Interaction of EVI1 with cAMP-responsive Element-binding Protein-binding Protein (CBP) and p300/CBP-associated Factor (P/CAF) Results in Reversible Acetylation of EVI1 and in Co-localization in Nuclear Speckles*

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EVI1 is a very complex protein with two domains of zinc fingers and is inappropriately expressed in many types of human myeloid leukemias. Using reporter gene assays, several investigators showed that EVI1 is a transcription repressor, and recently it was shown that EVI1 interacts with the co-repressor carboxyl-terminal binding protein 1 (CtBP1). Earlier, we showed that the inappropriate expression of EVI1 in murine hematopoietic precursor cells leads to their abnormal differentiation and to increased proliferation. Using biochemical assays, we have identified two groups of transcription co-regulators that associate with EVI1 presumably to regulate gene expression. One group of co-regulators includes the CtBP1 and histone deacetylase. The second group includes the two co-activators cAMP-responsive element-binding protein-binding protein (CBP) and p300/CBP-associated factor (P/CAF), both of which have histone acetyltransferase (HAT) activity. All of these proteins require separate regions of EVI1 for efficient interaction, and they divergently affect the ability of EVI1 to regulate gene transcription in reporter gene assays. Confocal microscopy analysis shows that in the majority of the cells, EVI1 is nuclear and diffused, whereas in about 10% of the cells EVI1 localizes in nuclear speckles. However, in the presence of the added exogenous co-repressors histone deacetylase or CtBP1, all of the nuclei have a diffuse EVI1 staining, and the proteins do not appear to reside together in obvious nuclear structures. In contrast, when CBP or P/CAF are added, defined speckled bodies appear in the nucleus. Analysis of the staining pattern indicates that EVI1 and CBP or EVI1 and P/CAF are contained within these structures. These nuclear structures are not observed when CBP is substituted with a point mutant HAT-inactive CBP with which EVI1 also physically interacts. Finally, we show that the interaction of EVI1 with either CBP or P/CAF leads to acetylation of EVI1. These results suggest that the assembly of EVI1 in nuclear speckles requires the intact HAT activity of the co-activators.

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The EVI1 gene was originally identified as a retroviral integration site leading to myeloid tumors in susceptible strains of mice (1). The gene is highly conserved through evolution and encodes a transcription repressor characterized by two DNA-binding zinc finger domains (2, 3). During murine embryogenesis, the gene is expressed in several organs, and homozygous disruption of EVI1 by gene targeting leads to embryonic death caused by severe malformation and defects in almost all of the developing organs (4). After birth and in the adult organism, EVI1 is detected in several organs but not in normal hematopoietic organs including bone marrow (5). The forced expression of EVI1 in cell lines and in murine embryonic stem cells reduces the cellular response to growth inhibition by transforming growth factor-β (6–8) and affects the normal hematopoietic differentiation of murine bone marrow precursors (9) and embryonic stem cells (7). In addition, the inappropriate expression of EVI1 in cell lines leads to up-regulation of cell replication (7). The mechanisms by which EVI1 disrupts normal cell replication and hematopoietic differentiation are not known, but it was suggested that they could be related to the ability of this protein to function as a transcription repressor (10, 11). Recently, it was shown that EVI1 interacts specifically with the co-repressor carboxyl-terminal binding protein (CtBP) in yeast (12) and in mammalian cells (13, 14). The inappropriate expression of EVI1 in hematopoietic cells has been associated with very aggressive chronic myelogenous leukemia and acute myeloid leukemia (AML) in mouse and in man (15, 16). In man, the inappropriate expression of EVI1 is triggered by chromosomal rearrangements that disrupt the 3q26 chromosomal region where the gene is located (17, 18). The most frequent rearrangements are the t(3;3)(q21;q26) and the inv(3)(q21q26) associated with very aggressive AML and myelodysplastic syndrome (19, 20). The inappropriate activation of EVI1 occurs also by unknown mechanisms, and chronic myelogenous leukemia patients frequently display EVI1 up-regulation, although their chromosome 3 appear normal by conventional cytogenetics.

Many oncoproteins involved in leukemia are activated and modified by chromosomal translocations. It is suggested that the altered oncoproteins acquire the ability to recruit transcription co-repressors (Sin3, CtBP, SMRT, and N-CoR) to promoters of genes that regulate cell cycle or hematopoietic differentiation, presumably leading to reconfiguration of the chromatin

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The abbreviations used are: CtBP, carboxyl-terminal binding protein; AML, acute myeloid leukemia; CBP, cAMP-responsive element-binding protein-binding protein; P/CAF, p300/CBP-associated factor; HAT, histone acetyltransferase; HDAC, histone deacetylase; HA, hemagglutinin; IP, immunoprecipitation; CAT, chloramphenicol acetyltransferase; PBS, phosphate-buffered saline.

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architecture and inappropriate gene regulation. Indeed, several investigators have shown that nuclear proteins such as AML1/ETO, PML/RARα, and TEL/AML1 interact with members of the co-repressor family (21–23). In addition, some co-activators that are expressed in most tissues, such as the cAMP-responsive element-binding protein–binding protein (CBP) and its homolog p300, are also involved in neoplastic transformation of cells following chromosomal translocations (24, 25). These and other co-activators, such as the p300/CBP–associated factor (P/CAF), are pleiotropic co-factors that often have histone acetyltransferase (HAT) activity. These enzymes regulate cell growth and differentiation in several systems including the hematopoietic system.

In this report we have analyzed the ability of EVI1 to interact with both co-repressors and co-activators. We have found that EVI1 interacts with both classes (class I and class II) of histone deacetylases (HDACs), and we confirm that it interacts with CtBP1. The interaction with CtBP1 or HDACs requires distinct nonoverlapping domains of EVI1 separated by ~500 amino acids. In addition, we found that EVI1 also specifically interacts in vivo with CBP and P/CAF through distinct nonoverlapping domains and that EVI1 is acetylated by CBP and P/CAF. The interaction of these co-regulators is specific and results in the opposite response of a reporter gene in vitro. By confocal microscopy analysis, we find that EVI1 is assembled in nuclear speckles in less than 10% of the cells. The addition of CtBP1 and HDAC1 abrogates the ability of EVI1 to be assembled in nuclear speckles, whereas the addition of CBP or P/CAF results in nuclear speckles that contain EVI1 and CBP or EVI1 and P/CAF. Finally, we have also determined that the region of EVI1 including the CtBP1-binding domain is required for up-regulation of the cell cycle in 32Dcl3 cells.

**EXPERIMENTAL PROCEDURES**

Epitope-tagged Full-length and Mutant EVI1 Plasmids—The expression vectors used in this study are MSCV (CLONTECH), pCMV-myc-nuc containing the Myc epitope and a nuclear localization signal (Invitrogen/Life Technologies, Inc.), pCMV-NotI containing the FLAG epitope (gift of Dr. D. Russell), and pCMV-HA containing the HA epitope.2 Differently epitope-tagged proteins were used for immunoprecipitation and confocal analysis. The entire open reading frame of EVI1 (EVI1–1051) was cloned in-frame in pCMV- NotI and pCMV-HA. To clone the first set of zinc fingers, the Neol-NotI fragment of EVI1 was subcloned into the Neol site of pCMV-myc-nuc. This region encodes the first 269 amino acids of EVI1 and was named pCMV-myc-nuc EVI1–269. A larger EVI1 region including the first EVI1 Pst site was inserted into the same vector and named pCMV-myc-nuc EVI1–546. pCMV-myc-nuc EVI1–736 was constructed by extending EVI1 to the second Pst site, just upstream of the second set of zinc fingers. Using a similar cloning strategy, we also generated the four FLAG-tagged plasmids pCMV-FLAG-EVI1–283, pCMV-FLAG-EVI1–514, pCMV-EVI1–653, and pCMV-FLAG-EVI1–1051. All cloning junctions were verified by sequencing to confirm the reading frame. The same vectors were subcloned in the MSCV-neo retroviral vector. CtBP1-FLAG was a gift of Dr. R. Baer (Columbia University). The HDAC-FLAG plasmid was a gift of Dr. S. L. Schreiber (Harvard University). Plasmids encoding HA-tagged CBP and a point mutant HAT-incompetent CBP were a gift of Dr. T. Kouzarides (CRC Cambridge, UK). The P/CAF-FLAG construct used in this study was obtained from Dr. Y. Nakatani (National Institutes of Health) (26).

Site-directed Mutagenesis—Site-directed point mutants were created by polymerase chain reaction using primers that convert aspartate-leucine (DL) to alanine-serine (AS). All of the polymerase chain reactions were performed using the high fidelity Pfu DNA polymerase (Stratagene). Each individual clone was sequenced to confirm the mutation.

**Acetylation of EVI1 and Assembly in Nuclear Speckles**

To identify potential co-repressors that interact with EVI1, we performed the yeast two-hybrid analysis using full-length EVI1, the two domains of zinc fingers, or the central domain of the protein. However, the assays showed no significant binding to any known co-repressor, and the selected segments act as portable activation domains in yeast and therefore they could not be used for this study (data not shown). We therefore used targeted co-IP strategy. To confirm that EVI1 interacts with CtBP1, we used co-IP analysis with several deletion mutants and point mutants shown in Fig. 1. After transient transfection, the proteins were

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immunoprecipitated with agarose-conjugated anti-FLAG antibody and were separated by electrophoresis. Full-length or mutant EVI1 proteins were visualized with EVI1 antiserum. The results confirmed that full-length EVI1 physically interacts with CtBP1 (Fig. 2A, top panel, lanes 1 and 5). To identify the region necessary for CtBP1 interaction, we used the deletion mutants EVI1–736 and EVI1–546. The results show that whereas EVI1–736 interacts with CtBP1 (Fig. 2A, top panel, lanes 1 and 5), the mutant EVI1–546 is unable to interact with CtBP1 (Fig. 2A, top panel, lanes 3 and 7). Thus, the 190-amino acid region upstream of the second set of zinc finger is necessary for the interaction between EVI1 and CtBP1. The expression of the transfected FLAG-CtBP1 was confirmed by stripping the blot and reprobing the blot with anti-FLAG antibody (Fig. 2A, bottom panel, lanes 5–7). Within this stretch of 190 amino acids, EVI1 contains a PLDLS CtBP1-binding consensus (amino acids 584–588) and a variation of this motif, PFDLT (amino acids 553–557). To determine whether both sites are necessary for interaction with CtBP1, we generated EVI1 point mutants in which either the first consensus sequence was mutated from PFDLT to PFAST (mutant A; Fig. 1) or the second consensus was mutated from PLDLS to PLASS (mutant B; Fig. 1). In addition, a double mutant with mutations of both sites (mutant C; Fig. 1) was generated. Each one of the point mutants was expressed at a high level in transiently transfected Cos-7 cells (Fig. 2B, top panel, lanes 7–9). Co-IP analysis indicated that mutation at the PFDLT site impaired the interaction as shown by the faint IP band (Fig. 2B, top panel, lane 2). However, mutation of the PLDLS site almost completely abrogated the interaction between the two proteins (Fig. 2B, top panel, lane 3). Mutation of both sites eliminates the interaction between EVI1 and CtBP1 (Fig. 2B, top panel, lane 4). Expression of transfected FLAG-CtBP1 was confirmed by stripping the blot and reprobing the blot with anti-FLAG antibody (Fig. 2B, bottom panel, lanes 6–9). These results confirm those recently reported by other investigators on the interaction between CtBP1 and EVI1 (13, 14).

The Amino Terminus Zinc Finger Domain of EVI1 Interacts with HDAC Enzymes

It was shown that transcription repressors often recruit HDAC enzymes at the promoter site either by direct interaction or through intermediate binding of an adaptor co-repressor such as Sin3, SMRT, or N-CoR (29–31). So far, two major classes of HDACs have been identified that contain highly homologous enzymatic domains (32, 33). Therefore, to determine whether EVI1 associates with HDAC enzymes, we used co-IP analysis of cells transiently transfected with EVI1 and either HDAC1 (class I) or HDAC4–6 (class II). The proteins were precipitated with monoclonal FLAG antibody (for FLAG-tagged HDACs), separated on gel, and analyzed with antiserum to EVI1. The results indicate that EVI1 is able to interact with members of both classes of HDACs; however, the strongest interaction was observed for HDAC1 and HDAC4 (Fig. 3, top panels, lanes 1 and 5). To identify the region of EVI1 that is required for the interaction with the HDACs, we used the EVI1 deletion mutants EVI1–736 (Fig. 3, top panels, lanes 2 and 6) and EVI1–269, which contains only the proximal domain of zinc fingers (Fig. 3, top panels, lanes 3 and 7). We found that the shortest EVI1 mutant is still capable of interacting with HDAC1 and HDAC4, indicating that the interaction occurs through the proximal domain of EVI1 zinc fingers. Thus, it appears that EVI1 contains two regions that interact separately with CtBP1 and HDAC and that CtBP1 bound to EVI1 is not the most efficient or major site for recruitment of an HDAC enzyme. To confirm the expression of transfected FLAG-HDAC1 and FLAG-HDAC4, the blots were stripped and reprobed with anti-FLAG antibody. The results (Fig. 3, bottom panels, lanes 5–7) indicate that both proteins were expressed in the transfected cells.

EVI1 Interacts Separately with the Co-activators CBP and P/CAF

The ability to interact with both co-activators and co-repressors is a feature shared by several transcription factors. CBP/p300 and P/CAF are co-regulators that have been implicated in neoplastic transformation (24, 25). To determine whether EVI1 physically interacts with CBP, we used co-IP analysis of proteins after co-transfection of 293T cells with HA-CBP and either the full-length or deletion mutants of FLAG-tagged EVI1. The proteins were immunoprecipitated with commercial antibody that recognizes the amino terminus of CBP. The precipitated proteins were separated by electrophoresis, transferred to a nylon membrane, and probed with anti-EVI1 antiserum. Analysis of the immunoprecipitated proteins showed that EVI1 interacts with P/CAF. Co-IP assays and identified by EVI1 antiserum. The results (Fig. 4A, top panel, lanes 1 and 6). The deletion mutants EVI1–653 (Fig. 4A, top panel, lanes 2 and 7) and EVI1–514 (Fig. 4A, top panel, lanes 3 and 8) are still capable of interaction with CBP. However, the deletion mutant EVI1–283 does not interact with CBP. Thus, the interaction between EVI1 and CBP requires the EVI1 region between amino acids 283 and 514 (Fig. 4A, top panel, compare lanes 3 and 4). A point mutant variant of CBP that has no HAT activity interacts with EVI1 as the wild type CBP, indicating that the HAT activity is not required for interaction (data not shown). It is well known that CBP is often found associated with P/CAF and several other proteins in large co-activator complexes. To determine whether the interaction of EVI1 with CBP could lead to the recruitment of P/CAF, we used co-IP assays of transiently transfected cells and Western blot analysis. The cells were transiently transfected with plasmids encoding full-length or mutant EVI1 proteins and with a plasmid encoding FLAG-tagged P/CAF. The proteins were immunoprecipitated with anti-FLAG antibody, separated by electrophoresis, and identified by EVI1 antiserum. The results (Fig. 4B, top panel) show that EVI1 interacts with P/CAF. Co-IP assays and
Western blot analyses with EVI1 deletion mutants show that the interaction requires the short deletion mutant EVI1–283 (Fig. 4B, top panel, lanes 2 and 5) and indicate that the interaction occurs through the proximal zinc finger domain of EVI1. Thus, the regions of EVI1 necessary for interaction with CBP and P/CAF are separate and nonoverlapping. The expression of CBP and P/CAF in the transiently transfected cells was confirmed by Western blot analysis of the stripped membranes (Fig. 4, bottom panels).

The Interaction with CtBP1 and CBP Divergently Affects the Transcription Activity of EVI1

CtBP1 Increases the Transcription Repression by EVI1—To evaluate the functional significance of the EVI1–CtBP1 interaction, we used reporter gene assays of transiently transfected NIH-3T3 cells. We used the pTAK-CAT promoter in the presence or absence of added CtBP1. It was shown that EVI1 binds directly to this promoter and that this promoter is repressed by EVI1 (10). In our assays we consistently find that EVI1 represses this promoter to about 60%–70% of its normal activity (Fig. 5, bars 1 and 2). In the presence of added CtBP1, the activity of the promoter was further reduced by EVI1 from 60% to about 45% (Fig. 5, bars 2 and 5). However, when the EVI1 mutant C (unable to interact with CtBP1) was used, the normal activity of the promoter was reduced to only about 80% and did not significantly change in the presence of added CtBP1 (Fig. 5, bars 3 and 6). In parallel, we used the same assays to observe the effect of HDAC1. However, we did not detect any further repression by the addition of exogenous CBP. Because the HDAC enzymes are abundant in the cell (in contrast to CtBP1 which is limiting in the cell), it is possible that the endogenous level of the combined two classes of HDAC enzymes is sufficient for the interaction with the transfected EVI1. This situation is similar to that of Sin3, which, when added in reporter gene assays, does not change noticeably the response of a promoter to a transcription factor because it is already very abundant in the cell. In contrast to what has been reported by others (13), these data show that CtBP1 affected directly the response of a promoter to which EVI1 binds through its zinc finger domain.

CBP Abrogates Repression and Induces Activation—We used reporter gene studies in NIH-3T3 cells to determine whether exogenous CBP has a significant effect on the transcription activity of EVI1. As shown in Fig. 5 (bars 2 and 8), the addition
The activity of the CAT reporter gene was normalized with the internal mutant CBP that has lost the HAT enzymatic function. The bar 10 dn. This activation is not observed with a dominant negative point mutant EVI1-C (bar 2). In contrast, co-transfection of CBP represses the reporter gene by full-length EVI1 but not by the point mutant EVI1-C (bar 6). In contrast, co-transfection of CBP reverses the function of EVI1 from repressor (bar 2) to weak activator (bar 8). This activation is not observed with a dominant negative (dn) point mutant CBP that has lost the HAT enzymatic function (bar 10). The activity of the CAT reporter gene was normalized with the internal control β-galactosidase. Each assay was carried out in duplicate and repeated at least two times.

Because the biological promoters regulated by EVI1 are not known, it is very difficult to state with certainty whether EVI1 is always and only a transcription repressor. It seems, however, that EVI1, as other transcription factors, could function in both ways, depending on the target genes and the need of the cell. To confirm that CBP collaborates with EVI1 in promoter activation, we used a dominant negative form of CBP that has no HAT activity. The results, shown in Fig. 5 (bars 8 and 10), suggest that the activation of the promoter by EVI1 requires the active HAT domain of CBP.

**Up-regulation of Cell Replication by EVI1 Requires the CtBP1-binding Region**

We earlier reported that the forced expression of EVI1 in 32Dc13 and embryonic stem cell lines leads to a significant up-regulation of cellular replication (7, 8). To determine the region of EVI1 that is necessary for cell cycle up-regulation, we generated stable 32Dc13 cell lines expressing the full-length EVI1 and the deletion mutants EVI1–736 and EVI1–546. We did not use the mutants (EVI1–269 or EVI1–283) because the part of EVI1 left in these mutants is too small to be informative. The cell replication rate was determined by daily counting of the total number of live cells for a period of 8 days. The results (Fig. 6) confirm our previous results that inappropriate expression of full-length EVI1 (EVI1–1051) results in a significant increase of cell replication. We observed a similar increase of cell proliferation with the longer mutant of EVI1 (EVI1–736) that contains the CtBP1-binding region. However, we observed that the deletion of the region that interacts with CtBP1 (EVI1–546) restores normal cellular growth, suggesting that CtBP1 could be involved in cell cycle regulation by EVI1.

**CBP and P/CAF Acetylate EVI1**

It is well known that HAT enzymes modify transcription factors in addition to the histone proteins. Therefore, to determine whether CBP and P/CAF have the ability to acetylate EVI1, we used co-IP and Western blot analyses of cells transfected with EVI1 and the co-activators CBP or P/CAF.
proteins were immunoprecipitated with antibody to EVI1 and separated by electrophoresis. After transfer to a polyvinylidene difluoride membrane, the immunoprecipitated proteins were probed with antibody to acetylated lysine. The results are shown in Fig. 7 (A, P/CAF; B, CBP). The cells were co-transfected with P/CAF and EVI1 (Fig. 7A, lanes 2 and 4). After co-IP with EVI1-antibody, the separated proteins were analyzed with antibody against acetylated lysine (lanes 1 and 2). We clearly observed a unique band in lane 2 but not in lane 1 (untransfected cells). This band corresponds in size to that detected with EVI1 antibody (Fig. 7A, lane 4). A similar assay was carried out for cells co-transfected with CBP and EVI1 (Fig. 7B, lane 5), with a dominant negative form of CBP and EVI1 (lane 6), or with nontransfected cells (lane 7).

Assembly of EVI1 in Nuclear Speckles Is Abrogated by Interaction with the Co-repressors HDAC and CtBP1

Confocal microscopy analysis of mammalian cells transfected with EVI1 indicates that in over 90% of the cells EVI1 is a nuclear protein with a diffused pattern. In the remaining cells, EVI1 is assembled in small speckles that are evenly distributed over the nucleus with the exclusion of the nucleoli (Fig. 8A). To determine whether the association of EVI1 with co-repressors alters the pattern of localization, we examined by confocal microscopy cells that were transiently transfected with EVI1 and with the co-repressors studied in this work. For these assays, we used transient transfection and epitope-tagged EVI1 because EVI1 antibody suitable for confocal microscopy analysis of the endogenous protein is not available.

Association of EVI1 with the Co-repressors CtBP1 or HDAC1 Abrogates the Nuclear Speckles—Fig. 8 (B–G) shows the results of confocal analysis of cells transfected either with EVI1 and CtBP1 (Fig. 8, B–D) or with EVI1 and HDAC1 (Fig. 8, E–G). The staining patterns were very similar, and in both cases all of the nuclei we examined were characterized by a diffused staining. No obvious speckle was observed in any of the analyzed cells. Thus, although EVI1 physically interacts with these co-repressors, as shown by co-IP assays, it appears that the interaction does not occur in defined nuclear regions. Indeed, because we observed a diffused nuclear staining in all of the co-transfected cells, it appears that the overexpression of EVI1 with either CtBP1 or HDAC1 leads to the complete abrogation of the nuclear speckles that we noted in about 10% of the cells transfected with EVI1 only (Fig. 8A).

EVI1 and CBP Co-localize in Nuclear Speckles—In cells co-transfected with EVI1 and CBP, the two proteins co-localize in clearly defined nuclear speckles (Fig. 8, H–J) similar to those noted for EVI1 only (Fig. 8A). However, the total number of nuclei containing the nuclear speckles increased from about 10% (for only EVI1) to about 25–30% (for EVI1 and CBP).

Assembly of EVI1 and CBP in Nuclear Speckles Requires HAT Activity—To determine whether the formation of the nuclear speckles was due solely to the physical interaction between the proteins or was related to the HAT enzymatic activity of CBP, we repeated the co-transfection and immunofluorescence analysis with a point mutant CBP that does not have HAT activity. The nuclear staining appeared homogeneously diffused (Fig. 8, K–M) and similar to that observed for HDAC1 and CtBP1. These results would suggest that the assembly of EVI1 and CBP in nuclear speckles requires the functional HAT activity of the enzyme.

EVI1 and P/CAF Co-localize in Nuclear Speckles—Immunofluorescence analysis of 293 cells co-transfected with EVI1 and P/CAF showed that the two proteins are found in distinct nuclear bodies in about 10% of the cells. However, there was not a complete co-localization of the two proteins within the nuclear bodies as we had seen for CBP. Analysis of the cells showed that whereas the majority of the speckles contained both EVI1 and P/CAF, some of them contained only one of the two proteins, either EVI1 or P/CAF (Fig. 8, N–P). Thus, it appears that CBP and P/CAF have similar but not completely identical patterns when they are co-transfected in cells with EVI1. Because at this time we do not have a HAT-inactive P/CAF, we could not determine whether the co-localization of P/CAF and EVI1 in nuclear speckles depends on the activity of this enzyme as it does for CBP.

DISCUSSION

After the identification of transcription co-regulators as critical modulators of gene expression, several investigators have shown that oncoproteins activated in human leukemia often acquire the ability to associate inappropriately with the transcription co-regulators, leading to the deregulation of genes that are involved in the normal hematopoietic differentiation. This hypothesis is particularly suitable to chimeric oncoproteins that derive from the inappropriate fusion of two truncated transcription factors following a chromosomal translocation. It was suggested that for some of these chimeric oncoproteins, such as AML1/ETO, TEL/AML1, and PML/RARα, the loss of domains that critically interact with the co-repressors could lead to the improper assembly of complexes with impaired transcription functions. In fact, it was proposed that the chimeric oncoproteins probably form large multiprotein complexes involved in the transcription deregulation of genes. This hypothesis is especially attractive because it could lead to the development of new cancer drugs that can selectively inhibit the enzymatic activity of specific co-regulators. Although EVI1 is not a chimeric oncoprotein, the results that we present in this report indicate that EVI1 belongs to a growing family of transcription co-regulators, leading to the deregulation of genes that are involved in the normal hematopoietic differentiation.
EVI1 and co-regulators (P) and were treated as described under B–N, in lanes 1 or CBP (lane 2) band corresponding in size to EVI1 is observed with anti acetylated lysine antibody in extracts of cells co-transfected with EVI1 and either P/CAF (lane 2) or CBP (lane 5). A dominant negative CBP that has lost HAT activity is unable to acetylate EVI1 (lane 6). Untransfected cells are shown in lanes 1, 3, and 7.

Our combined data suggest that the interaction with the co-repressors and co-regulators could result in periodic, reversible acetylation and deacetylation of EVI1 and in the assembly of acetylated EVI1 in nuclear speckles. This hypothesis is supported by the fact that cells that express only exogenous EVI1 have a number of speckles lower than cells that express also exogenous CBP or P/CAF, which are present in the cell in limiting amounts. In addition, it is likely that the deacetylation of EVI1 is mediated by the histone deacetylases with which EVI1 interacts. Thus, EVI1 belongs to a growing family of proteins that reside in a different subcellular location after covalent modification. There are several proteins that undergo post-translational covalent modification either depending on the cell cycle (TEL) (36) or for functional reasons (PML, p53, MDM2, etc.) (37–39), resulting in the assembly of nuclear speckles and altered localization. The nuclear speckles are considered subnuclear regions that perform specific roles and have been associated with cellular functions such as DNA replication, regulation of apoptosis, and gene transcription.

EVI1 is clearly a complex transcription factor with multiple functions, and this complexity is further demonstrated by the ability of EVI1 to interact with co-activators, specifically CBP and P/CAF, in addition to co-repressors. It is interesting to note that, as for HDAC and CtBP1, the interaction of EVI1 with the two co-activators requires very distinct domains of EVI1 that do not overlap. This is unusual in that CBP itself is able to interact with P/CAF, and one would therefore expect that P/CAF could be recruited to and be a participant of the activator complex anyway. At this time, it is not clear whether P/CAF bound to EVI1 retains the ability to interact with CBP at the same time. This would require that the interaction domains of P/CAF with either CBP or EVI1 are separate. The interaction of EVI1 with the co-activators has clearly two functional con-
sequences; one of them is the transition of EVI1 from a transcription repressor to a transcription activator (at least in reporter gene assays), and the second one is the assembly of EVI1 into well defined nuclear speckles that contain the co-activators as well.

In conclusion, since the first finding that the inappropriate expression of EVI1 has a dominant role in the development and progression of murine leukemia, there have been several reports linking chronic and acute myeloid human leukemia with the deregulation of this gene. Overall, several reports show that up to 70% of chronic myeloid leukemia cells overexpress EVI1. Whereas there is no doubt that this complex protein profoundly alters hematopoiesis and cell replication, there is very little information on the normal biological role of EVI1 or on the pathways that are affected by EVI1 in leukemia. Our results show that EVI1 is able to interact with functionally opposite transcription co-regulators leading to the reversible acetylation of EVI1 and to the translocation of EVI1 to defined nuclear speckles. We suggest that the localization of EVI1 in nuclear speckles depends on its acetylation states and could determine the functions of the protein, and we speculate that the acetylated and deacetylated state of EVI1 could have opposite functions. These results further increase the complexity of this protein and the difficulty of understanding its role in normal and transformed cells, but they might provide insights for the development of appropriate clinical drugs that can be successfully used to treat EVI1-caused leukemia.

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Interaction of EVII with cAMP-responsive Element-binding Protein-binding Protein (CBP) and p300/CBP-associated Factor (P/CAF) Results in Reversible Acetylation of EVII and in Co-localization in Nuclear Speckles
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