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TRIM28 Mediates Primer Binding Site-Targeted Silencing of Murine Leukemia Virus in Embryonic Cells

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SUMMARY

Moloney murine leukemia virus (M-MLV) replication is restricted in embryonic carcinoma (EC) and embryonic stem (ES) cells, likely to protect the germ line from insertional mutagenesis. Proviral DNAs are potently silenced at the level of transcription in these cells. This silencing is largely due to an unidentified *trans*-acting factor that is thought to bind to the primer binding site (PBS) of M-MLV and repress transcription from the viral promoter. We have partially purified a large PBS-mediated silencing complex and identified TRIM28 (Kap-1), a known transcriptional silencer, as an integral component of the complex. We show that RNAi-mediated knockdown of TRIM28 in EC and ES cells relieves the restriction and that TRIM28 is bound to the PBS in vivo when restriction takes place. The identification of TRIM28 as a retroviral silencer adds to the growing body of evidence that many TRIM family proteins are involved in retroviral restriction.

INTRODUCTION

Moloney murine leukemia virus (M-MLV) is a prototypical simple retrovirus and replicates efficiently in most dividing cells that express the virus receptor. Virus replication is restricted, however, in mouse embryonic carcinoma (EC) and embryonic stem (ES) cells (Barklis et al., 1986; Teich et al., 1977). Although infection of EC or ES cells by M-MLV results in the successful integration of the proviral DNA into the genome, transcription from the viral promoter in the long terminal repeat (LTR) is potently silenced. This transcriptional silencing is attributed in part to reduced transcription factor binding to the viral enhancers in the LTR (Hilberg et al., 1987; Linney et al., 1984) and in larger part to the presence of repressive *trans*-acting factors in these cell types (Akgun et al., 1991; Flanagan et al., 1989; Tsukiyama et al., 1989). A major target of this repression is the repressor binding site (RBS), a DNA element of 17 bp contained within the 18 bp encoding the primer binding site (PBS) near the 5′ end of the M-MLV RNA genome (Barklis et al., 1986; Feuer et al., 1989; Loh et al., 1987). The PBS of the M-MLV genome is complementary to 18 nucleotides at the 3′ end of the host proline tRNA and is a critical sequence for virus replication. The $tRNA_{pro}$ is annealed to the PBS in the RNA genome at the time of virus assembly and, upon infection, is used as the primer for minus-strand DNA synthesis during reverse transcription (Harada et al., 1979). The mechanism of RBS-mediated transcriptional repression is unknown but is thought to act as a DNA element in the context of the integrated provirus. Therefore, RBS function is thought to be distinct from the role of the PBS in tRNA annealing and priming on the viral RNA. Evidence has been obtained that the RBS is able to repress LTR-driven transcription in an orientation- and position-independent fashion and can function even when placed outside of the transcriptional unit (Loh et al., 1990; Petersen et al., 1991). Subsequent to this immediate transcriptional repression, the provirus is frequently further silenced by DNA methylation (Niwa et al., 1983). In EC cells the RBS sequence can repress nearby heterologous promoters such as the SV40 early and adenovirus major late promoters, and such repression is not blocked by a variety of known DNA insulator sequences (Modin et al., 2000b). The repression mediated by the RBS can be relieved by a single G to A base pair mutation, known as the B2 mutation, within the DNA sequence encoding the PBS (Barklis et al., 1986). The repression can also be relieved by replacing the PBS of M-MLV with a sequence complementary to another cellular tRNA such as glutamine (Gln) tRNA (Grez et al., 1990; Petersen et al., 1991).

There is strong evidence that the RBS mediates its repressive activity through a *trans*-activating DNA-binding factor. The repressive activity of the RBS is saturable by transfection of increasing amounts of DNA containing the RBS sequence (Loh et al., 1988). The presence of a binding factor has also been detected in EC cell nuclear extracts using a probe spanning the PBS sequence of M-MLV by exonuclease III protection assays (Loh et al., 1990). This exonuclease III protection footprint is reduced

upon either differentiation of the EC cells or the use of a probe containing the B2 mutation, strongly suggesting that the presence of this factor correlates with repression. Similar experiments performed by electrophoretic mobility shift assay (EMSA) using M-MLV PBS sequences as a probe have shown that DNA-binding activity was enriched in nuclear extracts from EC cells when compared with differentiated cell lines (Petersen et al., 1991).

RBS-mediated repression of M-MLV is not limited to mouse ES and EC lines but has been observed in early mouse embryos as well as primary stromal cells from mouse bone marrow (Haas et al., 2003; Vernet and Cebrian, 1996). Most intriguingly, it has also been observed to occur in a human hematopoietic cell line and primary human hematopoietic cells isolated from umbilical cord blood (Haas et al., 2003). This raises the possibility that viruses such as human T-lymphotropic virus type 1 (HTLV-1) that also use a $tRNA_{pro}$ PBS sequence might be targeted for repression in pluripotent cells. Other PBS sequences have also been shown to be specifically repressed in EC cells, such as the PBS for lysine-1,2 tRNA found in Mason-Pfizer monkey virus, visna virus, and spumavirus. This raises the possibility that specific cellular repressors may also target these viruses for silencing (Modin et al., 2000a; Yamauchi et al., 1995). There is a rationale for the evolution of repression at PBS elements: during reverse transcription, the PBS sequence on one strand of the viral DNA is actually synthesized by reverse transcription of the cellular tRNA itself. Thus, the viral PBS sequence is a uniquely effective target site for repression by the host since any escape point mutations in the PBS sequence would quickly revert to the original sequence during viral replication.

Previous attempts to purify the RBS binding factor by biochemical means have been unsuccessful due to the factor's apparent instability. However, it appeared that such purification might be possible if biochemical conditions were found under which the factor was stable (Yamauchi et al., 1995). We undertook a search for such conditions, partially purified the factor, successfully identified a cellular component of the complex, and demonstrated its requirement for RBS-mediated silencing.

RESULTS

Purification of the RBS Complex

To characterize the RBS binding factor, nuclear extracts were prepared from the repressing F9 EC cell line and differentiated control lines, and RBS binding activity was detected by electrophoresis mobility shift assays (EMSA) using a 28 bp ^{32}P-labeled probe containing the M-MLV PBS. This probe has been previously been used to detect RBS binding activity in F9 and other EC cell lines, and the resulting shift was shown to be sensitive to the B2 mutation and to competition by cold probe (Petersen et al., 1991; Yamauchi et al., 1995). Extracts of F9 cells contained significant binding activity, producing a single shifted band of very low mobility (Figure 1A). NIH/3T3 cells yielded very low levels of binding activity, and HeLa, RAT2, and 293A cells showed no detectable activity.

The F9 nuclear extracts were fractionated by a variety of procedures, and the fate of the RBS binding activity was monitored by EMSA. When the crude lysates were treated with increasing concentrations of ammonium sulfate, the majority of the RBS binding activity was recovered in the precipitate from the 40% ammonium sulfate fraction (Figure 1B). To purify the activity further, the precipitate was dissolved in 1 M ammonium sulfate and applied to a butyl sepharose hydrophobic interaction column, and the bound proteins were eluted with a decreasing salt concentration gradient. The bulk of the activity eluted in a relatively broad peak centered at 0.7–0.4 M ammonium sulfate (Figure 1C). Proteins in fractions 13–40 from this column were then concentrated and applied to a Sephacryl S-500 gel filtration column (Figure 1D). The activity eluted from this column in a single relatively tight peak, with the majority found in fractions 17–21 (Figure 1D). The behavior in this gel filtration step suggested that the RBS binding factor was large. Superose 6 gel filtration was performed to obtain a better estimate of the size of the complex. The RBS binding factor eluted from these columns in a broad peak, indicative of an apparent size ranging from about 2 to 0.7 MDa, and centered on approximately 1.3 MDa (see Figure S1A in the Supplemental Data available with this article online). The large size suggests that the activity probably resides in a multisubunit complex. These various chromatographic steps were assembled into a final purification scheme used routinely for preparation of the activity (Figure 1E).

Identification of TRIM28 as a Possible Component of RBS Complex

To identify possible components of the complex, the most highly purified fractions were probed by EMSA with the 28 bp M-MLV PBS DNA, and the shifted band defining the complex was excised from the gel. The proteins associated with the DNA were analyzed by mass spectroscopy (Table 1). The major proteins identified were tripartite motif protein 28 (TRIM28, Kap-1, Tif1-beta) and valosin containing protein (VCP, p97). VCP is an extremely abundant protein accounting for 1% of the total cellular protein, is predominantly localized to the cytoplasm, and is involved in a variety cellular processes including endoplasmic reticulum (ER)-associated degradation, apoptosis, and membrane fusion (Wang et al., 2004). Due to its cytoplasmic localization, VCP was unlikely to be biologically relevant to RBS-mediated repression and, because of its high abundance in the cell, was assumed to be a contaminant. The TRIM28 protein, 89 KDa in size, is a member of the tripartite motif (TRIM) family and contains three characteristic RING finger, B box, and coiled-coil motifs at its N terminus. TRIM28 functions as corepressor with the Krüppel-associated box (KRAB) zinc finger DNA-binding proteins (Friedman et al., 1996) and acts by bridging the KRAB domain of the finger proteins to several known transcriptional corepressors, including the NuRD histone deacetylase

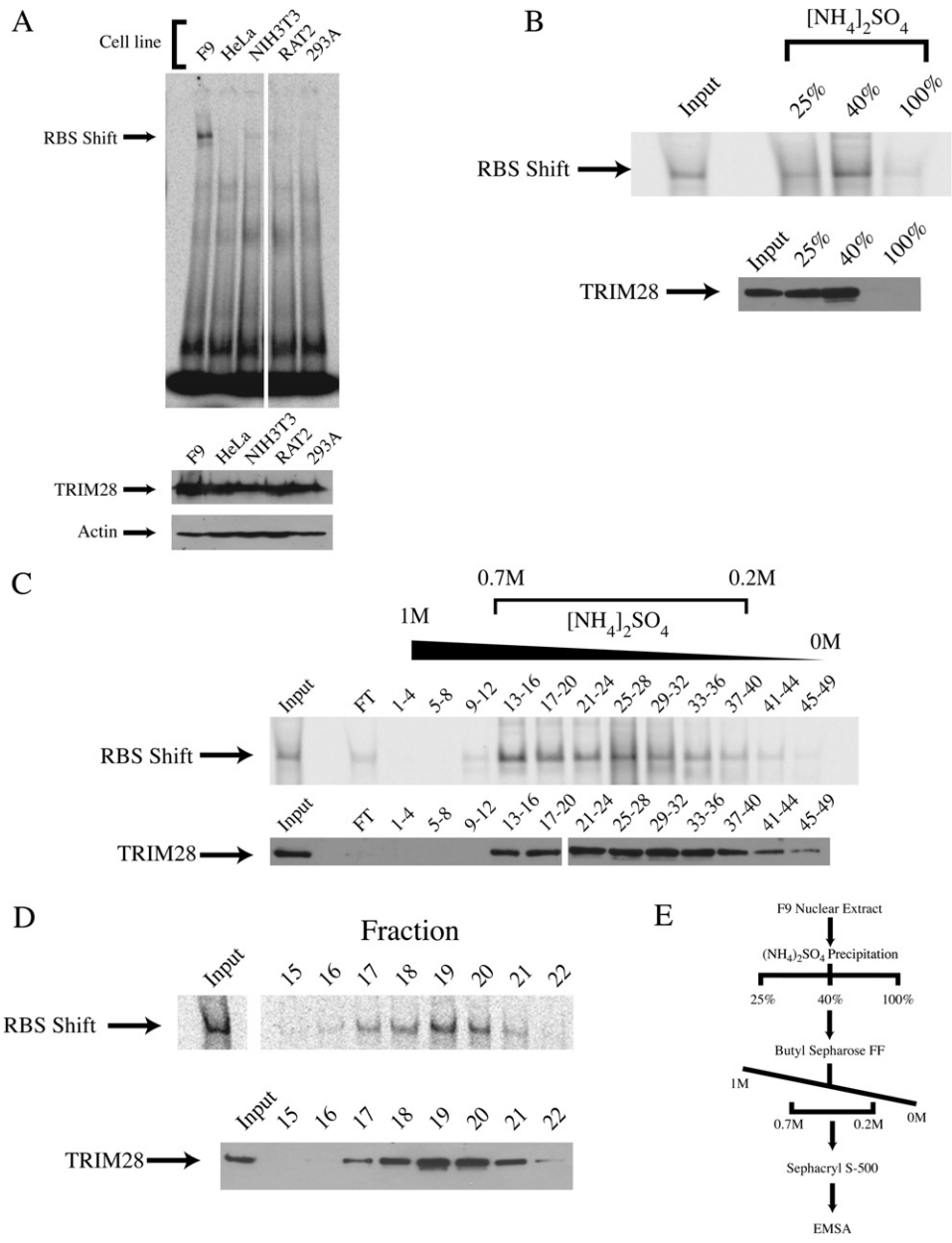

Figure 1. Biochemical Purification of the RBS Binding Complex from F9 Nuclear Extracts

(A) Upper panel: nuclear extracts from F9, HeLa, NIH 3T3, RAT2, and 293A were prepared and used in EMSA with a ^{33}P-labeled 28 bp probe corresponding to the WT M-MLV RBS sequence. Lower panels: western blots of the same nuclear extracts probed with anti-TRIM28 (ab22553) and anti-actin antibodies.

(B) Upper panel: EMSA performed as above, on 25%, 40%, and 100% ammonium sulfate fractions of F9 nuclear extracts. Lower panel: anti-TRIM28 western blot of same fractions.

(C) EMSA performed as above on fractions from butyl sepharose FF fractionation of 40% ammonium sulfate cut from (B). Lower panel: anti-TRIM28 western blot of same fractions.

(D) EMSA performed as above on Sephacryl S-500 fractions from fractionation of 13–40 from (C). Lower panel: anti-TRIM28 western blot performed on same fractions.

(E) Graphical representation of final purification scheme used.

complex, the histone H3 K9 methyltransferase ESET, and HP1 (Le Douarin et al., 1996; Schultz et al., 2001, 2002). Remarkably, the transcriptional repression induced by TRIM28 is heritable over many cell cycles, even after the KRAB box DNA-binding protein targeting TRIM28 to a promoter is no longer present, suggesting that TRIM28 is involved in the recruitment of proteins required for heritable gene silencing (Ayyanathan et al., 2003). TRIM28 is also

Table 1. List of Gene Products Identified by lc-ms-ms Mass Spectroscopy

Protein Identified	No. Peptides Matched
Tripartite motif protein 28 (TRIM28)	8
Valosin-containing protein (VCP/p97)	9
Albumin	1
Ribosomal protein L14	1

List of gene products identified from lc-ms-ms mass spectroscopy of excised EMSA band using 28 bp RBS sequence as probe. The number of different peptides of each protein identified is shown in right column. Proteins are listed in order of statistical probability of correct match.

required for the differentiation of EC cells, as F9 EC cells expressing a TRIM28 mutant defective for association with HP1 are not able to differentiate correctly (Cammas et al., 2004). TRIM28 is therefore an attractive candidate as a component of the RBS silencer complex.

To confirm that TRIM28 was a plausible member of the RBS complex, western blots were performed on the fractions collected during its biochemical purification to determine whether the presence of TRIM28 tracked with the presence of the RBS binding activity. In the ammonium sulfate precipitation, levels of TRIM28 correlated well with levels of binding activity, and both the majority of the RBS binding factor and the TRIM28 was recovered in the 40% fraction (Figure 1B). In the hydrophobic interaction column, the levels of TRIM28 and RBS binding activity correlated well, with both enriched in fractions 13–40 (Figure 1C). Finally, in the gel filtration column, the abundance of TRIM28 tracks nearly perfectly with the RBS binding activity, with fractions 17–21 having the majority of both TRIM28 and binding activity (Figure 1D). These results are consistent with TRIM28 being present in the complex.

TRIM28 Is an Integral Part of the RBS Binding Complex

To determine whether TRIM28 is a true component of the RBS binding complex, a series of EMSA reactions were performed using F9 nuclear extracts and the 28 bp PBS sequence as probe. To these binding reactions, an anti-TRIM28 or anti-FLAG control antibody was added in an attempt to supershift the RBS complex to a lower mobility species. In the presence of increasing TRIM28 antibody and not the control antibody, the complex was indeed supershifted (Figure 2A, lanes 5–7). Furthermore, competition with an excess of either WT or B2 mutated cold DNA showed that this supershift was specifically competed by the WT PBS sequence (Figure 2A, lanes 14–15). The supershift experiments demonstrate that TRIM28 is an integral component of the RBS complex. To extend this observation to other cell lines, supershifts were also performed using nuclear extracts from PCC4 cells, another EC line previously shown to possess RBS activity (Petersen et al., 1991), and from the ES cell line JM1. In both cases, the RBS complex was observed to be supershifted by an anti-TRIM28 antibody and thus contains TRIM28 (Figure S2).

To test whether TRIM28 was stably associated with the components of the RBS complex, the F9 nuclear extracts were immunodepleted by addition of anti-TRIM28 antibodies followed by removal of the antibody on protein A/G sepharose beads. Tests of the resulting depleted extracts showed that immunodepletion with polyclonal TRIM28-specific antisera, but not with control antisera, led to complete removal of RBS binding activity from F9 nuclear extracts (Figure 2B). To further test if the intact RBS complex was indeed bound to the TRIM28-specific antibodies, we asked whether RBS binding activity could be recovered from the protein A/G sepharose beads used in a successful TRIM28 immunodepletion. Such sepharose beads were added directly to an EMSA binding reaction and low levels of RBS binding activity were indeed detectable (Figure 2C). Presumably, TRIM28 and the complete RBS binding complex was able to dissociate from the TRIM28 antibody and bind to the DNA probe. This suggests that at least a portion of the TRIM28 remains stably associated with all the components of the RBS binding complex.

Differentiation of F9 Cells Leads to a Decrease of TRIM28 Levels and a Concomitant Loss of RBS Activity

Differentiated cell lines do not restrict M-MLV in an RBS-dependent manner (Petersen et al., 1991). In addition, differentiation of EC cells by retinoic acid (RA) followed by treatment with the DNA demethylating agent 5-azacytidine (azaC) leads to reactivation of silenced M-MLV proviruses, whereas azaC treatment alone does not (Niwa et al., 1983). These results suggest that differentiation of F9 cells should lead to loss of RBS binding activity. To test this notion, F9 cells were treated with RA at 1 μM concentration for either 2, 4, or 7 days. Nuclear extracts were prepared from these cells (as well as from time matched control cells), and these extracts were used in EMSA reactions with the 28 bp MuLV PBS probe. As RA-induced differentiation progressed, RBS binding activity decreased and, after 7 days, was completely absent (Figure 3A). It has been previously been shown that differentiation of F9 cells into primitive endoderm-like cells with RA can lead to a decrease in steady-state TRIM28 levels (Cammas et al., 2004). To test for this decrease in TRIM28 in differentiated F9 cells and to test whether the decrease of TRIM28 levels correlated with the disappearance of RBS binding activity, the same nuclear extracts were analyzed for TRIM28 levels by western blot (Figure 3A). As expected, the steady state levels of TRIM28 decreased during differentiation and, after 7 days, were almost completely absent. These results suggest that the loss of TRIM28 may be responsible for the loss of RBS activity in these cells.

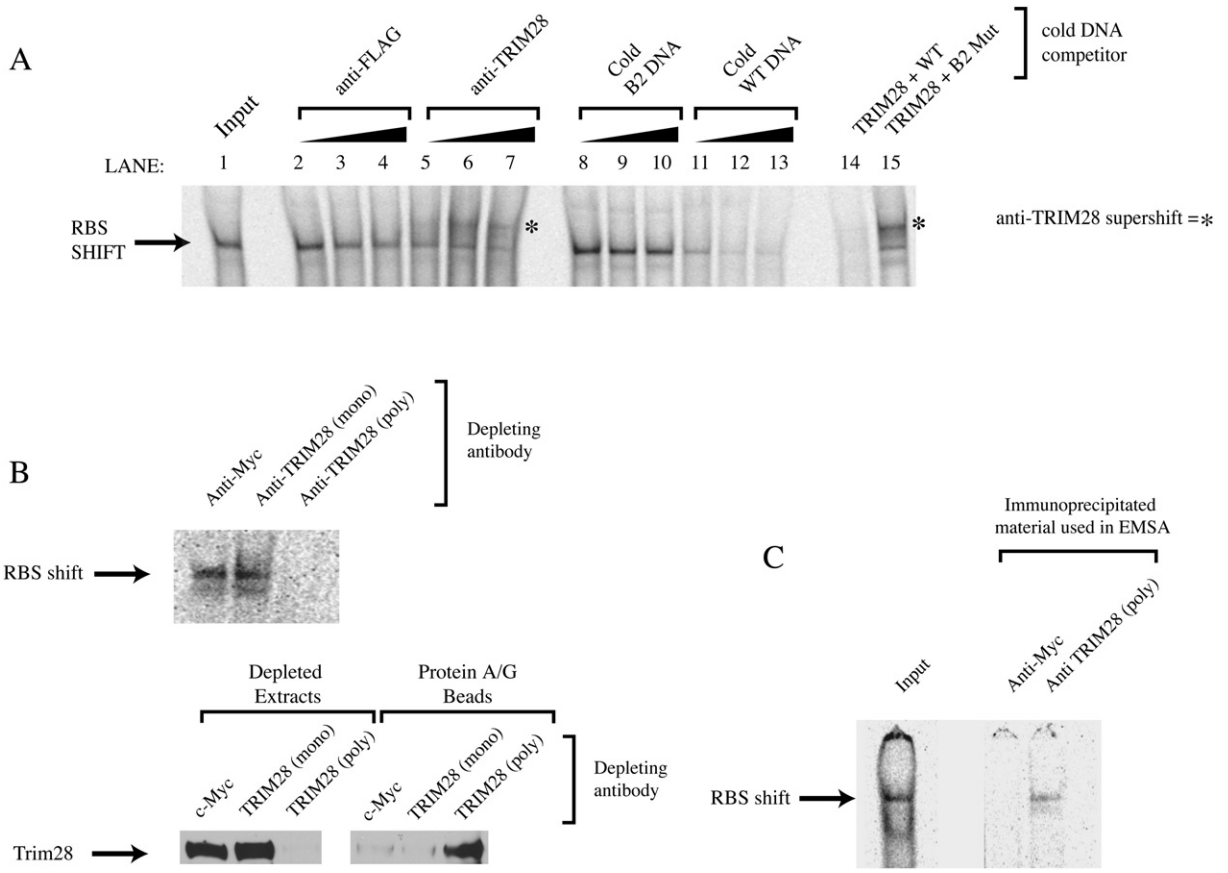

Figure 2. TRIM28 Is an Integral Component of the RBS Binding Complex

(A) EMSA performed with ^{33}P-labeled 28 bp probe corresponding to WT M-MLV RBS sequence on F9 nuclear extracts. Increasing amounts of anti-TRIM28 (lanes 5–7) or anti-FLAG (lanes 2–4) antibodies were included (1, 5, and 10 μg of antibodies as indicated; lanes 2 and 5, 3 and 6, and 4 and 7, respectively). Competitions with cold DNA were performed with 0.5, 1, and 2 μg of cold WT probe (WT; lanes 11, 12, and 13) or probe DNA containing B2 mutation (B2; lanes 8, 9, and 10) as indicated. Competitions with 2 μg of cold probe, either WT (lane 14) or B2 (lane 15) were also performed in presence of 5 μg of anti-TRIM28 antibody.

(B) Upper panel: F9 nuclear extract was immunodepleted with 3 μg TRIM28 polyclonal antibody, 3 μg TRIM28 monoclonal antibody (which is unable to immunoprecipitate TRIM28), or 3 μg anti-c-*myc* antibody. Extracts were used in EMSA reactions as above. Lower panel: LHS shows anti-TRIM28 western blot of depleted extracts used in upper panel. RHS shows anti-TRIM28 western blot of boiled protein A/G beads used in immunodepletions.

(C) EMSA performed as above on protein A/G beads from immunodepletion as in (B).

During the course of RA-induced differentiation, it was observed that the RBS shifted band subtly changed its mobility. This mobility change was also observed in the superose 6 fractionation of the RBS complex (Figure S1A). To determine if this change in mobility was due to a loss of TRIM28, supershifts with TRIM28 antibodies were performed on the extracts from cells treated with RA for 2 and 4 days, as well as a selection of fractions from the superose 6 experiment (Figure 3B; Figure S1B). In all cases, the RBS complex was supershifted by the TRIM28 antibody suggesting that TRIM28 is not lost from these complexes.

Knockdown of TRIM28 in ES and EC Cells Results in a Loss of RBS Binding Activity in These Cells

The correlation of TRIM28 levels with RBS binding activity during differentiation suggested that TRIM28 might be essential for RBS function. To test this notion, we asked whether RNAi-mediated knockdown of TRIM28 in EC and ES cells would lead to a loss of RBS binding activity. We used a retroviral RNAi system to target endogenous TRIM28 for stable knockdown in F9 and PCC4 EC lines, as well as the ES cell line JM1. Various primers targeted to the coding sequence of the mouse TRIM28 gene were used and pools of cells stably expressing various different RNAi constructs were analyzed for both expression of TRIM28 as well as RBS binding activity by EMSA (Figures 4A and 4B; Figure S3). In F9 cells, nearly complete knockdown of TRIM28 in Pool 6 lead to abrogation of RBS shift activity. Additionally, partial knockdown of TRIM28 correlated with a significant decrease in RBS binding activity (Pools 2 and 3). Complete knockdown of TRIM28, however, was found to be extremely detrimental to F9 cells, leading to growth

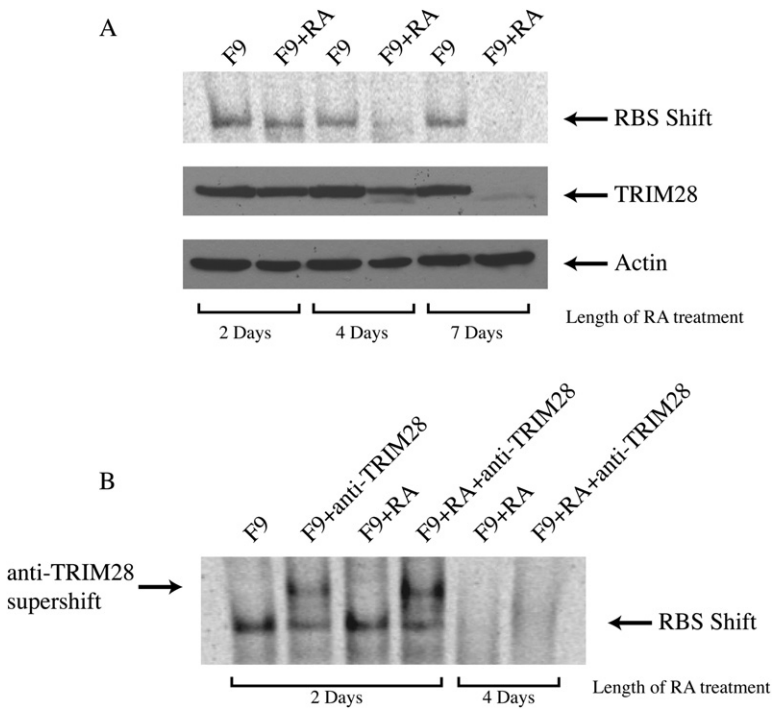

Figure 3. Differentiation of F9 Cells Leads to a Decrease of TRIM28 Levels and a Concomitant Loss of RBS Activity

(A) Upper panel: EMSA performed with nuclear extracts from F9 cells treated with 1 μM retinoic acid for 2, 4, or 7 days, or from time matched untreated controls, and with ^{33}P-labeled WT DNA probe. Lower panels show western blots of same extracts using anti-TRIM28 antibody and anti-actin antibody.

(B) EMSA performed with extracts from F9 cells treated with RA as above, but every second lane was preincubated with 5 μg anti-TRIM28 antibody.

arrest after a short number of cell cycles and a flattened morphology characteristic of cell-cycle arrest (data not shown). Surviving stable clones from Pool 6 were consistently shown to express higher levels of TRIM28, suggesting that complete knockdown of TRIM28 was selected against in cell lines which could form colonies. Therefore, to obtain populations with reduced levels of TRIM28, clonal cell lines from Pool 2 with partial knockdown were generated, along with clones expressing a scrambled siRNA control. One such line, Clone 2.3 (2.3), showed a robust knockdown of TRIM28 and no obvious gross morphological phenotype. As observed in the pools, the partial TRIM28 knockdown in this line correlated well with a decrease in RBS binding activity (Figure 4A). A possible mechanism for the decrease in RBS-mediated silencing in Clone 2.3 is that the knockdown of TRIM28 is causing these F9 cells to differentiate, as TRIM28 has a documented role in the differentiation of F9 cells (Cammas et al., 2004). To address this possibility, F9, Scram2, and Clone 2.3 cells were tested for two markers of their differentiation status. OCT3/4 is a well documented marker of pluripotency that has been shown to decrease upon F9 cell differentiation (Lenardo et al., 1989), and endo-A cytokeratin (TROMA-1) is known to only be expressed in F9 cells that are differentiated (Oshima, 1982). Both OCT3/4 and Endo-A cytokeratin showed little difference in levels among these cell lines, especially when compared to the dramatic change in their levels in the same cell lines after differentiation by treatment with RA (Figure S4). These results suggest that TRIM28 knockdown in Clone 2.3 did not induce a normal program of differentiation, nor did it affect the ability of the cells to differentiate correctly with RA.

Knockdown of TRIM28 was also attempted in an ES cell line to test for its effects on RBS activity. In JM1 ES cells, TRIM28 knockdown was even more detrimental to viability than in F9 cells. Partial knockdown of TRIM28 did correlate with decreased RBS activity (Figure S3), but pools of cells expressing the most efficient RNAi construct (Pool 6) were observed to have an extremely high rate of cell death (data not shown), and these ES lines were not used for further study.

Knockdown of TRIM28 in PCC4 cells, another EC line, was considerably more successful: all siRNA constructs led to a large decrease in TRIM28 levels and a near complete loss of RBS activity (Figure 4B). As in F9 cells, Pool 6 had the most efficient knockdown; however, in contrast to F9 cells, this pool was viable and showed no gross morphological differences from the control PCC4 cells. To ensure that the knockdown construct was not acting through an off-target gene, a myc/HIS-tagged TRIM28 construct designed to be resistant to siRNA knockdown was transiently reintroduced into Pool 6 by adenovirus transduction. This reexpression of TRIM28 resulted in recapitulation of RBS activity in this cell line (Figure 4B), whereas transduction with an empty adenoviral control vector did not. To further ensure that the reconstitution of RBS activity in this cell line was due to the exogenous expression of TRIM28-myc/HIS, a supershift was performed in these extracts using a c-*myc* antibody (Figure 4B). The c-*myc* antibody indeed supershifted the restored complex, confirming that the exogenously expressed TRIM28 had

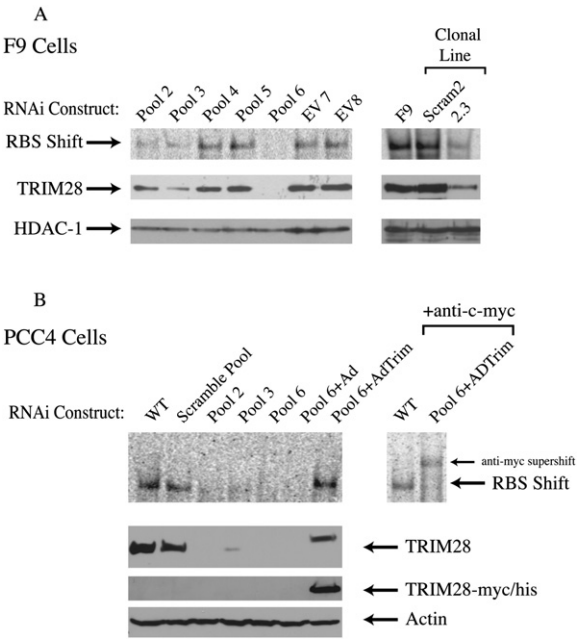

Figure 4. RNAi-Mediated Knockdown of TRIM28 Correlates with Loss of RBS Activity in F9 and PCC4 Cells

(A) Upper panel left, EMSA performed using ^{33}P labeled 28 bp probe corresponding to WT M-MLV RBS sequence and nuclear extracts prepared from F9 cells previously transduced with pSUPERRETRO-RNAi-puro construct and placed under puromycin selection. Each pool received a different siRNA sequence targeted to a different CDS sequence of TRIM28. Nuclear extract levels were equalized by Bradford protein assay. Upper panel right, same as above except nuclear extracts were prepared from clonal Scram2 and 2.3 cell lines. (2.3 is a clonal cell line derived from Pool 2) Lower panels: anti-TRIM28 and anti-HDAC-1 western blots performed on same extracts as in upper panel. HDAC-1 is used as a loading control. EV, empty vector.

(B) Upper panel EMSA performed (as above) with nuclear extracts from PCC4 cells targeted for TRIM28 RNAi knockdown using same constructs as above and also complementation of RBS activity by exogenously expressed TRIM28 in cell line Pool 6 using an adenoviral expression system. +AdTRIM denotes infection of cell line 24 hr prior to preparation of nuclear extract with an adenovirus expressing TRIM28 with a C-terminal myc/HIS tag. (MOI 100) +Ad denotes infection of cell line 24 hr prior to preparation of nuclear extract with a control adenovirus that does not express TRIM28 (MOI 100) Final two lanes show PCC4 WT nuclear extract and TRIM28-myc/HIS complemented Pool 6 nuclear extract incubated with 3 μg of anti-c-*myc* antibody, which supershifts RBS complex in TRIM28-myc/HIS expressing cells. Lower panels: anti-TRIM28, anti-c-*myc*, and anti-actin western blots performed on same extracts as in upper panel.

recapitulated a functional RBS complex. These results show that TRIM28 is required for RBS binding activity and that knockdown of TRIM28 can eliminate the RBS complex without inducing differentiation.

Knockdown of TRIM28 in F9 and PCC4 Cells Leads to an Attenuation of PBS-Mediated Silencing of M-MLV In Vivo

The observation that TRIM28 levels correlated with RBS binding activity suggested that knockdown of TRIM28 should relieve repression of M-MLV transcription. To analyze the effect of TRIM28 knockdown on PBS-mediated repression in F9 and PCC4 cells, two M-MLV-based retroviral vectors, LJ-PAdMLPEnh$^-$ (WT) and LJB2-ADMLPEnh$^-$ (B2), were used. These vectors both express the neomycin resistance marker from the adenoviral major late promoter, and are identical except that the former has a wild-type proline PBS and the latter a B2 mutated PBS (Modin et al., 2000b). M-MLV particles pseudotyped by the VSV G protein and containing either the LJ-PAdMLPEnh$^-$ or the LJB2-ADMLPEnh$^-$ vectors were produced, and the infectivity of each of these virus preparations was determined by colony formation assays in medium containing G418. The WT vector is repressed by RBS binding activity and the B2 vector is not, and therefore, the ratio of the titers of these viruses in a particular cell line is a measure of the RBS activity in that cell line.

Rat2 cells lack RBS-mediated repression activity, and therefore, the WT and B2 viruses infect these cells with very similar efficiencies. To correct for variations in titers between virus preparations, we chose to use this cell line as the standard, and the ratios of the titers of the B2/WT viruses were all normalized to the ratio of titers in this cell line. In F9 and PCC4 cells, the titer of the WT virus was repressed 40- and 70-fold, respectively, over that of the B2 virus, demonstrating that these cells possess a strong RBS-silencing activity (Figure 5). In the F9 Scram2 cell line and PCC4 Scramble pool cells, which both express a control scrambled siRNA hairpin, an even greater level of repression was observed; the titer of the WT virus was repressed nearly 60-fold in F9 cells and over 200-fold in PCC4 cells when compared to the B2 virus (Figure 5). This increase in repression over the parental line was consistently observed in cell lines stably expressing control siRNA hairpins and may be due to nonspecific effects of these siRNA hairpins on the cell, such as activation of the interferon response (Bridge et al., 2003). In Clone 2.3 cells, where TRIM28 levels were modestly reduced by RNAi, a decrease in the fold repression from the parental cell line of 8.5-fold was observed (Figure 5). In PCC4 Pool 6 cells, where a better knockdown was observed, there was a more dramatic effect, with a decrease in the fold repression from the parental cell line of nearly 12-fold (Figure 5). (The results for the individual experiments are shown in Figure S5). We note that despite the high efficacy of the RNAi knockdown of TRIM28 in PCC4 Pool 6, the ratio of the titers of the WT virus compared to the B2 virus does not return to 1 as in RAT2 cells, This could be due to the very low level of TRIM28 that is still present in these cells (data not shown) Taken together, these results confirm that knockdown of TRIM28 causes a significant decrease in RBS-dependent silencing of M-MLV and strongly suggests that TRIM28 is required for this process.

TRIM28 Is Present at the PBS In Vivo and Its Presence Is Correlated with Viral Silencing

If TRIM28 is indeed critical for RBS binding and activity, it should be bound to the RBS in vivo when an integrated

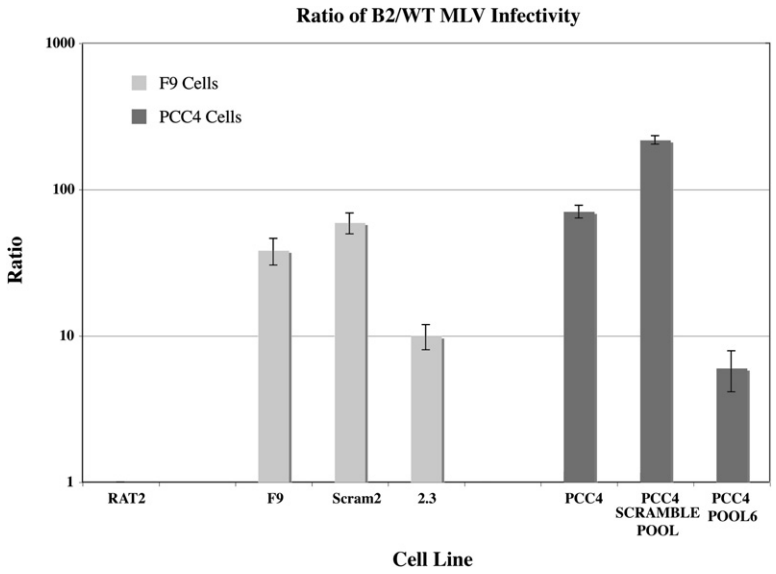

Figure 5. RNAi-Mediated Knockdown of TRIM28 Relieves PBS-Mediated Repression of M-MLV

RAT2, F9, Scram2, 2.3, PCC4, PCC4 SCRAMBLE pool and PCC4 Pool 6 cell lines, were infected with VSV-G pseudotyped M-MLV containing either LJ-PAdMLPEnh$^-$ (WT) or LJB2-ADMLPEnh$^-$ (B2) constructs. Infection efficiency in each cell line was monitored by colony count after 2 weeks of G418 selection. Graph shows ratio of B2/WT infection efficiency in each cell line, normalized with RAT2 = 1. Error bars show ± standard error, with n = 3 (F9 data) and n = 2 (PCC4 data).

M-MLV provirus is silenced and absent when it is not. Transcriptional corepressors that interact with TRIM28 and mediate its activity could also be bound at the RBS in repressing cells. To investigate these predictions, chromatin immunoprecipitation (ChIP) experiments were performed on two previously characterized F9 cell lines that contain a single copy of an M-MLV-based vector integrated into the same genomic locus (Berwin and Barklis, 1993). One cell line (R6A4) has a wild-type PBS and the other (R6C2) has a PBS carrying the B2 mutation (Figure 6A). The R6A4 line does not express the β-galactosidase reporter gene from the viral genome since the LTR is silenced by the wild-type PBS and also has been further silenced by subsequent DNA methylation (Berwin and Barklis, 1993); the R6C2 line does express β-galactosidase, as the vector contains the B2 mutation (Figure 6A). Chromatin immunoprecipitations were performed on both R6C2 and R6A4 cell lines using antibodies specific for TRIM28 and a histone H3 dimethyl Lysine 9 (diMeH3 K9) antibody. The diMeH3 K9 antibody was selected as the histone H3 K9 methyltransferase ESET is known to associate with TRIM28, and histone H3 lysine 9 methylation has been shown to correlate with loci silenced by TRIM28 (Schultz et al., 2002). The ChIP assay showed that both TRIM28 and diMeH3K9 are highly enriched at the PBS in the R6A4 as compared to the R6C2 cell line (Figure 6B). At the same time, the levels of binding to the housekeeping GAPDH gene were tested, and no difference between the cell lines was observed (Figure 6B). Thus, the increased binding of TRIM28 and the increased presence of histone H3 lysine 9 methylation at the proviral loci with the wild-type PBS over the B2 PBS are site specific. These results provide evidence that TRIM28 is indeed selectively bound at the wild-type PBS of an integrated M-MLV genome and that the B2 mutation decreases that enrichment of TRIM28 at this site, thereby attenuating its repressive influence. In order to confirm this observation, this ChIP assay was also performed with an alternative TRIM28 antibody as well as antibodies to the TRIM28 interaction partners HP1α, HP1γ ESET, and HDAC1. These results confirm that TRIM28 is enriched at the WT PBS in the R6A4 cell line and suggest that HP1γ is also specifically recruited to a site of PBS-mediated repression by TRIM28 (Figure S6). These results are consistent with observations suggesting that the ability of TRIM28 to repress transcription is linked to its binding to HP1 (Sripathy et al., 2006).

DISCUSSION

It has been known for over 30 years that murine leukemia proviruses are silenced in ES and EC cells (Teich et al., 1977). This restriction of proviruses in pluripotent cells is likely to have evolved to protect the embryo from the reactivation of endogenous retroviruses and retrotransposons that could cause damaging mutations in the germ line and early progenitor cells. These elements might well otherwise be expressed after the genome-wide DNA demethylation that occurs at several stages during the development of the mammalian embryo (Morgan et al., 2005). Early studies suggested that this silencing was mediated by the binding of a factor to the PBS to repress transcription from the integrated provirus (Barklis et al., 1986). The identity of the cellular factors involved in this process, however, has remained unknown. In this paper, we provided evidence that the cellular factor TRIM28 is a integral component of the RBS binding complex found in EC and ES cells (Figures 1 and 2; Figure S2). TRIM28 is a known transcriptional corepressor, acting as a bridge between KRAB-domain zinc finger DNA binding factors and other transcriptional repressors such as the NuRD deacetylase complex, the histone H3 K9 methyltransferase ESET, and HP1 (Le Douarin et al., 1996; Schultz et al., 2001, 2002).

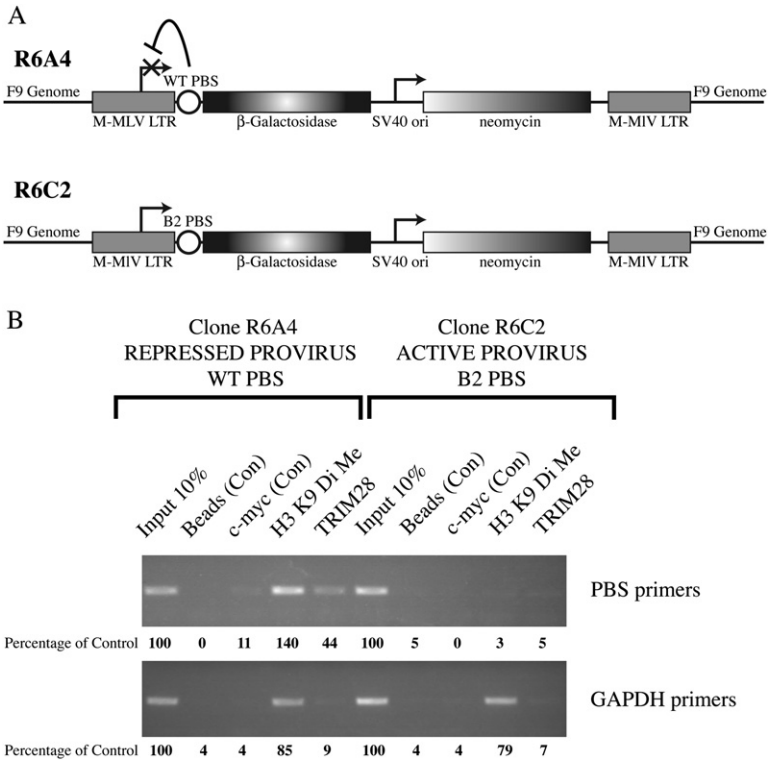

Figure 6. TRIM28 Is Present at the PBS of M-MLV In Vivo when Silencing Takes Place

(A) Cartoon depicting proviral constructs stably integrated in the same genomic location in cell lines R6A4 and R6C2. Cell line R6A4 has a WT PBS sequence that leads to silencing of the adjacent LTR, and R6C2 has a B2 mutated PBS, and therefore, the adjacent LTR remains unsilenced.

(B) Chromatin immunoprecipitations using anti-TRIM28, anti-histone H3 di-methyl K9 (H3 K9 Di Me), and anti-c-Myc antibody as a control. Upper panel shows PCRs performed using primers specific to PBS region of M-MLV; lower panel shows control primers directed to endogenous GAPDH gene. One-hundred percent control is arbitrarily defined as signal of 10% Input PCR of ChIP. Dilutions of all PCR products were performed to confirm that PCR was in linear range of amplification (data not shown).

Through these interactions, TRIM28 is recruited to cellular promoters and induces heritable gene silencing.

We found that knockdown of TRIM28 results in a specific alleviation of the repression in F9 and PCC4 cells (Figure 5) and that TRIM28 is present at the PBS of integrated M-MLV proviruses when repression takes place (Figure 6; Figure S6). This suggests that TRIM28 has an essential role in orchestrating the PBS-mediated silencing of integrated M-MLV proviruses in EC and ES cells, much as it does in silencing other target genes. While TRIM28 is apparently required for silencing, we note that it is not sufficient. Many differentiated cell lines that do not silence M-MLV still express high levels of TRIM28 (Figure 1A), and so other factors must regulate its function or otherwise be limiting determinants of its silencing activity.

TRIM28 may play an important role in early development, especially in ES and other pluripotent cells. During differentiation of EC cells, the intracellular distribution of TRIM28 protein changes from a diffuse nuclear staining to discrete foci colocalizing with heterochromatin, and its steady-state levels decrease considerably (Cammas et al., 2004; Figure 3). In early embryonic cells, TRIM28 has been shown to reside in complexes with a variety of early pluripotent markers including Nanog and Dax-1 (Wang et al., 2006). Interactions with these same factors may well be important in regulating TRIM28-mediated silencing of proviruses in these cells. Retroviral repression through the RBS element, however, may also involve specific and proprietary cofactors.

As no DNA-binding activity has been demonstrated for TRIM28 and as recombinant GST-TRIM28 appears to be unable to recapitulate a mobility shift of the wild-type PBS sequence (data not shown), we expect that the RBS binding complex contains at least one additional factor besides TRIM28. A logical candidate for such a factor would be one of the KRAB-box containing zinc finger proteins found expressed in both ES and EC cells. There are approximately 290 found in the mammalian genome (Urrutia, 2003), and such a protein would have the requisite ability to bind the RBS sequence and concomitantly recruit TRIM28 to induce silencing. In addition, these zinc finger proteins are among the few that are capable of recognizing an asymmetric DNA sequence as long as 17 bp, the size of the critical RBS element. We have been unable to identify such a factor in our purified preparations to date despite numerous attempts. This may be because the factor is present at very low abundance; it is thought that the KRAB domain can bind a trimer or possibly a hexamer of TRIM28, and if so, the zinc finger protein would be stoichiometrically underrepresented in the complex (Peng et al., 2000). We note that many KRAB box zinc finger proteins are able to dimerize (Urrutia, 2003) and that if each KRAB box were to bind a hexamer of TRIM28, the complex would be approximately the size of the RBS binding activity as judged by size exclusion chromatography.

A growing body of evidence suggests that many members of the TRIM family of proteins constitute an antiviral (especially antiretroviral) defense mechanism for the cell (Nisole et al., 2005). TRIM proteins have been shown to restrict multiple stages of the viral life cycle. TRIM5α has been shown to be responsible for HIV-1 and N-tropic M-MLV restriction in Old World monkeys and many other

mammals. TRIM5α restriction appears to take place in the cytoplasm and predominantly occurs before reverse transcription (Nisole et al., 2005). TRIM22 and TRIM32 have also been implicated in the restriction of HIV-1 (Nisole et al., 2005). These proteins appear to target HIV-1 transcription from the LTR by either binding to the Tat transactivator or through an unspecified mechanism. TRIM19 (better known as PML) has also been implicated in the restriction of several RNA viruses including vesicular stomatitis virus (VSV), influenza A, HIV-1, and human foamy virus (HFV) (Nisole et al., 2005). The finding that TRIM28 has a specific role in inhibiting M-MLV in pluripotent cells strengthens the argument that the TRIM protein family possibly evolved to protect cells from viral infection. It should also be noted the PBS-mediated silencing of retroviruses may be common to many mammals; human hematopoietic cells are also able to restrict M-MLV in a PBS specific manner (Haas et al., 2003). Further, embryonic cells may repress other retroviruses through their distinct PBS sequences (Yamauchi et al., 1995).

The existence of a natural mechanism for the silencing of MLV proviruses may serve as a model for the engineering of similar silencing of human viruses of clinical importance (Ellis, 2005). Thus, it may prove possible to activate the silencing machinery in more mature cells that would otherwise be susceptible to infection. In addition, several groups have used artificially engineered KRAB domain-containing transcription factors (which recruit TRIM28 to silence transcription) to induce silencing of integrated HIV proviruses (Eberhardy et al., 2006; Pengue et al., 1995). Consistent with our proposed model, one of these studies showed that an engineered KRAB box transcription factor is able to mediate transcriptional silencing of an integrated HIV-1 provirus when it is targeted to the PBS of HIV-1 (Eberhardy et al., 2006).

EXPERIMENTAL PROCEDURES

Cell Culture and Stable RNAi-Expressing Cell Line Production and Transduction

F9 cells were cultured in DMEM with 10% FBS; RAT2 and 293T cells were cultured in DMEM with 10% FetalcloneII (Hyclone). All cells were cultured at 37°C in 5% CO_2. RNAi knockdown was performed as described before (Leung et al., 2006). For primers used and more detailed description see the Supplemental Data. For viral transduction assays, viruses were prepared as for RNAi knockdown using either LJ-PAdMLPEnh$^-$ or LJB2-ADMLPEnh$^-$ vectors (Modin et al., 2000b). Retroviral preparations were then serially diluted (for titer determination) and added to F9 and RAT2 cells (seeded at [F9] 3.5 × 10^3 and [RAT2] 2 × 10^3 cells per cm^2 the day prior to transduction) in the presence of 8 μg of Polybrene/ml. G418-containing selective media was added 48 hr posttransduction at 1 mg/ml for RAT2 and at 0.5 mg/ml for F9 and PCC4 cells (for PCC4 cells, media was changed to 1 mg/ml after 10 days), and colonies were counted after 14–18 days of selection. Each experiment was performed in triplicate.

Preparation of Nuclear Extracts

Nuclear extracts were prepared from F9 cells essentially as described in Yamauchi et al. (1995). See the Supplemental Data for details.

Purification of RBS Complex

All procedures were performed at 4°C. F9 nuclear extract in buffer C was dialyzed against Dignam D (20 mM HEPES pH 7.9, 100 mM KCl, 0.2 mM EDTA) for 24 hr. By gradual addition of powdered ammonium sulfate, samples were raised to 25% saturation. Precipitated proteins were removed by centrifugation and discarded. Extract was then raised to 40% ammonium sulfate saturation, and after 20 min on ice, the precipitated material was collected and dissolved in Dignam D containing 1 M $(NH_4)_2SO_4$. This extract was applied to a HiTrap butyl FF column (Amersham) equilibrated with Dignam D containing 1 M $(NH_4)_2SO_4$. Proteins were eluted using a linear gradient of decreasing ammonium sulfate concentration. Fractions collected from column were concentrated and buffer exchanged into Dignam D using Amico-Ultra4 100-kDa spin columns (Millipore). Fractions containing RBS binding activity, as monitored by EMSA, were then applied to a Sephacryl S-500 gel filtration column equilibrated with Dignam D (plus 150 mM KCl). Samples were concentrated and monitored for activity as before. Positive fractions were used in an EMSA gel and the shifted DNA band excised.

Chromatin Immunoprecipitations

ChIP protocol was adapted from Fuks et al. (2003). See the Supplemental Data for details.

Electrophoretic Mobility Shift Assay

Double-stranded DNA probes were labeled using [γ-^{33}P] ATP and a T4 polynucleotide kinase kit (Amersham) and purified with G-25 Sephadex (Roche). DNA sequences of the probes are shown in the Supplemental Data. Binding reactions were performed using Modified Thornell binding buffer (Yamauchi et al., 1995) (25 mM HEPES [pH 7.9], 1 mM EDTA, 10% [v/v] glycerol, 5 mM DTT, 25 ng poly(dI-dC) per μl, 5 mM NaCl, 5 mM KCl, 3 mM $MgCl_2$, and 0.1 mM $ZnCl_2$ [no FBS was added]) (Yamauchi et al., 1995). Probes (50,000 CPM) were incubated with nuclear extract (prepared in buffer C; 10 μl per reaction) for 25 min at 30°C in a total volume of 20 μl. For supershifts and cold DNA competitions, antibody/cold DNA was added at same time as probe. Binding reactions were analyzed by electrophoresis on 4% native acrylamide gels (cast in 75 mM Tris pH 8, 75 mM boric acid, 1.5 mM EDTA) with reduced salt running buffer (35 mM Tris pH 8, 35 mM boric acid, 1 mM EDTA), dried down, and visualized using a phosphorimager screen.

Immunodepletions

Sixty microliters of F9 nuclear extract in 1× Modified Thornell binding buffer was used per reaction. Three micrograms of each antibody was added per reaction and incubated at 4°C for 2 hr. Twenty microliters of a slurry of protein A/G sepharose beads was added to each reaction and incubation was continued a further 2 hr. Reaction mixes were then spun in a centrifuge at 13K rpm for 1 min, 20 μl of supernatant was removed, and 50,000 CPM of standard EMSA DNA probe was added. EMSA was then performed as above.

Protein Identification by Mass Spectrometry

Gel slices were excised and proteins subjected to tryptic digest followed by peptide identification by lc-ms/ms using a hybrid high-resolution quadrupole time-of-flight electrospray mass spectrometer. Results were analyzed using MASCOT database search tool (Matrix Science).

Adenoviral Production/Infections

Adenoviral production and infections were performed as described in Ng et al. (2000), except infections were performed in 10%FBS.

Supplemental Data

The Supplemental Data include six supplemental figures and Supplemental Experimental Procedures and can be found with this article online at http://www.cell.com/cgi/content/full/131/1/46/DC1/.

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