantitative PCR were 32 cycles of 95°C for 30 s, 60°C for 1.5 min, and 72°C for 2 min. PCR primers were as follows. Primer
1, 5'-cctatttgaaccaatcag-3'; Primer 2, 5'-gccagatacagacttag-3'; Primer 3, 5'-aatgaagccacccacgtga-3' and primer 4, 5'-ggcgactcagtcaatcggag-3'. PCR products were visualized with SYBR Green I after 5% polyacrylamide gel electrophoresis or 1.2 % agarose gel electrophoresis. The band density was semi-quantified by densitometry (ATTO Printgraph).

RESULTS

Rapid Retroviral Gene Silencing in Human Brm-Deficient Tumor Cell Lines--We previously reported that transduction efficiency by VSV-G pseudotyped vector is quite high in cell lines originated from human solid tumors as well as murine fibroblasts (17, 18). Throughout this work, MOI (multiplicity of infection) is defined as the ratio of input infectious units (titrated on the corresponding cell line) to the number of cells used for the transduction. With the LacZ virus, we showed that the proportion of LacZ-expressing cells was dependent upon vector dosage following the equation $1-e^{1/MOI}$ in 3Y1, NIH3T3, and MIA PaCa-2 as well as several other human solid tumors including MDA-MB435 (17, 18 and our unpublished observations). Interestingly, however, we observed that in C33A and SW13 cells, the observed dose dependency differed from that predicted by the equation and
leveled off before the entire culture population expressed LacZ.

To closely examine why a significant cellular population in these C33A and SW13 cultures failed to express LacZ, these cells as well as MDA-MB435 (as positive control) were transduced with LacZ virus at a low MOI (about 0.4) to minimize the introduction of multiple proviral copies into a single cell. The transduced cell cultures were grown for 1 day to complete the retroviral integration and then, seeded at a low cellular concentration for single colony formation. The colonies formed 3 days after seeding were stained for LacZ to assess LacZ expression. An MDA-MB435 cell that was transduced with LacZ virus formed a colony in which all the progeny cells express lacZ (Fig. 1A). We define such colony as a “positive colony” (Fig. 1B). The cell that escaped from the transduction formed a colony that is exclusively composed of expression-negative cells. We define such colony as a “negative colony” (Fig. 1B). Surprisingly, most of the colonies formed by LacZ-virus transduced C33A and SW13 cells were composed of mixed populations of positive and negative cells (Fig. 1A). Such a colony was defined as a “mosaic colony” (Fig. 1B). When several C33A colonies were isolated in penicillin cups, separated into single cells by trypsinization, and cloned into 96 well plates, the cellular colonies produced either all provirus-positive progeny clones or all provirus-negative progeny clones as
judged by genomic PCR. These results negate the possibility that the mosaic colonies were formed by a mixed population of proviral-positive and -negative cells; the difference in LacZ expression in the mixed colonies are likely to represent retroviral gene silencing that occurred in a discontinuous and stochastic manner during cell proliferation as schematically illustrated in Fig. 1B.

To semi-quantify the extent of gene silencing, we tentatively defined “mosaic colony ratio” of a seeded culture after the transduction by dividing the number of mosaic colony by the sum of the number of positive colony and the number of mosaic colony. We determined the mosaic colony ratios for C33A, SW13, and MDA-MB435 and several other human tumor cell lines as well as murine fibroblast cell lines using the same LacZ virus. In most cell lines examined, including MDA-MB435, H1299, and SW620, HeLa S3 and 3Y1, the mosaic colony ratios were low and ranged from 0.02 to 0.15. C33A and SW13 cells, however, exhibited a high mosaic colony ratio (Fig. 2A). Two other cell lines, Saos2 and G401, showed similar properties to C33A and SW13 cells, although the mosaic colony ratio was lower.

Comparison of gene expression patterns among these cell lines might reveal host factors that inhibit or accelerate retroviral gene silencing. We were interested in Brm and
BRG1, which are the essential subunits of SWI/SNF chromatin remodeling complex and have DNA-dependent ATPase activity, as candidates for inhibitory factors. Expression of both proteins is reported to be absent or very low in both C33A and SW13 cells (19-22). Therefore, we screened the cell lines listed in Fig. 2A for Brm and BRG1 expression. BRG1 mRNA levels were assessed by semi-quantitative RT-PCR. BRG1 mRNA was nondetectable in both C33A and SW13 cells under this condition (30 cycles) (Fig. 2B), whereas BRG1 mRNA became detectable only in C33A by increasing the PCR amplification to 40 cycles (data not shown). These findings are consistent with the results of Western blotting analysis; BRG1 protein was not detected in SW13 cells and was low in C33A cells. All other cell lines were positive for BRG1 mRNA expression; however, H1299 cells were negative for BRG1 protein. These observations are consistent with a recent report that H1299 cells have a deletion in both BRG1 alleles that causes a frame shift in the coding region (23). The RT-PCR primer pair used here does not cover the deleted region.

Brm mRNA was not detected in C33A, SW13, Saos2, or G401 cells, but it was detected in the other cell lines examined here by PCR that amplification of the coding region of the Brm gene (Fig. 2B) as well as the 3’ non-coding region (data not shown).
Saos2 cells were previously reported to produce Brm by Western blotting analysis with a monoclonal antibody raised against Brm (21), and we were able to reproduce this finding. Since we found this monoclonal antibody to be weakly cross-reactive with BRG1, we used a non-cross-reactive goat polyclonal antibody and confirmed that these cell lines expressed no detectable Brm. Therefore the rapid retroviral gene silencing appears to be correlated with the absence of endogenous Brm expression (Fig. 2A), whereas loss of BRG1 does not appear to be related directly to retroviral gene silencing.

Vectors Encoding Brm, BRG1, and LacZ Can Initiate Exogenous Gene Expression in SW13 and C33A Cells, but Only Brm Vector Shows Prolonged Expression--Because C33A and SW13 cells have high mosaic colony ratios, we tested the possibility that the Brm deficiency in these cells causes retroviral gene silencing. If initial processes in the retroviral infectious cycle including viral entry, proviral integration, and initiation of LTR-driven exogenous gene expression are not disturbed in these host cells, a retrovirus vector encoding Brm would be expected to prolong viral RNA expression by the Brm protein produced initially in an early stage of infection. An autoregulatory loop would support continuous expression of Brm thereafter. To test this possibility, C33A and SW13 cells were transduced with Brm virus or control virus at a low MOI. One day after
transduction, cultures were trypsinized and seeded at a low cell number for single colony formation. When viral mRNA levels in C33A cells were examined by in situ hybridization 7 days after transduction with Brm virus using the gag probe (Fig. 3) or the Brm probe (data not shown), viral mRNA-positive cells were found predominantly in positive colonies (mosaic colony ratio of 0.32). In contrast, control virus yielded primarily “mosaic colonies” (mosaic colony ratio of 0.71) and BRG1 virus yielded predominantly “mosaic” colonies (mosaic colony ratio of 0.89) when detected by the gag probe. It is also noteworthy that most of the mosaic colonies formed by Brm virus-transduced C33A mainly composed of transgene-expressing cells, while control virus- (or BRG1 virus-) transduced C33A has much lower population of transgene-expressing cells (Fig. 3). These observations further confirm that Brm expression recovers the retroviral gene silencing very efficiently.

The above findings support the hypothesis that Brm expression in host cells is necessary to overcome retroviral gene silencing. Similar results were obtained in SW13 cells; the mosaic colony ratio of SW13 cells transduced with Brm virus and control virus was 35% and 79%, respectively, 7 days after transduction (Fig. 3). BRG1-expressing SW13 cells assumed a flat cellular morphology and formed colonies of less than 10 cells
and therefore mosaic colony ratio was not accessed. This observation is consistent with the previous report that BRG-1 has strong growth inhibitor effects in SW13 (23).

**Retroviral Gene Silencing in SW13 or C33A Cells Can Be Partially Released by Inhibitors of HDAC but Not DNA Methyltransferase**—To elucidate the molecular mechanisms involved in retroviral gene silencing in Brm-deficient cell lines, we used several inhibitors of HDACs (CHAP31 and TSA) or DNA methyltransferase (5-aza-C or 5-azadC). When SW13 cells were treated with CHAP31 for 2 days after transduction with LacZ virus, the percentage of positive colonies in the total colonies increased in a CHAP31-dose-dependent manner and the percentage of mosaic colonies in the total colonies was slightly increased (Fig. 4). As schematically shown in Fig.1B, if the retroviral gene silencing were to be partially alleviated by this HDAC inhibitor, “mosaic colony” would become “positive colony”. At the same time, an “apparently negative colony” (where all the component cells ceased expressing transgene within three circle of cell division) would be present, it will become “mosaic colony”. Only slight increase in percentage of mosaic colony observed in Fig 4 would be explained by this scheme. PCR analysis indicated that 47% of cellular clones derived from a parallel culture of LacZ virus-transduced SW13 cells harbored proviral DNA (Fig. 4, arrow). This observation
indicates that not all the SW13 cells harboring the provirus can express LacZ even in the presence of 4 nM of CHAP31, further confirming that this inhibitor did not completely released the gene silencing.

Similar results were obtained when cells were treated with 60 nM TSA; however strict quantitation was hampered by the cytotoxic effect of the reagent on diluted cellular clones. On the other hand, treatment with 5-azadC (0.5 µM) or 5-aza-C (2 µM) for 2 days did not increase the percentage of either positive or mosaic colonies among the total colonies. In C33A cells, similar release from gene silencing was also detected by the treatment with 4 nM of CHAP31 but treatment with 5-azadC (0.5 µM) or 5-aza-C (2 µM) caused no changes in the percentage of either “mosaic colony” or “positive colony” as was shown in SW13 cells. These findings suggest that histone acetylation is involved in retroviral gene silencing in Brm-deficient cell lines and that the process does not involve a mechanism mediated through CpG methylation.

*Brm-Containing SWI/SNF Complex Inhibits Recruitment of YY1 Complex and Enhances Acetylation of Specific Lysine Residues in Histone H4 in the 5’LTR Region--Since* HDAC inhibitors recovered retroviral gene silencing in Brm-deficient cell lines, it might be possible that SWI/SNF complex normally induces histone acetylation in the 5’LTR region
of the provirus. Since targeted acetylation of histone H4 tails is likely to be one of the major factor in the regulation of either cellular (24-27) or viral (8) gene expression, we next analyzed whether the acetylation pattern of histone H4 at this locus can be modulated by the exogenous expression of Brm in these cells by using chromatin immunoprecipitation (ChIP)-PCR assay (Figure 5). In Brief, C33A cells transduced with either control virus or Brm virus were fixed by crosslinking with formaldehyde. Cells were isolated and lysed, and chromatin was sonicated to generate fragments with average size around 500 base pairs. Chromatin fragments were then immunoprecipitated with the antibody against tetra-acetylated histone H4 (pan acetylated H4) or with antibodies specific for individually acetylated lysine residues (K5, K8, or K12) of histone H4. After protein-DNA crosslinks in the immunoprecipitates were reversed, DNA was extracted and analyzed for the presence of 5’LTR using a set of primers that do not detect the 3’LTR (primers 1 and 2). Using the antibody against tetra-acetylated histone H4, no significant difference was detected between Brm expressing and non-expressing cells. Using antibodies of individually acetylated lysine residues, significant enhancement in acetylation on K5 and K8 was observed in Brm virus transduced C33A, while acetylation on K12 was not significantly modulated by the retrovirally induced Brm expression.
Considering the possibility that SWI/SNF complex counteracts the Pc-G protein complex to suppress gene silencing, we next examined whether recruitment of Pc-G protein complexes to the LTR is modulated by Brm-containing SWI/SNF complex. Among mammalian Pc-G, only YY1 is currently known to have specific DNA-binding activity, and an YY1 binding site is present in the 5′-region of the MuLV-LTR (28) (Fig. 5). By ChIP-PCR assay using the same cellular extracts described above, we examined whether the amount of YY1 in the 5′LTR is reduced by the expression of Brm. In the immunoprecipitates with anti-YY1 antibody, there was a lower amount of 5′LTR sequence in C33A cells transduced with Brm virus compared with C33A cells transduced with control virus.

Since HDAC1 and HDAC2 have been reported to form large Pc-G protein complexes with YY1 (29,30), we next analyzed these two proteins in the 5′LTR. In Brm virus-transduced C33A cells, the amounts of HDAC1 and HDAC2 were also reduced. Since the PCR product of the primer pair 1 + 2 does not include the YY1 recognition site, another primer pair 3 + 4 was used to cover the YY1 recognition site, although it can not distinguish the 5′-LTR and 3′-LTR. The results were similar to those obtained with the primer pair 1 + 2(Fig.5). The linker histone, histone H1, known to be associated with
inactive chromatine, also reduced by the introduction of *Brm* (Fig. 5). These data are consistent with the notion that when the provirus MuLV-LTR integrates into C33A cells, Pc-G protein complexes including YY1, HDAC1, and HDAC2, are efficiently recruited to silence retroviral gene expression by deacetylating specific lysine residues in histone H4 and this suppressive effect can be efficiently counteracted by functional SWI/SNF complex.

In the U3 region of the MuLV-LTR, there are binding sites for transcriptional factors such as C/EBP (31) and c–Myc (32), which are reported to recruit SWI/SNF complex. Therefore, we attempted to detect recruitment of SWI/SNF complex subfamily members to the LTR. For chromatin immunoprecipitation analysis, we used anti-BAF60a antibody to precipitate SWI/SNF complex because BAF60a was reported to be present in both Brm-containing and BRG1-containing SWI/SNF complexes and because similar levels of this protein were detected in both C33A cells transduced with control virus and with Brm virus. We detected greater amounts of LTR sequence in immunoprecipitates of Brm virus-transduced C33A cells than in those from control virus-transduced cells. However, recovery of LTR sequences with anti-BAF60a was much lower than that with Pc-G protein complexes (unpublished data). This may be partly because the interaction between the SWI/SNF complex and DNA is not direct and is mediated through transcription factors.
DISCUSSION

Human tumor cell lines deficient in Brm allow retrovirus vectors to enter, integrate, and initiate exogenous gene expression, yet rapid retroviral gene silencing is induced. The Brm-containing SWI/SNF subfamily inhibited recruitment of Pc-G complexes composed of YY1, HDAC1, and HDAC2 to the LTR region of the integrated provirus, leading to the enhancement of histone H4 acetylated on lysine 5 and lysine 8. Consistent with these findings, HDAC inhibitor treatment partially released the retroviral gene silencing observed in Brm-deficient cell lines. Although the biological significance of this phenomenon is not fully clear at this moment, the observation that Brm but not BRG1 is undetectable in mouse embryonic stem cells (33) may provide a clue. Since retroviral gene expression is strongly suppressed by both DNA methylation-dependent and -independent pathways in mouse embryonic stem cells (6), it is tempting to speculate that the lack of Brm gene expression in this cell type contributes to gene silencing in a DNA methylation-independent manner.

Gene silencing was observed as a reduction in the ratio of cells expressing exogenous genes in transduced cultures and not as a reduction in the average expression
levels in the entire culture, showing that silencing occurs in a discontinuous and stochastic manner as schematically illustrated in Figure 1B. In C33A and SW13 cells, the mosaic colony ratios were reduced when cells were to transduce with the LacZ virus at higher MOIs (unpublished observation), suggesting that proviral copy number rather than cellular physiology determines the degree of transcriptional down-regulation. Therefore, we believe the variegated gene silencing observed here reflects drastic structural changes that occurred around the proviral LTR and that these structural changes are the all-or-none result of the opposing actions of trx-G and Pc-G protein complexes.

Considering the fact that the retroviral gene silencing observed in Brm deficient cell lines was efficiently suppressed by Brm introduction (Fig. 3), it is worth pointing out that the silencing was not fully recovered by the treatment with the HDAC inhibitor, CHAP31 alone (Fig.4). This might suggest the possibility that Brm have some additional biochemical roles other than counteracting HDAC activity as a component of SWI/SNF complex in the chromosome (Fig.5). The search for such biochemical functions of Brm, an ATPase with a putative helicase motif, would be important.

Retrovirus vectors have been designed recently to overcome chromosomal position effects (34,35). For example, the chicken hypersensitive site 4 (cHS4) of the
chicken globin LCR, which acts as an insulator, was cloned into MuLV-LTR. In some cell lines, the probability of integrated proviral gene expression increased, and the level of \textit{de novo} methylation of the 5'LTR in the cells decreased. Insulators are thought to protect adjacent DNA sequences from gene silencing induced by heterochromatin, which is associated with the products of the HP-1 gene family (36). However, this gene family does not usually overlap with Pc-G or trx-G. Moreover, Pc-G proteins, unlike the HP-1 family proteins, do not appear to favor areas in the nucleus where there are visible compactions of chromatin (37). These observations suggest that the molecular mechanisms involving HP-1 family proteins and Pc-G proteins are different. Our finding that the counteraction between Pc-G protein complex and trx-G protein complexes contributes to retroviral gene silencing is likely to provide information for designing new types of retrovirus vectors for long-term expression.

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FOOTNOTES

The abbreviations used are: Pc-G, Polycomb-group; trx-G, trithorax-group; HDAC, histone deacetylase; CHAP, Cyclic hydroxamic-acid-containing peptide; TSA, trichostatin A; 5-aza-C, 5-azacytidine; 5-azadc, 5-azadeoxycytidine; RT-PCR, reverse transcription - polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ChIP, chromatin immunoprecipitation; MOI, multiplicity of infection.

FIGURE LEGENDS

Fig. 1. Rapid silencing of retroviral gene expression was observed in some human cell lines. (A) Cells were transduced by retrovirus at a low MOI (0.4) to minimize the introduction of multiple proviral copies. One day after the transduction, the transduced culture was trypsinized and seeded at a very low cell density for the single colony formation. C33A and SW13 cells transduced with LacZ virus formed mosaic colonies as evidenced by LacZ expression on day 3 after seeding. In LacZ-transduced MDA-MB435
cells, primarily positive colonies were observed. The bar indicates 0.1 mm.

(B) A schematic presentation of a model of retrovirus gene silencing observed in (A).

Bold arrows indicate the virus transduction to a single cell. Black circles indicate cells expressing the exogenous gene introduced by retroviral vector, while white circles show non-expressing cells. Cellular divisions of three cycles were illustrated here. In most cell lines (such as MDA-MB435), the transduced cells keep expressing the exogenous gene even after the cell divisions as shown in A. Such a cellular colony is expected to be exclusively composed of transgene-expressing cells and is defined as a “positive colony”. A cell that escaped from the transduction, of course, will give rise to a colony where all the cells are not expressing the transgene (defined as a “negative colony”). However, the transduced SW13 (or C33A) formed colonies that are composed of both transgene-expressing cells and transgene-non-expressing cells. We defined this colony as a “mosaic colony”. We hypothesized that in SW13 or C33A, the transgene expression was silenced in a discontinuous and stochastic manner. For semi-quantification of the extent of gene silencing, we tentatively defined “mosaic colony ratio” of a seeded culture after the transduction by dividing the number of “mosaic colony” by the sum of the number of “positive colony” and the number of “mosaic colony”.

Fig. 2. **Mosaic colony ratios of several cell lines derived from human tumors and their expression levels of Brm and BRG1.** (A) A culture of each cell line was transduced with LacZ virus at a low MOI, seeded 1 day after transduction, and stained with LacZ 3 days after seeding. The mosaic colony ratio was calculated by dividing the mosaic colony number by the sum of the mosaic colony number and positive colony number. Bars represent SD. Expression status for Brm and BRG1 evaluated in (B) is summarized at the right. (B) Expression levels of *BRG1* and *Brm* mRNA as estimated by semi-quantitative RT-PCR and expression levels of BRG1 and Brm protein as estimated by Western blotting of total cellular proteins. PCR products were separated by PAGE and stained with SYBR Green. Arrows indicate the expected sizes of the PCR products; 638 bp for *BRG1*, 633 bp for *Brm*, and 431 bp for *GAPDH*. Arrowhead indicates the position of BRG1 (190k Da) and Brm (190k Da).

Fig. 3. **Viral mRNA expression in colonies derived from C33A cells transduced with control virus, Brm virus, and BRG1 virus.** Cultures were transduced with each virus
at an MOI of approximately 0.4, seeded 1 day after transduction for single colony formation, and fixed on day 7 after seeding for in situ hybridization. Viral gene expression was assessed by in situ hybridization (blue), and cell cytoplasm was stained by eosin (pink). Only clones that contain viral mRNA-expressing cell(s) are shown. P and M indicate the colonies judged as “positive colony” and “mosaic colony”, respectively. Bar, 0.1 mm.

Fig. 4. Effect of CHAP31 treatment on percentage of positive, mosaic and negative colonies in the total colonies formed by SW13 cells transduced with LacZ. Transduced cultures were seeded as described in the legend of Fig.2 (A). Colony status was determined day 3 after seeding. CHAP31 was added at the indicated concentrations 48 hrs before the LacZ staining. The arrow indicates the percentage of clones harboring proviral DNA in the total colonies formed by the same transduced culture (47%).

Fig. 5. ChIP assay of C33A cells transduced with control virus or Brm virus. Upper panel, a schematic representation of LTRs shown together with the two pairs of PCR
primers (1 and 2; 3 and 4) and the positions of enhancers in the U3 region. Y; YY1, C/E; C/EBP; T; TATA box, and PBS; primer binding site. The PCR products, 407 bp (for 1 and 2) and 478 bp (for 3 and 4) were separated by PAGE and by agarose gel, respectively and stained with SYBR Green. Input corresponds to 12% of cell lysates used for the immunoprecipitation. C and M indicates samples from control virus-transduced C33A and Brm virus-transduced C33A, respectively. M/C indicates the ratio of the band density of Brm virus-transduced C33A as compared with that of control virus-transduced C33A. For this quantification, the average ratios of the band density were calculated from two independent experiments from the cellular extract preparation.
(A) 

| Cell Line      | Colonies                |
|----------------|-------------------------|
| C33A           | Negative colony         |
| SW13           | Positive colony         |
| MDA-MB435      | Mosaic colony           |

(Gene Silencing)

No Gene Silencing

Addition of HDAC inhibitors

Positive colony

Negative colony

Apparent Negative colony

Mosaic colony

(B) 

![Diagram](http://www.jbc.org/)

Addition of HDAC inhibitors
### Mosaic colony ratio (%)

| Cell line | 0  | 20 | 40 | 60 | 80 | 100 | Expression |
|-----------|----|----|----|----|----|-----|------------|
| C33A      |    |    |    |    | +  |     | ±          |
| SW13      |    |    |    |    | +  |     | -          |
| Saos2     |    |    |    |    | +  |     | + M       |
| G401      |    |    |    |    | +  |     | +          |
| H1299     |    |    |    |    | +  |     | +          |
| SW620     |    |    |    |    | +  |     | +          |
| MDA-MB435 |    |    |    |    | +  |     | +          |

### Expression

- **Brm**:
  - C33A: +
  - SW13: ±
  - Saos2: ±
  - G401: +
  - H1299: +
  - SW620: +
  - MDA-MB435: +

- **BRG1**:
  - C33A: ±
  - SW13: -
  - Saos2: ±
  - G401: ±
  - H1299: +
  - SW620: +
  - MDA-MB435: +

### (A)

#### RT-PCR

- **Brm**: C33A, SW13, Saos2, G401, H1299, SW620, MDA-MB435

- **BRG1**: C33A, SW13, Saos2, G401, H1299, SW620, MDA-MB435

- **GAPDH**: C33A, SW13, Saos2, G401, H1299, SW620, MDA-MB435

### (B)

#### Western blotting

- **Brm**: C33A, SW13, Saos2, G401, H1299, SW620, MDA-MB435

- **BRG1**: C33A, SW13, Saos2, G401, H1299, SW620, MDA-MB435
| Virus | Control | Brm | BRG |
|-------|---------|-----|-----|
|       | ![Control Image](image1) | ![Brm Image](image2) | ![BRG Image](image3) |

**Fig. 3**

- Control
- Brm
- BRG
Fig. 4

The graph shows the colony status (%) in relation to CHAP31 concentration (nM). The x-axis represents CHAP31 concentration ranging from 0 to 4 nM, while the y-axis represents colony status ranging from 0 to 50%. Three types of colonies are plotted:

- **Negative colony**
- **Mosaic colony**
- **Positive colony**

The graph indicates an increase in colony status with increasing CHAP31 concentration, particularly for the positive colony.
Maintenance of integrated proviral gene expression requires Brm, a catalytic subunit of SWI/SNF complex
Taketoshi Mizutani, Taiji Ito, Mitsue Nishina, Nobutake Yamamichi, Akiko Watanabe and Hideo Iba

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