Transcriptional Repression Mediated by the PR Domain Zinc Finger Gene RIZ

(Received for publication, April 10, 1997, and in revised form, August 11, 1997)

Ming Xie, Gang Shao‡, Inge M. Buyse§, and Shi Huang¶

From the La Jolla Cancer Research Center, The Burnham Institute, La Jolla, California 92037

The RIZ (G3B or MTB-Zf) zinc finger gene is structurally related to the myeloid leukemia gene, MDS1-EVI1, and the transcription repressor/differentiation factor, PRDI-BF1/BLIMP1, through a conserved amino-terminal motif, the PR domain. Similar to MDS1-EVI1, RIZ gene normally produces two protein products that differ by the PR domain. The smaller protein RIZ2 lacks the PR domain of RIZ1 but is otherwise identical to RIZ1. Here we show that RIZ proteins bind to GC-rich or Sp-1 binding elements and repress transcription. Both RIZ1 and RIZ2 repressed the herpes simplex virus thymidine kinase (HSV-TK) promoter, one of the best characterized eukaryotic promoters. Recombinant RIZ1 proteins were able to bind to HSV-TK promoter. This binding was mediated by the GC-rich Sp-1 elements of the promoter and the first three zinc finger motifs of RIZ1. RIZ also encodes a repressor domain that was mapped to the central region of the protein. Fusion of this region to the GAL4 DNA-binding domain generated GAL4 site-dependent transcriptional repressors. We also show that RIZ1 protein can efficiently repress the simian virus 40 (SV40) early promoter, which primarily consists of Sp-1 sites; RIZ2, however, only weakly repressed this promoter, suggesting a role for PR in modulating RIZ protein function. The data have implications for a role of RIZ proteins in the regulation of cellular gene promoters, many of which are characterized by GC-rich elements.

The RIZ zinc finger gene was isolated in a functional screening for proteins that can interact with the retinoblastoma tumor suppressor protein (1, 2). The biological role of this interaction remains unclear because of the limited understanding of RIZ gene function. The predicted rat and human RIZ proteins are of 1706 and 1719 amino acids respectively, and are highly homologous (84% amino acid identity). RIZ gene normally produces two different protein products, RIZ1 and RIZ2, that are widely expressed (3). An internal promoter generates RIZ2, which is identical to RIZ1 except that it lacks the RIZ1 PR domain that defines a subclass of Krüppel-like family of zinc finger genes. This RIZ gene structure is remarkably similar to a related PR domain gene MDS1-EVI1 involved in human and murine leukemia (4). An internal promoter within MDS1-EVI1 gene generates the EVI1 myeloid transforming gene product that lacks PR but is otherwise identical to MDS1-EVI1 (5). The PR domain of MDS1-EVI1 is a common target of viral insertions and chromosomal translocations in leukemogenesis (6–8), suggesting it might play an important biological function. EVI1 has been shown to bind to DNA specifically and can function either as a repressor or a weak activator of transcription (9, 10). The function of MDS1-EVI1 remains uncharacterized. It is of considerable interest to determine whether EVI1 and MDS1-EVI1, or RIZ1 and RIZ2, may function differently because of the PR domain.

Another PR domain gene is the PRDI-BF1/BLIMP1 transcription repressor/differentiation factor (11). It can repress the β-interferon gene promoter (12) and can induce B-lymphocyte maturation (13). BLIMP1 maps to human chromosome band 6q21-q22.1, a region often deleted in B cell non-Hodgkin lymphoma, suggesting that it might serve as a candidate tumor suppressor for this B cell tumor (14). In support of this notion, BLIMP1 has recently been shown to be a physiological transcriptional repressor of the c-myc oncogene (15).

Like its related PR domain genes, RIZ also display properties of transcription factor with a potential role in cell growth and tumorigenesis. RIZ gene products encode DNA-binding as well as transcription factor-binding activities as evidenced by the independent isolation of RIZ as an retinoblastoma-binding protein (RIZ), a DNA-binding protein (MTB-Zf), or as a GATA3 transcription factor binding protein (G3B) (1, 16, 17). MTB-Zf is essentially identical to RIZ2 (3), and binds to the MTE DNA element GTCATATGAC of human heme-oxygenase-1 gene and can weakly activate transcription (16). RIZ gene maps to human chromosome band 1p36, a region thought to harbor one or more tumor suppressor genes for a variety of human cancers including those of neurocrist, colon, liver, and breast tissues (16, 18). Abnormal RIZ gene expression has been observed in human brain tumors (16). RIZ1 mRNA was commonly found absent or at reduced levels in tumor cell lines and tissues while RIZ2 was always found expressed, consistent with RIZ1 as a potential tumor suppressor.

Better characterization of RIZ gene function is clearly needed to elucidate its potential role in transcriptional regulation and in cell growth control. Here, we have found that RIZ proteins function as transcription repressors and can bind to GC-rich Sp-1 elements. Using the herpes simplex virus thymidine kinase (HSV-TK)2 promoter as a model system (19), we

---

* This work was supported in part by National Institutes of Health Grant R01CA57496 and the United States Army Breast Cancer Research Program Grant RP851914 (to S. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Present address: Ligand Pharmaceuticals, Inc., San Diego, CA 92121.
§ Recipient of a Postdoctoral Fellowship from the D. Collen Research Foundation, Belgium.
¶ Present address: The Canadian Red Cross Society, Faculty of Medicine, University of Ottawa, Ottawa, Ontario, K1G 4J5, Canada.

1 L. Liu, I. Buyse, S. Simon, G. Brodeur, and S. Huang, manuscript in preparation.

2 The abbreviations used are: HSV-TK, herpes simplex virus thymidine kinase; SV40, simian virus 40; CAT, chloramphenicol acetyltransferase; PCR, polymerase chain reaction; kb, kilobase; GST, glutathione
show that RIZ proteins repress this promoter and can bind to it in vitro. Repression requires DNA-binding domain as well as a repressor domain that is transferable to a heterologous DNA-binding domain. We also show that RIZ proteins repress the simian virus 40 (SV40) promoter, which is primarily governed by GC-rich Sp-1 elements (19). Given that many cellular gene promoters, especially those of growth regulated genes, are characterized by GC-rich elements (20), these results have implications for a potential role of RIZ in the transcriptional regulation of a broad spectrum of cellular genes.

MATERIALS AND METHODS

Cell Culture and Transfections—NIH 3T3 cells were grown in Dulbecco’s modified Eagle’s medium plus 10% calf serum. For transfections of these cells, a calcium phosphate precipitation procedure was used (21). One day before transfection, the cells were split to a density of 2 × 10^5 cells/60-mm dish. The cells were given fresh media 0.5 h prior to transfection. The standard co-transfection included 1.5 μg of reporter gene, 8 μg of expression construct, and 1 μg of a β-galactosidase expression construct driven by the CMV promoter (pCMVβ) (22). The DNA precipitate was left on the cells for 16–20 h, and then the medium was changed. Cells were processed 24 h after withdrawal of DNA for immunoblot and chloramphenicol acetyltransferase (CAT) analysis. Equivalent amounts of cell extracts, made by freeze-thaw lysis, were incubated with acetyl-CoA and [14C]chloramphenicol for 1–2 h, and percent conversion of the chloramphenol to the acetylated form was measured by thin layer chromatography followed by quantitation on a phosphorimager radioanalytic imaging system scanner. Luciferase activity in cell extracts was determined by measurement of chemiluminescence using β-luciferin-potassium salt as substrate (Analytical Luminescence Laboratory and the MicroLumat LB96P microplate lumimeter system (EG&G Berthold)). The cell extracts were also assayed for β-galactosidase activity using standard procedures (23). All CAT or luciferase values were normalized based on the respective β-galactosidase activities. The data represent the average of at least three independent experiments. Multiple independent transfections did not show any correlation between RIZ protein expression and β-galactosidase activities, showing that RIZ did not affect CMV promoter, which drives β-galactosidase expression.

Immunoblot and Immunoprecipitation Analysis—GAL4 (1–147) an-tiserum (Santa Cruz Biotechnologies, Inc.) was used for immunoblot analysis to verify the expression of GAL4-RIZ fusion proteins. Monoclonal antibodies 2D7 and P4E1 were used for immunoblot analysis to verify expression of RIZ proteins (1, 3). Nuclear extracts were prepared from transfected cells by rapidly suspending cells in lysis buffer for 3 min (0.5% Nonidet P-40, 0.15 mM NaCl, 10 mM Tris-HCl, pH 7.9, and 1 mM EDTA). Nuclei were then pelleted at 2000 g for 5 min and washed once with lysis buffer without Nonidet P-40. Nuclear proteins were then extracted with high salt buffer (0.2 M NaCl, 0.2 M NaF, and 0.10 mM EGTA; pH 7.9, 0.2 mM DTT, 0.5 mM EDTA, 1.5 mM MgCl₂, 1.0 mM dithiothreitol, 1.0 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin, and 1 mM aprotonin) by shaking at 4°C for 30 min. The extracts were then cleared by centrifugation at 10,000 × g for 10 min at 4°C, which were then analyzed by SDS-gel electrophoresis. Immunoblot was performed on Immobilon P filters (Millipore) using RIZ or GAL4 antibodies and alkaline phosphatase-conjugated goat anti-mouse or anti-rabbit IgG. Immunoprecipitation of nuclear extracts was performed using 2D7 or P4E1 antibody and protein A-Sepharose as described (3).

Plasmid Constructions—pBLCAT2 contains the herpes simplex virus thymidine kinase promoter sequence (515 to +51) linked to the CAT gene (24). pGL3-promoter (Promega) contains SV40 early promoter linked to luciferase gene. pGL3-control (Promega) contains SV40 early promoter plus enhancer linked to luciferase gene. RIZ1 and RIZ2 protein expression plasmids pRIZRz and pRIZRzKK were previously described (3). For carboxyl-terminal-half deletion mutant p3RIZZB (deleting amino acid 901–1706), pRIZRz plasmid was linearized by NotI digestion. The linearized plasmid was treated with Klenow and self-ligated. The elimination of NotI site generated a frameshift and a stop codon at the deleted NotI site. For mutant p3RIZRR (deleting amino acids 659–1706), pRIZRz plasmid was linearized by XbaI followed by Klenow treatment and self-ligation generating a frameshift and a stop codon. For deletion of zinc finger 1–3 region in p3RIZRzB (deleting amino acids 216–575), the fragment from StuI to SpeI was deleted from p3RIZRzB to generate p3RIZRzBΔZf1–3.

The plasmid pGAL4-TK-CAT contains five copies of GAL4 DNA binding site in front of the HSV-TK promoter-CAT cassette in pBLCAT2 (generous gift of Dr. Yang Shi) (25). For GAL4 (amino acids 1–147)-RIZ fusion protein construct, pSG424 vector was used that expresses the GAL4 DNA-binding domain (amino acids 1–147) (26). A KpnI-BamHI 5’ fragment of rat RIZ cDNA was first cloned into the KpnI/XbaI sites of pSG424 to generate pSGNTRr. The unique EcoRI site in pSGNTRr was then eliminated by Klenow treatment to generate the in-frame fusion construct pSGNTR. For plasmids pGRIZ1(18–901) for pGRIZ1(18–659) fragment or pRIZRA was cloned into the KpnI-Stul sites of pSGNTR. To construct pGRIZ1(738–948), a PCR fragment of amino acid 738–948 region was cloned into pSG424. The PCR primers were RP68 (5’TCT CCA CAC GAC CCT CTT G–3’) and RP32 (5’GGG TAA GGA GGC TGC CTG C–3’). To make pGRIZ1(K755N) (point mutation in the GT3 motif), single nucleotide substitution was achieved by using the primer RP89 (5’ATG GGA AAG CATG CATG–3’). All PCR-derived constructs were confirmed by DNA sequence analysis.

To construct pGST-RIZ1-Zf1–3, the SpI to Stul fragment of RIZ cDNA (amino acid 300–520) containing zinc finger motifs 1–3 was cloned into pgEX-KG vector. For pGST-RIZ1-Zf4–6 plasmid, a PCR fragment of rat RIZ cDNA (amino acid 1114–1260) was amplified using primers RP72 (5’TGG TAC GAA AAG AAG TGC TCC–3’) and RP51 (5’TGG TAA CCT TCT CAG C–3’) and cloned into pGEX-KG vector. For pGST-RIZ1-Zf7–8 fusion plasmid (amino acid 1310–1524), an NcoI 0.26-kb fragment of human RIZ cDNA was first cloned into pGEX-KG vector to generate pGKHIZ20.3k. The 0.17-kb SalI-SclI fragment of this plasmid was then replaced with a 0.57-kb SalI-SclI fragment of rat cDNA to make pGST-RIZ1-Zf7–8.

Expression and Purification of Recombinant Protein—Plasmids bearing rat or human cDNA were transfected into E. coli XL1-blue cells, and 0.4 mg lysate was used with primers L745P.S (5’TCC AGT CTC GAC GGG CAT GGC TGC CTT G–3’) and T7 promoters (pT7-7). Pan-1, and expressed using the 2.0 g/ml isopropyl-β-d-thiogalactopyranoside (isopropyl-β-thiogalactopyranoside) for 3 h at 30°C. Protein extract was made and recombinant GST fusion protein was purified as described in (23).

Electrophoretic Mobility Shift Assay—The HSV-TK promoter probe was prepared by isolating the 0.1-kb fragment produced from BamHI and MluI digestion of pBLCAT2 plasmid. The isolated fragment was end-labeled using Klenow enzyme and [γ-³²P]ATP. Low ionic strength polyacylamide gel electrophoresis was performed as described (23). The binding buffer contains 3 mM MgCl₂, 0.1 mM ZnCl₂, 100 mM NaCl, 0.5 mM EDTA, 0.4% Nonidet P-40, 20 mM Hepes, pH 7.5, 50 μg/ml salmon sperm DNA, and 100 μg/ml bovine serum albumin. Oligonucleotides used for competition experiments were as follows: Sp-1 consensus oligonucleotide, 5’-ATT CGA TCG GGG GGG GGC GAC C-3’; GAL4 (Santa Cruz Biotechnologies, Inc.): Sp-1 mutant oligonucleotide, 5’-ATT CGA TCG GGG GGG GGC GAC C-3’; CTF, 5’-CTT GTC ATT GGC TAA CAC C-3’; and E-box, 5’-CTA GAC GAC CAA GAT G CAG T-3’.

DNA Immunoprecipitation Assay—This assay was performed essentially as described (27). The 100-base pair TK promoter probe, as used in EMSA, and protein A-Sepharose-bound RIZ (p3RIZRz) immunoprecipitation products were incubated for 1 h at 4°C in 200 μl of binding buffer as described for EMSA. The reaction was washed three times with binding buffer, processed as described (27), and analyzed by electrophoresis in a 5% polyacrylamide TBE gel or 1% agarose TBE gel, followed by autoradiography.

RESULTS

During the course of experiments examining the transcriptional regulatory function of RIZ proteins, we found that a commonly used reporter plasmid (pBLCAT2) was consistently repressed by RIZ gene products in transient transfections in 3T3 cells (21). Both RIZ1 and RIZ2 proteins can similarly repress the promoter in a dose-dependent manner. The plasmids (p3RIZRz and p3RIZRzKK) used for transient expression of RIZ1 and RIZ2 proteins have been previously described; the amount of RIZ1 protein produced from plasmid p3RIZRz is similar to that of RIZ2 from plasmid p3RIZRzKK (3). The construct pBLCAT2 contains the −105 to +51 sequences of the HSV-TK

S-transferase; EMSA, electrophoretic mobility shift assay; TBE, Tris-borate-EDTA.
promoter (24). Inspection of the sequence revealed no MTE element (GTCATATGAC), the known binding site for RIZ/MTB-Zf. The data suggest that RIZ can repress transcription independent of binding to the MTE DNA element.

RIZ proteins contain eight zinc finger motifs of the C2-H2 and C2-HC classes that are well known DNA-binding motifs; one such motif can be sufficient for sequence-specific DNA binding (28). It is likely that the MTE site might represent only one of the DNA-binding sites to which RIZ could bind. We thus examined whether RIZ proteins could directly bind to HSV-TK promoter, which might, at least in part, be responsible for the repression in Fig. 1. For these experiments, several GST fusion proteins containing various RIZ zinc finger motifs were expressed in E. coli and purified as shown in Fig. 2B. GST fusion protein of zf7–8 migrated slower than predicted in SDS gel; this is likely a result of anomalous migration that is also displayed by full-length RIZ proteins (1).

We next examined the DNA-binding activities of these GST fusion proteins using HSV-TK promoter probe. As shown in Fig. 2C, dose-dependent binding was observed for GST-ZF1–Zf3 protein containing the amino-terminal three zinc finger motifs. No binding activity was observed for zinc finger 4–6 or 7–8 GST fusion proteins. The results show that the amino-terminal three zinc finger motifs of RIZ can directly bind to HSV-TK promoter. To examine whether full-length RIZ1 protein could also bind to HSV-TK, we performed DNA immunoprecipitation assay. Nuclear extracts of 3T3 cells transfected with p3RIZr or pcDNA3 vector were immunoprecipitated with P4E1 antibody or M73 E1A antibody. Proteins bound to protein A-Sepharose with p3RIZr or pcDNA3 vector were immunoprecipitated with P4E1 antibody or M73 E1A antibody. Proteins bound to protein A-Sepharose were incubated with HSV-TK probe. Lane 1 contains 5% of the total input counts used for each of the other samples. Bound DNA was analyzed on 1% agarose TBE gel.

Each site was used to compete for binding to HSV-TK promoter probe in EMSA assays (Fig. 3B). A consensus Sp-1 site oligonucleotide inhibited binding in a dose-dependent manner. A mutant oligonucleotide with a “GG” to “TT” substitution in the Sp-1 binding site weakly inhibited binding only at high dose. Both the CTF and the E-box oligonucleotides derived from HSV-TK did not significantly inhibit binding. The results show that GC-box Sp-1 site mediates binding of HSV-TK promoter to RIZ. Binding of RIZ protein to Sp-1 site was also more directly demonstrated by EMSA assays using Sp-1 oligonucleotide as probe (Fig. 3C).

A typical transcriptional repressor contains both a DNA-binding domain and a repressor domain. Results described above mapped a GC-rich DNA-binding domain to zinc finger 1–3 of RIZ. To determine whether RIZ1 protein also encodes a repressor domain, we made several deletion mutants of RIZ1. Mutant p3RIZrB (amino acid 1–901) expressing the amino-terminal half of RIZ1 protein (amino acid 1–901) showed 2–4-fold less repression activity than p3RIZrB (amino acid 216–575) from p3RIZrB. This deletion completely abolished repression activity (Fig. 4A). The result suggested that amino acid 659–901 might encode transcriptional repressor domain. To show that repression by p3RIZrB also requires DNA-binding, we constructed mutant p3RIZrΔzf1–3 that deleted zinc finger 1–3 region (amino acid 216–575) from p3RIZrB. This deletion completely abolished repression activity (Fig. 4A). Similar levels of expression of these mutant proteins were confirmed by immunoblot analysis (Fig. 4B). The DNA binding activities of these mutant proteins were also examined by DNA immunoprecipitation assay using antibody P4E1, which can immunoprecipitate all these mutant proteins. As expected, mutant p3RIZrA and p3RIZrB retained DNA binding but p3RIZrΔzf1–3 showed no activity (Fig. 4C). The results suggest that repression by RIZ1 requires an intact DNA-binding domain as well as a repressor domain.
We next asked whether the repressor domain can also function when linked to a heterologous DNA-binding domain. The GAL4 system for mapping transcriptional regulatory motifs was used (26). Several GAL4-(1–147)-DNA-binding domain fusion proteins of RIZI were constructed. These fusion protein constructs were assayed by using pGAL4-TK-CAT construct as reporter, which contains five copies of GAL4 binding sites linked to the HSV-TK promoter. Similar to results of Fig. 4, pGRIZ1-(18–901) fusion protein strongly repressed transcription but pGRIZ1-(18–659) did so only weakly (Fig. 5A). Amino acids 738–948 were found to be sufficient to confer repression function to GAL4-(1–147) protein, demonstrating that this region encodes repressor activity. This region has been noted to contain putative GTPase and SH3 motifs (1). To examine whether some of these conserved sequence motifs might play a role in repression function, two point mutations were generated. The point mutation K755N altered a conserved residue in the G1 motif of the putative GTPase domain and did not affect repression. The point mutation L745P that alters the SH3 motif completely disrupted repression activity. Immunoblot analysis of transfected cells showed that these different GAL4 fusion constructs expressed similar levels of proteins (Fig. 5B). The results suggest that sequences encoding the putative SH3 motif but not the GTPase motif might be involved in repressor function.

The results described above suggest that RIZ proteins may function to repress promoters containing GC-rich Sp1 sites. To test a distinct promoter regulated by Sp1, we analyzed the effect of RIZ on the SV40 early promoter, which is primarily controlled by Sp1 sites (19, 29). Two reporter constructs were tested: pGL3-promoter, which contains the SV40 early promoter linked to the luciferase gene, and pGL3-control, which also contains the SV40 enhancer in addition to the promoter. As shown in Fig. 6A, RIZ1 efficiently repressed both reporters (3–4-fold), whereas RIZ2 repressed weakly (1–1.5-fold). Immunoblot analysis showed that similar levels of RIZ1 and RIZ2 proteins were produced by transient transfection (Fig. 6B). The results suggest a role for PR in regulating the transcriptional repressor function of RIZ proteins.

**DISCUSSION**

The predicted protein structure and the ways through which the RIZ/G0B/MTB-Zf gene was isolated suggest that this gene might function as a DNA-binding transcription factor. MTB-Zf or RIZ2 has previously been shown to bind to the MTE element GTCATATGAC and to function as a weak transcriptional repressor. The results described above suggest that RIZ proteins may function to repress promoters containing GC-rich Sp1 sites. To test a distinct promoter regulated by Sp1, we analyzed the effect of RIZ on the SV40 early promoter, which is primarily controlled by Sp1 sites (19, 29). Two reporter constructs were tested: pGL3-promoter, which contains the SV40 early promoter linked to the luciferase gene, and pGL3-control, which also contains the SV40 enhancer in addition to the promoter. As shown in Fig. 6A, RIZ1 efficiently repressed both reporters (3–4-fold), whereas RIZ2 repressed weakly (1–1.5-fold). Immunoblot analysis showed that similar levels of RIZ1 and RIZ2 proteins were produced by transient transfection (Fig. 6B). The results suggest a role for PR in regulating the transcriptional repressor function of RIZ proteins.

The predicted protein structure and the ways through which the RIZ/G0B/MTB-Zf gene was isolated suggest that this gene might function as a DNA-binding transcription factor. MTB-Zf or RIZ2 has previously been shown to bind to the MTE element GTCATATGAC and to function as a weak transcription activator (16). Here, we provide evidence that RIZ gene

**FIG. 3. RIZ binding to HSV-TK promoter is mediated by Sp1.** A, DNA sequence of HSV-TK promoter. The sequence of HSV-TK promoter (−105 to +51) in pBLCAT2 plasmid is shown here flanked by BamH1 and XhoI sites. Sp1, TF, E-box, and TATA box sites are marked. MluI enzyme site is also underlined. Transcription start site is marked +1. B, Sp1 consensus oligonucleotide competes for RIZ binding to HSV-TK promoter. EMSA assays using pGST-RIZ1-Zf1–3 protein (50 ng) and HSV-TK promoter probe were performed in the absence (lane 1) or presence of various oligonucleotides representing Sp1, mutant Sp1, TF, E-box, and TATA box elements as indicated. The amounts of each competitor used was in 25-, 100-, and 400-fold excess over the probe. C, RIZ binding to Sp1 consensus oligonucleotide. Sp1 oligonucleotide probe was incubated with GST protein (lane 1) or GST-RIZ1-Zf1–3 protein (lane 2) and analyzed by EMSA assays.

**FIG. 4. Mutational analysis of RIZ transcriptional repression function.** A, analysis of transcriptional activity of RIZ mutant proteins. 3T3 cells were transfected with the reporter plasmid pBLCAT2, internal control plasmid pCMV-β, and RIZ mutant plasmids p3RIZrB, p3RIZrA, or p3RIZrBΔZf1–3, whose structures are schematically shown. CAT activity obtained by co-transfection of pBLCAT2 and pcDNA3 was set at 100%. B, immunoblot analysis of RIZ mutant proteins. 3T3 cells were transfected with RIZ mutant protein expression constructs. Nuclear extracts prepared from transfected cells were resolved on SDS gel followed by immunoblot analysis using RIZ antibody P4E1. Lanes 1–4 represent extracts from mock, p3RIZrBΔZf1–3, p3RIZrB, and p3RIZrA transfected cells, respectively. Stars mark the positions of expressed proteins. C, DNA immunoprecipitation assay. Nuclear extracts of 3T3 cells transfected with p3RIZrA (lane 2), p3RIZrB (lane 3), p3RIZrBΔZf1–3 (lane 4), and pcDNA3 vector (lane 5) were immunoprecipitated with P4E1 antibody. Proteins bound to protein A-Sepharose were incubated with HSV-TK probe. Bound DNA was analyzed on 0.5% polyacrylamide-TBE gel. Lane 1 contains 5% of the total input counts used for each of the other samples."
products can bind to a different type of DNA element and function to repress transcription. We show that RIZ gene products can efficiently repress the transcriptional function of HSV-TK promoter, one of the best characterized eukaryotic promoters. The Sp-1 and CTF sites are well established essential elements for basal expression of this promoter (19). Disruptions of either element impaired promoter function (30). The mechanisms of repression of HSV-TK by RIZ probably involves direct binding of RIZ proteins to the Sp-1 sites of the promoter. We show that RIZ proteins could directly interact with this promoter which can be blocked by consensus Sp-1 oligonucleotide. We also show that the DNA-binding motif of RIZ is required for repression of HSV-TK. Definitive proof for a role of binding to GC-rich elements requires demonstration of loss of RIZ-mediated repression as a result of Sp-1 site mutation. Here, the requirement of Sp-1 sites for basal expression as demonstrated previously (30) could make such an experiment uninformative. An impaired basal activity caused by Sp-1-site mutation may obscure RIZ repression effects. Nevertheless, demonstrating site dependence represents an important future study. It should be noted that several factors other than Sp-1 can also bind to GC-rich motifs such as E2F1 (31) and others (20).

If DNA binding is directly involved in RIZ repression of HSV-TK, a simple mechanism for repression might be through competition with Sp-1 proteins for binding sites. While our results do not rule out this passive mechanism, two lines of evidence suggest that RIZ exerts repression through an active mechanism. First, truncation mutant p3RIZrA showed significantly impaired repression relative to truncation mutant p3RIZrB. While they differ by the central region of RIZ which encodes repressor activity, both mutant proteins contain zinc finger motifs 1–3, which were sufficient for binding to HSV-TK. The observation suggests that DNA-binding alone cannot convey maximal repression.

Second, conforming to the commonly observed modular nature of transcriptional regulatory domains, the repressor region of RIZ (amino acid 738–948) could act in a heterologous context. Fusion of this region to the GAL4-DNA-binding domain generated a GAL4-binding site-dependent repressor. As noted previously (1), this region of RIZ bears sequence similarity to the consensus sequence of SH3 and GTPase motifs. However, functional significance of this similarity remains unclear. It appears that a GTPase function is unlikely involved in repressor function. The mutant protein p3RIZrB-(1–901), lacking part of the conserved GTPase structure (missing the G4 motif, TQPD), retained full repressor function. A point mutation (K755N), changing the conserved Lys residue in the G1 motif of GTPase domain, did not affect repressor function but is known to disrupt GTP binding of other GTPases (32). In contrast, another point mutation (L745P) outside the GTPase domain completely abolished repressor function. Although substituting to Pro may appear to represent a drastic change, this region is Pro-rich to begin with (Fig. 5C). The result seems to implicate a role for SH3 sequence because the mutated residue is conserved among different SH3 motifs. Evidence for a role of

FIG. 5. Repressor activity of GAL4-RIZ fusion proteins. A, 3T3 cells were transfected with reporter plasmid pGAL4-TK-CAT or pBLCAT2, internal control plasmid pCMV-β, and various GAL4 fusion proteins of RIZ. CAT activity obtained by co-transfection of reporter and pcDNA3 plasmids was set at 100%. B, immunoblot analysis of GAL4-RIZ fusion proteins. 3T3 cells were transfected with GAL4 fusion protein expression plasmids. Nuclear extracts prepared from transfected cells were resolved on 5% (lanes 1–3) or 10% (lanes 4–7) SDS gel followed by immunoblot analysis using anti-GAL4 serum. Lanes 1–3 represent extracts from pGRIZ1-(18–901), pGRIZ1-(18–659), and mock-transfected cells, respectively. Lanes 4–7 represent extracts from mock-, pGRIZ1-(738–948), pGRIZ1(L745P), and pGRIZ1(K755N)-transfected cells, respectively. Stars mark the position of the expressed proteins. C, partial amino acid sequence of SH3 and GTPase motifs of the region is shown here starting from amino acid (aa) residue 738, which is linked to GAL4 protein. SH3 and GTPase motifs are underlined. The mutated residues are circled.
obtained by co-transfection of reporter and pcDNA3 plasmids was set at 100%. Each lane as determined by Coomassie Blue staining of a portion of the gel (not shown). Similar amounts of total protein extracts were loaded in 2D7 and P4E1. Similar amounts of total protein extracts were loaded in each lane as determined by Coomassie Blue staining of a portion of the gel (not shown).

These observations suggest that PR domain might be important for either basal expression or for activation in response to growth factors, and housekeeping enzymes (20). In many cases where it has been studied, the GC-rich element appears to be essential for the formation of positive factors for DNA binding sites. It has also been shown recently to contain a repressor domain that is required for its transforming activity (10). It will be important to examine whether MDS1-EV1 might function as a better or worse repressor relative to EV1, which might provide a molecular rational for the specific disruption of MDS1-EV1 PR in leukemogenesis. The repressor domain of EV1 bears no similarity to that of RIZ and has been noted to be proline-rich (10), which is a common feature of some repressor domains (37). The repressor region of RIZ has multiple prolines but is clearly not the most proline-rich region of the protein. It is intriguing that the RIZ repressor region resembles SH3 domain, which is known to bind to proline-rich peptides. As transcriptional activation or repression domains are known to act through protein-protein contact, it is not inconceivable that a SH3-proline-type interaction could be involved in the mechanisms of transcriptional repressor domain action.

Because GC-rich elements are extremely common in cellular gene promoters, the results presented here suggest a role for RIZ proteins in the regulation of a potentially large number of cellular genes. In particular, many growth-regulated genes are characterized by GC-rich and TATA-less promoters including oncogenes, growth factors, and their receptors, transcription factors, and housekeeping enzymes (20). In many cases where it has been studied, the GC-rich element appears to be essential for either basal expression or for activation in response to growth regulatory signals. Future investigations will be needed to determine whether RIZ proteins might repress cellular growth-regulated genes and in turn exert control over cell growth.

Acknowledgments—We thank Dr. Yang Shi for plasmid pGAL4-TK-CAT. We thank Drs. Graig Hauser and Amy Yee for critically reviewing the manuscript and Crystal Herndon for secretarial assistance.



**REFERENCES**

1. Buyse, I. M., Shao, G., and Huang, S. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 4467–4471
2. Buyse, I. M., and Huang, S. (1997) J. Virol. 71, 6200–6203
3. Liu, L., Shao, G., Steele-Perkins, G., and Huang, S. (1997) J. Biol. Chem. 272, 2894–2901
4. Fears, S., Mathieu, C., Zeleznik-Le, N., Huang, S., Rowley, J. D., and Nucifora, G. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 1642–1647
5. Bartholomew, C., and Ile, J. N. (1993) Mol. Cell. Biol. 13, 1820–1828
6. Morishita, K., Parker, D. S., Mucenski, M. L., Jenkins, N. A., Copeland, N. G., and Ile, J. N. (1998) Cell 94, 831–840
7. Morishita, K., Parganas, E., William, C. L., Whittaker, M. H., Drahkin, H., Oral, J., Taele, R., Valentine, M. B., and Ile, J. N. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 3937–3941
8. Nucifora, G., Begy, C. R., Kobayashi, H., Roulston, D., Claxton, D., Pedersen-Jensen, J., Parganas, E., Ile, J. N., and Rowley, J. D. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 4004–4008
9. Morishita, K., Suzukawa, K., Taki, T., Ile, J. N., and Yokota, J. (1995) Oncogene 10, 1961–1967
10. Bartholomew, C., Kibey, A., Clark, A. M., and Walker, M. (1997) Oncogene 14, 569–577
11. Huang, S. (1994) Cell 76, 9
12. Keller, A. D., and Maniatis, T. (1991) Genes Dev. 5, 868–879
13. Turner, C. A., Jr., Mack, H. D., and Davis, M. M. (1994) Cell 77, 297–306
14. Mock, B. A., Liu, L., LeFashler, D., and Huang, S. (1996) Genomics 37, 24–28
15. Lin, Y., Wong, K.-k., and Calame, K. (1997) Science 276, 596–598
16. Murasaca, Y., Takahashi, K., Yoshizawa, M., and Shibahara, S. (1996) Eur. J. Biochem. 235, 471–479
17. Shapiro, V. S., Lee, P., and Winoto, A. (1995) Gene (Amst.) 163, 329–330
18. Buyse, I. M., Takahashi, E., and Huang, S. (1996) Genomics 34, 119–121
19. McKnight, S., and Tjian, R. (1986) Cell 46, 795–805
20. Azizkhan, J. C., Jensen, D. E., Pierce, A. J., and Wade, M. (1993) Crit. Rev. Eukaryotic Gene Expression 3, 229–254
21. Chen, C. A., and Okayama, H. (1988) BioTechniques 6, 632–638
22. MacGregor, G. R., and Caskey, C. T. (1989) Nucleic Acids Res. 17, 2365
23. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. B., Seidman, J. G., Smith,
Transcription Repression by RIZ

31. Shin, E. K., Tevosian, S. G., and Yee, A. S. (1996) J. Biol. Chem. 271, 12261–12268
32. Bourne, H. R., Sanders, D. A., and McCormick, F. (1991) Nature 349, 117–127
33. Feller, S. M., Ren, R., Hanafusa, H., and Baltimore, D. (1994) Trends Biochem. Sci. 19, 453–458
34. Muraosa, Y., and Shibahara, S. (1993) Mol. Cell. Biol. 13, 7881–7891
35. Goodrich, J. A., Cutler, G., and Tjian, R. (1996) Cell 84, 825–830
36. Kreider, B. L., Orkin, S. H., and Ihle, J. N. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 6454–6458
37. Cowell, I. G. (1994) Trends Biochem. Sci. 19, 38–42