

PRH Represses Transcription in Hematopoietic Cells by at Least Two Independent Mechanisms*

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PRH (proline-rich homeodomain protein) is strongly expressed in the hematopoietic compartment. Here we show that PRH is a repressor of transcription in hematopoietic cells. A fragment of PRH that includes the homeodomain can bind to TATA box sequences *in vitro* and can also bind to the TATA box-binding protein. PRH represses transcription from TATA box-containing promoters in intact cells but does not repress transcription from a promoter lacking a TATA box. A mutation in the PRH homeodomain that blocks binding to DNA but that has little or no effect on binding to the TATA box-binding protein significantly reduces the ability of the protein to repress transcription and provides the first clear demonstration that a homeodomain can bring about transcriptional repression *in vivo* by binding to a TATA box. However, we also show that mutation of the PRH homeodomain does not block the ability of PRH to repress transcription when this protein is tethered upstream of the TATA box via a heterologous DNA-binding domain. PRH also contains an N-terminal proline-rich repression domain that is separate from the homeodomain. Deletion mapping suggests that this repression domain contains at least two regions that both independently contribute to transcriptional repression.

Homeodomain proteins are a family of transcription factors that are important regulators of gene expression in all eukaryotes (1). The homeodomain is a 60-amino acid motif that mediates sequence-specific binding to DNA (2). The homeodomain can also make protein-protein contacts that modulate the ability of the homeodomain proteins to enter the nucleus (3), bind DNA (4), and regulate transcription (5, 6). PRH (proline-rich homeodomain protein) was first identified in differentiated avian hematopoietic and liver cells (7) and was subsequently found to be conserved in human, *Xenopus*, mouse, and rat where it has also been called XHex or Hex (8–10). PRH is expressed in the anterior endoderm in developing *Xenopus* and mouse embryos (11, 12) and is present in fetal liver, thyroid, and lung (13). It is a member of the tinman family of homeodomain-containing proteins that includes several transcription factors essential for the development of cardiac tissue (14). Within the hematopoietic compartment PRH mRNA is ex-

pressed in B-cells, myelomonocytic cells, and erythroid cells but not in T-cell lineages (7, 15), and in general, PRH mRNA levels are down-regulated as hematopoietic cells differentiate (15, 16). Several studies suggest a role for PRH in the regulation of cell proliferation and differentiation. PRH is transiently expressed in vascular endothelial cells in *Xenopus* embryos and overexpression of *Xenopus* PRH disrupts developing vascular structures and brings about an increase in the number of these cells (8). In addition, overexpression of PRH in Myb-Ets transformed multipotential hematopoietic cells inhibits cell growth, whereas the expression of truncated PRH derivatives alters the ability of these cells to differentiate (16). Finally, PRH interacts with PML (promyelocytic leukemia protein), a growth control protein, in several leukemic cell lines (17) and is up-regulated in some B-cell leukemias (18).

PRH has recently been reported to repress transcription in liver cells (10). However, the mechanism or mechanisms whereby this protein brings about transcriptional repression have yet to be determined. Transcriptional repression mechanisms can be broadly classified into four main categories: steric hinderance, quenching, direct repression, and the modulation of chromatin structure. Many repressor proteins hinder sterically the binding of transcription activators or general transcription factors, and this is often referred to as passive repression. For example, the *Drosophila* homeodomain protein Engrailed (En) blocks the action of the activator protein Fushi tarazu by competing for a common binding site on DNA (19). *In vitro* studies have demonstrated that En can also repress transcription by directly competing with the TATA box-binding protein (TBP)¹ for binding to a TATA box (20). Similarly, *in vitro* studies with Even-skipped (Eve), another *Drosophila* homeodomain protein, have suggested that Eve can repress transcription by a mechanism that involves the cooperative binding of Eve protein to low affinity Eve binding sites that lie adjacent to TATA box sequences or activator protein binding sites. This results in Eve sterically blocking DNA binding by TBP or the activator protein Zeste (21, 22). In quenching repressor proteins mask the activity of locally bound activator proteins. For example, the *Drosophila* protein Kruppel can bind to Sp1 and prevent this protein activating transcription (23). In addition, repressor proteins can interfere with the targets of the activators, such as components of the core transcription machinery, a process known as direct repression. Kruppel has been shown to bind *in vitro* to the small subunit of TFIIE (24), and a number of repressor proteins have been shown to bind to TBP including: Eve (25), the mouse homeodomain protein Msx-1 (26), the

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¹ The abbreviations used are: TBP, TATA box-binding protein; DBD, DNA-binding domain; bp, base pair(s); aa, amino acid(s); GST, glutathione S-transferase; NTA, nitrilotriacetic acid; PAGE, polyacrylamide gel electrophoresis; TK, thymidine kinase.

unliganded thyroid hormone receptor (27), and the global repressor proteins Dr1 (28) and Mot1 (29).

Several repressor proteins interact with chromatin stabilizing factors or chromatin assembly complexes. The best understood chromatin stabilizing proteins are probably the histone deacetylases. Histone deacetylation is thought to tighten the nucleosome-DNA interaction with the consequence that the access of transcription factors to their binding sites is hindered. The mammalian protein YY1 interacts directly with a number of proteins involved in transcription, including mRPD3, a histone deacetylase (30). Many other transcriptional repressors interact with deacetylases indirectly via corepressor proteins. For example, the Max-Mad repressor complex and the unliganded thyroid hormone receptor protein interact with mSin3a/mSin3b and the closely related corepressors N-CoR and SMRT, respectively. These corepressors then effect the repression of transcription through the recruitment of histone deacetylases (31, 32).

Several repressor proteins have been shown to use more than one mechanism to bring about transcriptional repression. For example, the En homeodomain protein appears to utilize at least four different mechanisms: En can compete with activators (19), compete with TBP for binding to DNA *in vitro* (20), interact directly with the *Drosophila* corepressor protein Groucho (33, 34), and, in addition, En contains a repression domain that is separate from the Groucho interacting region and that works by an as yet unknown mechanism (35). In this study we examine the ability of PRH to regulate transcription in hematopoietic cells, we investigate the PRH repression domains, and we explore the mechanisms whereby PRH might bring about transcriptional repression.

MATERIALS AND METHODS

Plasmid DNAs Used in This Study

Reporters—The pTK luciferase reporter construct has been described previously (36). The pTK-GAL luciferase construct was made by cloning five GAL4 binding sites between the *EcoRI* and *BamHI* sites of pTK. The pTK-PRH luciferase construct was made by cloning five PRH binding sites identified in site selection experiments (7) between the *HindIII* and *SmaI* sites of pTK. The HS1-luciferase reporter is described in (37) and was supplied by Dr. K. Gaston (University of Bristol, Bristol, UK). The SV40 promoter-luciferase reporter is the pGL-3 promoter vector from Promega. The transfection control plasmids are pSV- β -galactosidase control vector (pSV-*lacZ*) from Promega and the pRSV- β -galactosidase (pRSV-*lacZ*), which contains the Rous sarcoma virus long terminal repeat placed upstream of the *lacZ* gene.

Expressors—pCMV-PRH was made by cloning the 1.5-kilobase PRH cDNA fragment described in Ref. 7 into the *EcoRI* site of pCMV (Promega). pSG424 has been described previously (38). The N-terminal glutamine-rich Sp1 activation domain (base pairs 1–510) and full-length HIV-1 TAT (base pair 831–1091 of the HIV genome) were cloned in frame with GAL4 DBD into the multiple cloning site of pSG424. These plasmids were kindly supplied by Dr. M. Dickens (University of Leicester, Leicester, UK). pMLV-GAL147plink has been described previously (39). GAL4-VP16 was made by cloning a polymerase chain reaction fragment encoding the viral VP16 acidic activation domain (base pairs 1680–1923) in frame between the *SalI* and *SpeI* sites of pMLV-GAL147plink. The plasmid pcDNA3-TLE1 expresses human TLE1 (40) and was kindly supplied by Dr S. Stifani (McGill University, Montreal, Canada). To construct the pBlueScript-TBP plasmid used for *in vitro* transcription and translation experiments, an *EcoRI-HindIII* fragment encoding the TBP cDNA was isolated from the plasmid pGEXKG-TBP (41) kindly supplied by Dr D. Hornby (University of Sheffield, Sheffield, UK). This fragment was cloned between the *EcoRI* and *HindIII* sites of pBlueScript II (Stratagene).

GAL4-PRH Fusion Proteins—The PRH cDNA described in Ref. 7 was cloned into the *EcoRI* site in pBlueScript (Stratagene) to create pBSK-PRH16.3. This plasmid was cut with *SalI* (which is located 5' to the first PRH ATG codon) and *NarI* (98 bp after the ATG), and an oligonucleotide corresponding to the coding region from the ATG to the *NarI* site was cloned between the *SalI* and *NarI* sites. A *SmaI-SmaI* fragment was then removed from the 3'-noncoding region, and the vector was

self-ligated to create pBSK-PRH18.3. For GAL4-PRH 1–277, a *SalI-SpeI* fragment from pBSK-PRH18.3 was cloned into pMLV-GAL147plink between the *SalI* and *SpeI* sites. GAL4-PRH 1–141 was constructed from pBSK-PRH18.3 by cutting with *NotI* and cloning in a linker with a stop codon and a *SpeI* site to create pBSK-PRH18.341; the 423-bp *SalI-SpeI* fragment was then transferred to pMLV-GAL147plink. For GAL4-PRH 28–141, plasmid GAL4-PRH 1–141 was cut with *SalI* and *PstI* (removing bp 1–84) blunted with T4 polymerase and religated. For GAL4-PRH 81–141, an *AclI* fragment was cut from pBSK-PRH18.341. This corresponds to amino acids (aa) 81–141 and some pBlueScriptKII vector sequence. The *AclI* sites were filled in with Klenow and cloned into the pMLV-GAL147plink *SalI* and *SpeI* sites that had been cut and filled in by T4 polymerase. This recreates the *SalI* site, but the *SpeI* site is lost. For GAL4-PRH 107–141, GAL4-PRH 1–141 was cut with *SalI* (aa 1) and filled in and then cut with *ApaI* (aa 105), trimmed back with T4 polymerase, and religated. Thus, the 1–107 *SalI* to *ApaI* fragment was removed, leaving the 107–141 PRH fragment and the vector on religation recreated the 5' *SalI* site. For GAL4-PRH 1–105, an *ApaI* fragment from pBSK-PRH18.3 was cloned into the *SmaI* site of pBSK to make pBSK-PRH18.305. The *ApaI* fragment contained the 5' *SalI* site and aa 1–105 of PRH. The 315-bp *SalI-SpeI* PRH fragment from this vector was cloned into pMLV-GAL147plink. For GAL4-PRH 28–105, pBSK-PRH18.305 was cut with *PstI* and *SalI*, blunted with T4 polymerase, and religated to itself, thus removing aa 1–28 of PRH and the 5' *SalI* site to create pBSK-PRH18.3205. An *XhoI-SpeI* PRH fragment from this vector was cloned into pMLV-GAL147plink. The *XhoI-SpeI* PRH fragment has three extra codons 5' to aa 1 in PRH, so GAL4-PRH 28–105 contains three extra codons between the GAL4 DBD and aa 1 of PRH. For GAL4-PRH 1–125, an *ApaI* (aa 105–141)-*SpeI* fragment was removed from GAL4-PRH 1–141 leaving aa 1–105. An oligonucleotide linker coding for amino acids 105–125 (from the *ApaI* site) and including a *SpeI* site was cloned into this plasmid. For GAL4-PRH 107–277, the *ApaI SpeI* 510-bp PRH fragment from pBSK-PRH18.3 was isolated after the *ApaI* site was blunted with T4 polymerase. This fragment was cloned into the *SalI SpeI* site of pMLV-GAL147plink after the *SalI* site in the vector was filled in with Klenow. This creates GAL4-PRH 107–277 and recreates the *SalI* site at the 5' end. For GAL4-PRH 49–141, GAL4-PRH 143–277, GAL4-PRH 143–210, and GAL4-PRH 202–277 were made by polymerase chain reaction from GAL4-PRH 1–277 using 5' oligonucleotides carrying a *SalI* site and a 3' oligonucleotides carrying a *SpeI* site. The polymerase chain reaction fragments were cloned into the *SalI* and *SpeI* sites of pMLV-GAL147plink: 49–56, 5'-GTGACCCCGCCCC-CTCCTGCCCCGCC-3'; 61–65, 5'-GTCGACACGCTGCCGTGCCAAC-3'; 143–148, 5'-GTCGACAAGAGGAAGGGTGGCCAG-3'; 202–208, 5'-GTCGACCTGAAGCAGGGAGAACCCAGG-3'; 125–120, 5'-ACTAGTGCTCTGGCGGATCAGTGC-3'; 141–136, 5'-ACTAGTCAGCGGCCGCTGGATGA-3'; 210–204, 5'-ACTAGTGGTGGCTGGGGGTTCTCCTG-3'; and 277–270, 5'-ACTAGTGCCTGTGGCGCTGTAGAA-GCCTTT-3'.

Bacterial Expression Plasmids—The histidine-tagged PRH expression vectors were created by cloning the *NotI-EcoRI* fragment from pBSK-PRH16.3 containing sequences encoding the PRH homeodomain and C terminus, together with an *XhoI-NotI* linker, between the *XhoI* and *EcoRI* sites of pTrcHisA (Invitrogen) to create pTrcHisA-PRH 137–277. A *NotI-SmaI* PRH fragment carrying the N194A mutation described below was transferred from the pBSK-PRH_{HDM} 18.3 clone into pTrcHisA-PRH 137–277, replacing the wild type PRH sequence and creating pTrcHisA-PRH_{HDM} 137–277.

The GST-PRH 1–141 expression vector was created by cloning the DNA sequence encoding the PRH N terminus (amino acids 1–141), as a *SalI-SpeI* fragment from pGAL4-PRH 1–141 into pGEX20T that had been cut with *XhoI* and *SpeI*, creating pGEX20T-PRH 1–141. pGEX20T is a derivative of pGEX2T (Amersham Pharmacia Biotech) and contains unique *XhoI* and *SpeI* restriction sites in the polylinker downstream of the GST moiety. The GST-tagged human PRH N terminus was a gift from Dr. G. Manfioletti (University of Trieste, Trieste, Italy). Briefly, the human PRH N terminus (amino acids 1–131) was cloned as an *EcoRI* fragment into pGEX3X (Amersham Pharmacia Biotech). All constructs were checked by DNA sequencing.

Cell Culture and Transient Transfection Assays

BM2 cells were grown in RPMI 1640 (Life Technologies, Inc.) 25 mM HEPES medium supplemented with 10% tryptose phosphate, 10% glutamine, 10% fetal calf serum, and 5% chicken serum to a density of $\sim 1 \times 10^6$ cells/ml. The cells were collected by centrifugation and then resuspended in RPMI 1640 25 mM HEPES plus 10% fetal calf serum to

a density of 5×10^7 cells/ml. 1×10^7 cells plus $5 \mu\text{g}$ each of the luciferase and lacZ reporter plasmids and the amount of expressor plasmid indicated under "Results" were electroporated using a Bio-Rad GenePulser (0.25 V, 960 microfarads). Cells were rested for 10 min and then incubated overnight in 10 ml of supplemented medium. After 24 h the cells were harvested, and luciferase activity was assayed using the Promega luciferase assay system according to the manufacturer's instructions. β -Galactosidase assays were performed as an internal control for transfection efficiency. After subtraction of background, the luciferase counts were normalized against the β -galactosidase value. QT6 cells were grown in Dulbecco's modified Eagle medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum and 2% chicken serum. 3×10^6 QT6 cells were transfected with $5 \mu\text{g}$ of each of the reporter plasmids and $1 \mu\text{g}$ of expressor plasmid by calcium phosphate precipitation. Cell extracts were made 18 h after transfection as described for BM2 cells. At least two independent batches of expressor and reporter constructs were assayed in multiple experiments, and the results were averaged.

Site-directed Mutagenesis

Mutagenesis of PRH asparagine 194 to alanine was carried out in pBSK-PRH18.3 using a mutant oligonucleotide: 5'-AAAACGTGGTTC-CAGGCCCGCAGAGCCAAATG-3' and a QuikChange mutagenesis kit (Stratagene) according to the manufacturer's instructions. The underlined positions mismatch the template and introduce the desired sequence change. After sequencing the mutated clone (pBSK-PRH_{HDM} 18.3), a *NotI*-*SpeI* PRH fragment carrying the mutation was transferred from pBSK-PRH_{HDM} 18.3 into pMLV-GAL147plink-PRH (GAL4-PRH), replacing the wild type PRH sequence and creating pGAL4-PRH_{HDM}.

Proteins Used in This Study

Histidine-tagged fusion proteins were purified from bacterial lysate by chromatography on a nickel (Ni^{2+} -NTA-agarose) column (Qiagen) essentially according to the manufacturer's instructions. The eluted proteins were assayed for purity by SDS-PAGE followed by staining with Coomassie Blue and quantified using the Bio-Rad phosphoric acid protein assay. GST fusion proteins were purified over glutathione-Sepharose 4B beads (Sigma) and assayed for purity and concentration as above.

Circular Dichroism

Circular dichroism spectra of pTrcHisA-PRH 137–277 and pTrcHisA-PRH_{HDM} 137–277 proteins (3.3 and 6.4 μM , respectively, in 25 mM phosphate buffer, pH 7.9) were obtained using a JY CD6 CD spectrometer with 5-mm-pathlength cells. Spectra were collected at 1-nm increments, using a 20-s integration time. The spectra have been corrected for concentration differences.

Electrophoretic Mobility Shift Assays

Single stranded oligonucleotides (100 ng) were 5'-end labeled with [^{32}P]ATP using T4 polynucleotide kinase. After annealing to the complementary oligonucleotide free label was removed using Sephadex G50 spin columns. Labeled oligonucleotides (20,000 cpm) were incubated with purified proteins in the quantities indicated in the figures in binding buffer (10% glycerol, 1 mM MgCl_2 , 20 mM Tris, pH 8.0, 1 mM dithiothreitol, 50 mM NaCl, 0.04 mg/ml dI-dC, 0.1 mg/ml bovine serum albumin). After 30 min on ice, complexes were resolved on 6% non-denaturing polyacrylamide gels run in $1 \times$ TBE and visualized using a PhosphorImager.

In Vitro Binding Assays

Transcription and translation was carried out using a TNT kit (Promega) according to the manufacturer's protocol. Approximately 50–100 μg of affinity-purified histidine-tagged fusion protein (PRH 137–277 or PRH_{HDM} 137–277) was bound to 100 μl of nickel-NTA beads (Qiagen) under the conditions recommended in the manufacturer's protocol. To assay for specific interactions 10 μl of [^{35}S]methionine-labeled *in vitro* translated TBP was added and incubated in binding buffer (20 mM HEPES, pH 7.8, 200 mM KCl, 5 mM MgCl_2 , 0.5 mM dithiothreitol, 0.5% Nonidet P-40, 50 ng/ μl bovine serum albumin) with gentle agitation for 60 min at 4 $^\circ\text{C}$. The beads were washed six times with 1 ml of binding buffer. Bound proteins were eluted with elution buffer (250 mM imidazole, 300 mM NaCl, 50 mM NaH_2PO_4) and analyzed by SDS-PAGE and fluorography.

Western Blotting

QT6 cells were harvested after transient transfection and lysed in luciferase assay lysis buffer (Promega). The supernatant (which contains no detectable signal with antibodies against GAL4 1–147) was removed and used for β -galactosidase and luciferase assays. The chromatin pellet was resuspended in 20 μl of 100 mM HEPES, pH 7.9, 5 mM MgCl_2 , and 1 mM CaCl_2 containing sufficient DNase I to digest the sample in ~ 5 min at 20 $^\circ\text{C}$. After the DNase I digestion, 20 μl of $2 \times$ SDS loading buffer was added, and the sample was boiled for 2 min. A proportional amount of each sample was separated by SDS-PAGE and blotted onto an Immobilon membrane. GAL4 fusion proteins were then detected using a mixture of two monoclonal antibodies 2GV3 and 3GV2 (a gift from Professor P. Chambon, (42) and an ECL kit (Amersham Pharmacia Biotech).

RESULTS

PRH Is a Repressor of Transcription in Hematopoietic Cells—The PRH protein consists of three regions (Fig. 1A). The N-terminal region (aa 1–143) is 20% rich in proline residues and 13% rich in alanine residues and is separated by the homeodomain (aa 144–203) from a C-terminal region (aa 204–277) 25% rich in acidic residues. Both proline/alanine rich regions and acidic regions are commonly found in the regulatory domains of transcription factors. The PRH cDNA was originally cloned from an avian cDNA library derived from BM2 cells, an avian myeloblastosis virus-transformed hematopoietic cell line (7). To investigate whether PRH functions as a transcription factor in BM2 cells, we performed transient cotransfection assays.

Previous site selection experiments using a truncated PRH protein (consisting of the PRH homeodomain and C terminus) determined the consensus PRH binding site to be 5'-(C/T)(A/T)ATT(A/G)-3' (7). Five copies of a PRH binding site identified in these experiments were placed upstream of the luciferase gene under the control of the thymidine kinase minimal promoter (TK) to create the reporter plasmid pTK-PRH (Fig. 1A). Plasmid pCMV-PRH expresses PRH under the control of the cytomegalovirus promoter. This expression plasmid was transiently cotransfected into BM2 cells along with pTK-PRH. The effect of pCMV-PRH on luciferase activity was compared with the effect of the empty expression vector pCMV. To normalize for transfection efficiency, a constant amount of a control plasmid containing the *lacZ* gene under the control of the SV40 promoter and enhancer was also cotransfected. As can be seen from the data shown in Fig. 1B, increasing amounts of the pCMV-PRH plasmid bring about a dramatic decline in luciferase activity. As little as 10 ng of the PRH expression plasmid is sufficient to bring about a 50% decrease in TK promoter activity, suggesting that PRH is a potent repressor of transcription.

A number of homeodomain proteins have been shown to repress transcription even in the absence of their cognate binding sites. To determine whether repression of the pTK-PRH promoter by PRH is dependent upon the presence of PRH binding sites, we cotransfected pCMV-PRH with the pTK reporter lacking PRH binding sites. Increasing amounts of pCMV-PRH bring about increasing repression of this reporter (Fig. 1C). However, in this case a 10-fold higher amount of pCMV-PRH (100 ng) is required to bring about a 50% repression of promoter activity. Thus, PRH is also capable of repressing transcription in the absence of PRH binding sites.

PRH Represses Activated Transcription—In the experiments described above PRH was able to repress transcription from a TK promoter lacking PRH binding sites. To establish whether PRH could repress activated transcription from this promoter, five copies of a GAL4 binding site were inserted upstream of the minimal TK promoter to create pTK-GAL (Fig. 2A). Plasmid pSG424 carries the yeast GAL4 DBD and was used to express a number of activator-GAL4 fusion proteins (38). Transcription activation domains from the Sp1 and HIV-1 TAT

FIG. 1. PRH is a repressor of transcription. *A*, the *top line* is a schematic representation of avian PRH. The *second* and *third lines* are schematic representations of the reporter plasmids pTK and pTK-PRH, respectively. The reporter plasmids contain the minimal TK promoter (from -109 to +52) located upstream of the luciferase gene. pTK-PRH contains five PRH binding sites upstream of the TK promoter. *B*, the *graph* shows the relative promoter activity found in BM2 cell extracts 24 h after transient cotransfection with increasing amounts of the PRH expression plasmid pCMV-PRH, 5 μ g of the pTK-PRH reporter plasmid, and 10 μ g of the β -galactosidase expression plasmid pSV-*lacZ*. The amount of PRH expression plasmid added is shown on a logarithmic scale. Relative promoter activity is the ratio of luciferase activity in the presence of pCMV-PRH to luciferase activity in the presence of an equal amount of the empty pCMV vector. The luciferase values were normalized with respect to transfection efficiency using the cotransfected β -galactosidase plasmid. Each transfection was performed a minimum of three times, and the values shown represent the means and S.D. *C*, BM2 cells were transiently cotransfected with increasing amounts of pCMV-PRH, 5 μ g of the pTK reporter plasmid, and 10 μ g of pSV-*lacZ*. Details are as in *B*.

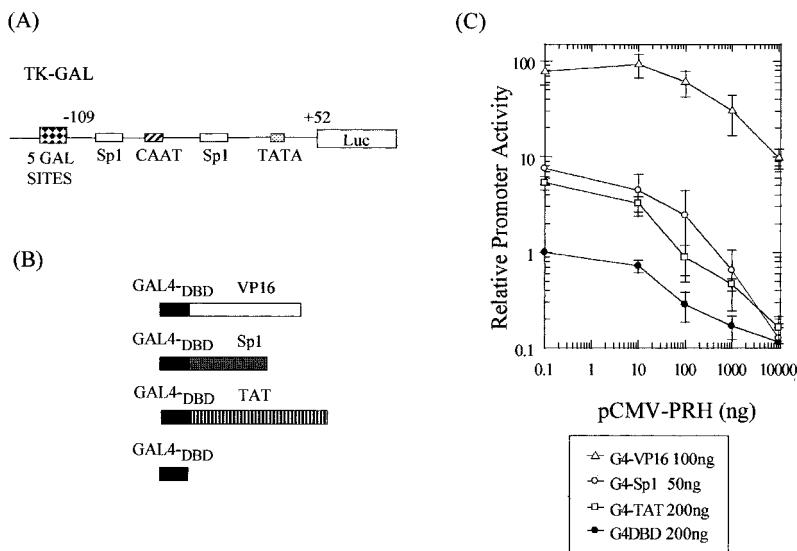
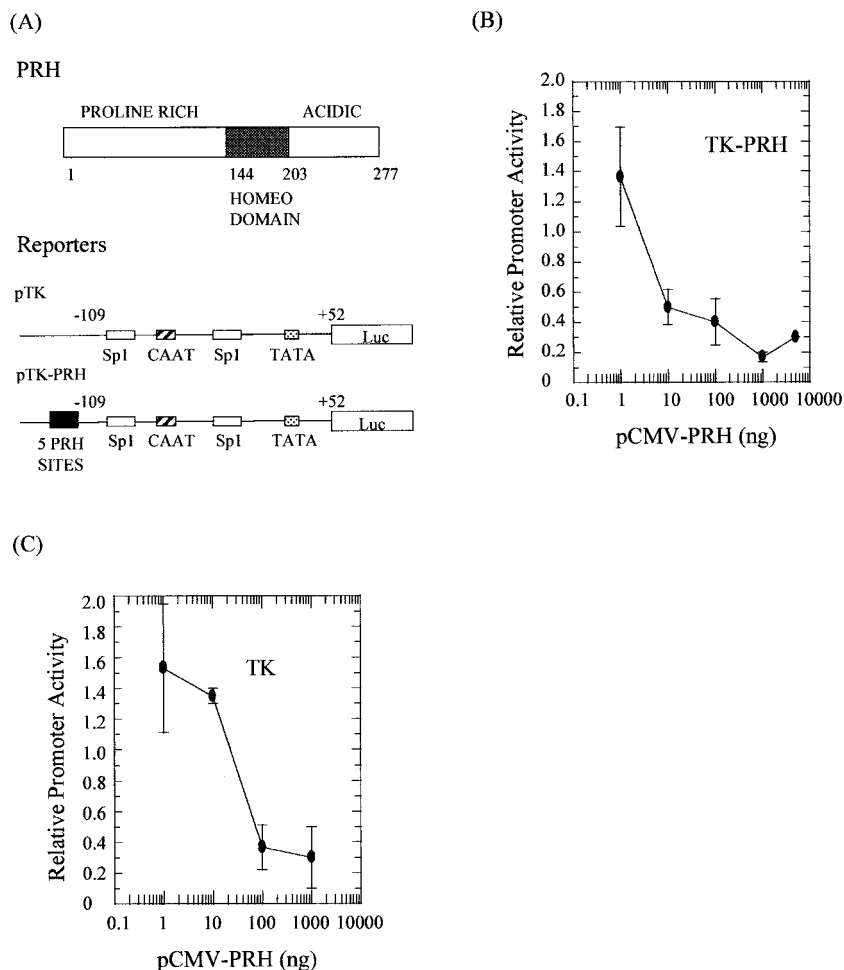


FIG. 2. PRH represses activated transcription. *A*, a schematic representation of the pTK-GAL reporter plasmid. Five GAL4 binding sites were placed upstream of the TK promoter in plasmid pTK to create pTK-GAL. *B*, a schematic representation of the GAL4-activator fusion proteins used in this study. Activation domains from VP16, Sp1, and HIV TAT were cloned in frame with the GAL4 DBD (for details see "Materials and Methods"). *C*, BM2 cells were transiently cotransfected with increasing amounts of pCMV-PRH, 5 μ g of the pTK-GAL reporter plasmid, 10 μ g of pSV-*lacZ*, and either GAL4-VP16 (100 ng), GAL4-Sp1 (50 ng), GAL4-TAT (200 ng), or GAL4 DBD (200 ng). Relative promoter activity is shown on a logarithmic scale. All other details are as described in Fig. 1*B*.

proteins were fused in frame with the GAL4 DBD present in pSG424. The HSV-1 VP16 acidic activation domain was also expressed as a GAL4 DBD fusion using the expression vector pMLV-GAL147plink (Fig. 2*B*). Between 50 and 200 ng of each expression plasmid or equivalent amounts of the empty vectors were transiently cotransfected into BM2 cells along with the pTK-GAL reporter (Fig. 2*C*). In each case plasmids expressing the activator-GAL4 fusion proteins increase luciferase activity. Activation levels range between 6- and 85-fold above basal TK-GAL promoter activity depending on the activator (Fig.

2*C*). Increasing amounts of pCMV-PRH were then cotransfected into BM2 cells along with each activator-GAL4 expression plasmid. In each case, PRH is able to repress activated transcription (Fig. 2*C*). Because each fusion protein contains a different class of activation domain (Sp1 is glutamine-rich, VP16 is acidic, and TAT does not contain a predominance of a single kind of amino acid), these data suggest that binding site-independent repression by PRH is not activator-specific.

PRH Strongly Represses TATA Box-dependent Promoters—In vitro the homeodomain protein En can bind directly to

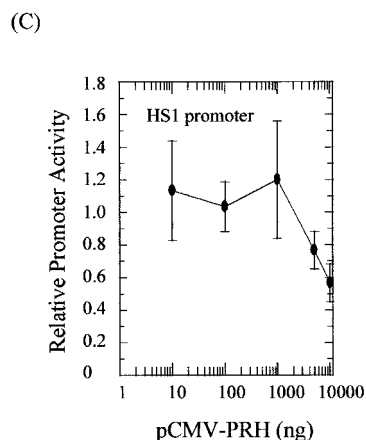
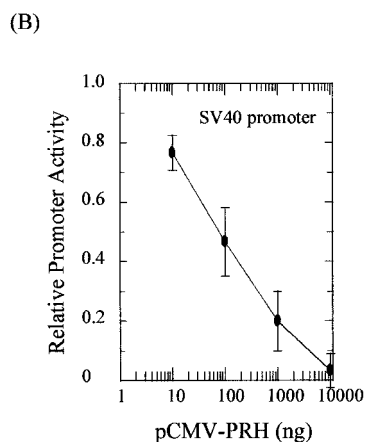
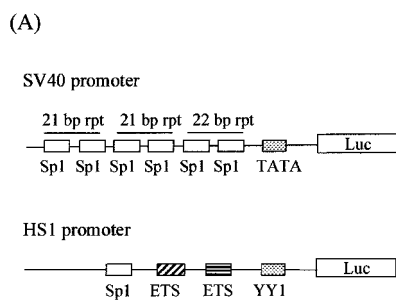


FIG. 3. PRH strongly represses TATA box dependent promoters. *A*, the *top line* is a schematic representation of the pGL-2 Promoter vector (Promega). This reporter plasmid contains the SV40 promoter upstream of the luciferase gene. The *bottom line* is a schematic representation of the HS1 reporter plasmid. This construct contains sequences from the HS1 promoter (from -180 to +20) cloned upstream of the luciferase gene in pGL-2 basic. *B*, BM2 cells were transiently cotransfected with increasing amounts of pCMV-PRH, 5 μ g of the pGL-2 Promoter vector (SV40), and 10 μ g of the β -galactosidase expression plasmid pRSV-*lacZ*. Details are as in Fig. 1*B*. *C*, BM2 cells were transiently cotransfected with increasing amounts of pCMV-PRH, 5 μ g of the HS1 reporter plasmid, and 10 μ g of pRSV-*lacZ*. Details are as in Fig. 1*B*.

the TATA box and block the binding of TBP (20). To investigate whether the TATA box is an important element in binding site-independent transcriptional repression by PRH, we examined the effect of PRH on transcription from the two promoters shown in Fig. 3*A*. The enhancer-less SV40 early promoter consists of a TATA box and six Sp1 binding sites (43) and is present upstream of the luciferase gene in the pGL-2 Promoter vector (Promega). The human Surf-1 promoter (HS-1) is a TATA-less housekeeping promoter, which consists of an Sp1 binding site, two binding sites for members of the ETS family of transcription factors, and a YY1 binding site and is present upstream of

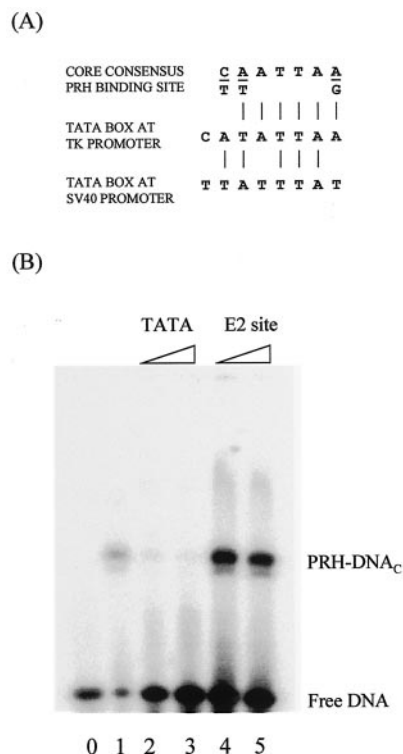
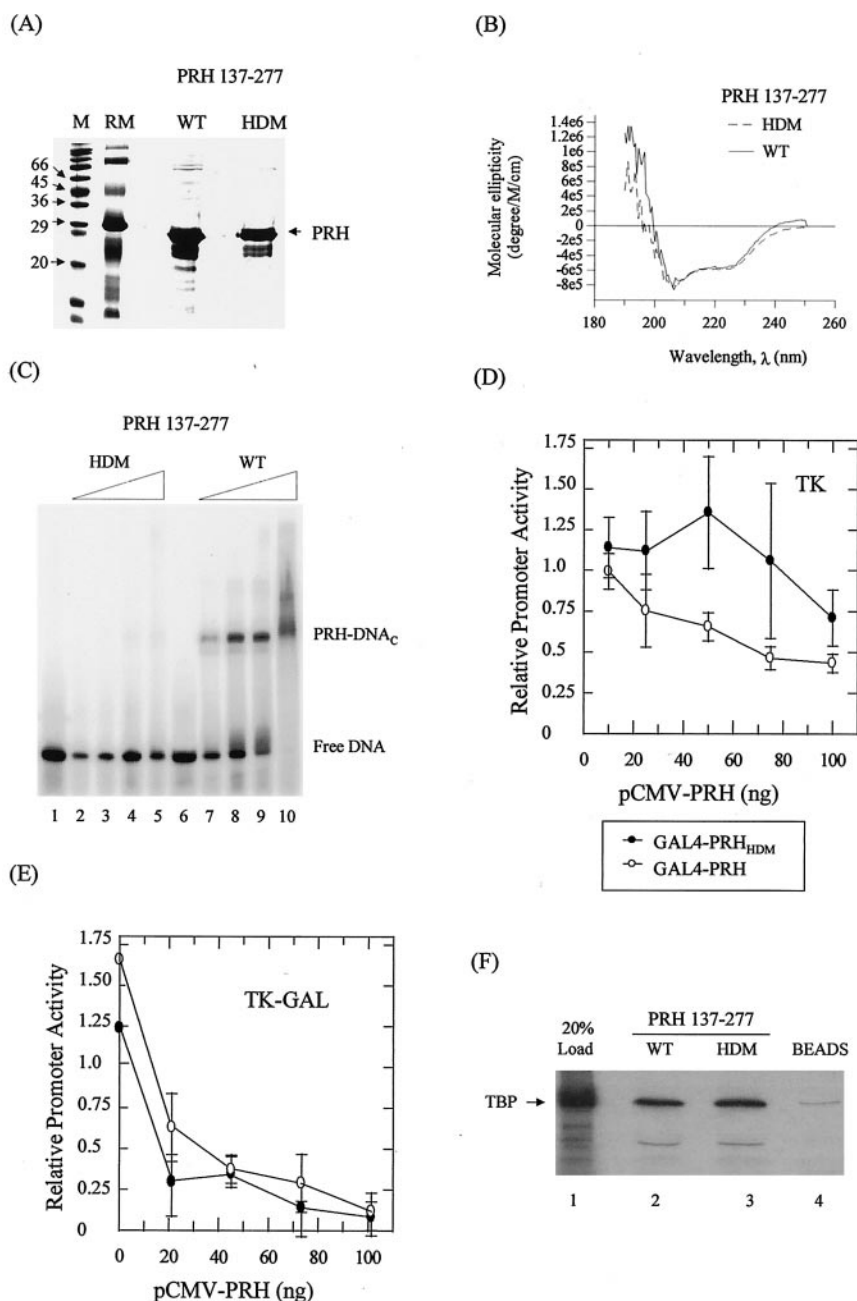


FIG. 4. PRH binds to the TK TATA box. *A*, an alignment of the core consensus PRH binding site (*top line*) with the TATA boxes from the TK promoter (*middle line*) and SV40 promoter (*bottom line*). *B*, a labeled oligonucleotide carrying the TK TATA box was incubated with histidine-tagged PRH137-277 under the conditions described in the text. Free and bound DNA was resolved on a 6% polyacrylamide gel and visualized using a PhosphorImager. The addition of 100 ng PRH results in the formation of a retarded complex (PRH-DNA_C). The complex was competed away by the addition of 500 ng or 1000 ng of unlabeled TK TATA box (*lanes 2 and 3*, respectively) but not by equal amounts of an unrelated oligonucleotide carrying an HPV 16 E2 binding site (*lanes 4 and 5*, respectively).

the luciferase gene in the pGL2-Basic vector (37). These two reporters were transiently transfected into BM2 cells along with increasing amounts of pCMV-PRH (Fig. 3, *B* and *C*, respectively). As in the case of the TATA box containing TK promoter, 100 ng of pCMV-PRH is sufficient to bring about a 50% repression of SV40 promoter activity. In contrast, the same amount of pCMV-PRH has no effect on the TATA-less HS1 promoter and even 50–100-fold higher amounts of pCMV-PRH bring about only partial repression. Taken together with the data shown in Fig. 1, these experiments show that PRH is capable of strongly repressing two different TATA box containing promoters but is very inefficient at repressing a promoter that lacks a TATA box. These results suggest that the sensitivity of the TK and SV40 promoters to repression by PRH might be due to the TATA element.

PRH Binds to the TK TATA Box—An alignment of the consensus PRH binding site with the TK and SV40 TATA boxes shows that these sequences are very similar (Fig. 4*A*). The TK and SV40 TATA boxes deviate from the core consensus PRH binding site at 1 of 7 and at 2 of 7 positions, respectively. To determine whether PRH is capable of binding to the TK TATA box, we expressed a histidine-tagged fragment of PRH comprising the homeodomain and C-terminal region (amino acids 137–277) in bacterial cells. The histidine-tagged PRH137–277 protein was purified by chromatography over a nickel column (see “Materials and Methods”). Fig. 4*B* shows the results of an electrophoretic mobility shift assay in which PRH137–277 was added to labeled DNA carrying the TK TATA box. After 30 min at 4 °C, protein-DNA complexes were separated from free DNA

FIG. 5. The homeodomain mediates repression in the absence of upstream PRH binding sites. *A*, histidine-tagged PRH 137–277 (WT) and histidine-tagged PRH_{HDM} 137–277 containing the N194A mutation (HDM) were purified over an Ni²⁺-NTA-agarose column and the eluted proteins analyzed by SDS-PAGE. M, markers; RM, rainbow markers. *B*, circular dichroism was used to determine whether the presence of the N194A mutation affected the folding or stability of PRH_{HDM} 137–277. *C*, a labeled oligonucleotide carrying the TK TATA box was incubated with 16, 80, 400, or 2000 ng of histidine-tagged PRH_{HDM} 137–277 (lanes 2–5) or equal amounts of the histidine-tagged PRH137–277 (lanes 7–10) under the conditions described in the text. Free and bound DNA was resolved on a 6% polyacrylamide gel and visualized using a PhosphorImager. *D* BM2 cells were transiently cotransfected with increasing amounts of pGAL-PRH (empty circles) or pGAL-PRH_{HDM} (filled circles), along with 5 μ g of the pTK reporter plasmid and 10 μ g of pSV-*lacZ*. Other details are as in Fig. 1B. *E*, the experiment shown in *D* was repeated using the pTK-GAL reporter plasmid. *F*, *in vitro* transcribed and translated TBP (lane 1) was incubated with Ni²⁺-NTA-agarose beads carrying histidine-tagged PRH 137–277 (lane 2), Ni²⁺-NTA-agarose carrying histidine-tagged PRH_{HDM} 137–277 (lane 3), or Ni²⁺-NTA-agarose beads alone (lane 4) under the conditions described in the text. After extensive washes, bound TBP was removed from the beads using imidazole, run on an SDS-PAGE gel, and visualized using a PhosphorImager.



by electrophoresis on a 6% nondenaturing polyacrylamide gel and visualized by autoradiography. The addition of PRH results in the formation of a protein-DNA complex (Fig. 4B, lane 1). To investigate the specificity of this complex, we added competitor oligonucleotides to the binding reaction. Addition of an unlabeled oligonucleotide carrying the TK TATA box abolishes the binding of PRH to the labeled DNA (Fig. 4B, lanes 2 and 3). In contrast, addition of an unlabeled oligonucleotide carrying an unrelated DNA sequence did not compete away the PRH-TATA box complex (Fig. 4B, lanes 4 and 5). These data show that PRH is capable of binding to the TK TATA box and is thus capable of binding to the TK promoter even in the absence of upstream PRH binding sites.

The Homeodomain Mediates PRH Binding Site-independent Repression—The experiments described above strongly implicate the homeodomain in the ability of PRH to repress transcription from the TK promoter in the absence of upstream PRH binding sites. To determine whether the binding of PRH to the TATA box brings about transcriptional repression, the

homeodomain of PRH was mutated to abrogate its DNA binding activity. One of the invariant amino acids in all homeodomains is asparagine 51. This amino acid in the En homeodomain makes bidentate hydrogen bonds to adenine 13 of the E binding site, and this contact is crucial for DNA binding (44). The equivalent residue within the PRH homeodomain (asparagine 194) was mutated to an alanine using site-directed mutagenesis. To confirm that the N194A mutation blocks DNA binding, we first introduced this mutation into the histidine-tagged PRH137–277 protein. The resulting histidine-tagged PRH_{HDM}137–277 protein was expressed in bacteria and purified exactly as described above (Fig. 5A). CD was used to determine whether the N194A mutation altered the folding of the PRH_{HDM}137–277 protein. The CD spectra for PRH137–277 and PRH_{HDM}137–277 are very similar, implying that the mutation has no effect on protein folding or stability (Fig. 5B). Increasing amounts of PRH137–277 and PRH_{HDM}137–277 were added to labeled DNA carrying the TK TATA box, and DNA binding activity was assayed as described above. The

PRH_{HDM}137–277 protein shows little DNA binding activity (Fig. 5C, lanes 2–5), whereas at equal protein concentrations, PRH137–277 binds tightly to the labeled fragment (Fig. 5C, lanes 7–10).

To determine the effects of the N194A mutation on the ability of PRH to repress transcription from the TK promoter, this mutation was introduced into the full-length PRH cDNA in the context of a GAL4-PRH fusion. The resulting GAL4-PRH_{HDM} and GAL4-PRH expression plasmids were cotransfected into BM2 cells along with the TK reporter. The effects of GAL4-PRH_{HDM} and GAL4-PRH on transcription were compared with the effect of the GAL4 DBD alone, and transfection efficiency was measured as before. GAL4-PRH brings about a significant repression of transcription from the TK promoter when as little as 50 ng of expression plasmid is cotransfected (Fig. 5D). In contrast, equivalent amounts of the GAL4-PRH_{HDM} expression plasmid have little or no effect on the TK promoter (Fig. 5D). However, with higher amounts of cotransfected GAL4-PRH_{HDM} expression plasmid (100 ng), TK promoter activity is partially repressed. These data are consistent with the idea that PRH represses transcription at the TK promoter by binding to the TK TATA box. It is possible that the transcriptional repression seen in the presence of high levels of the GAL4-PRH_{HDM} expression plasmid results from the low level of TATA box binding activity shown by the mutated homeodomain (Fig. 5C, lane 5).

To determine whether binding to the TATA box is the only mechanism whereby PRH can repress transcription, we next looked at the ability of the GAL4-PRH_{HDM} protein to repress transcription when tethered upstream of the TATA box at GAL4 binding sites. The GAL4-PRH and GAL4-PRH_{HDM} expression plasmids were cotransfected into BM2 cells along with the TK-GAL reporter, and promoter activity was assayed as before. As can be seen from the data shown in Fig. 5E, there is little if any difference in the ability of these proteins to repress the TK-GAL promoter. Therefore, PRH must repress transcription using at least two mechanisms, one of which is dependent upon the DNA binding activity of the homeodomain and one of which is independent of the homeodomain-DNA interaction. In addition, because both of these GAL4 fusion proteins repress the TK-GAL promoter equally, this experiment shows that the wild type protein and the mutated protein are probably expressed at equivalent levels.

The Eve homeodomain is involved in binding to TBP (45). To determine whether the PRH homeodomain can interact with TBP, we performed *in vitro* binding assays. The PRH137–277 and PRH_{HDM}137–277 proteins described above were incubated with *in vitro* transcribed and translated TBP. Labeled TBP binds strongly to beads coated with either PRH137–277 or PRH_{HDM}137–277 but binds only very weakly to uncoated beads (Fig. 5F). Thus, PRH can bind directly to the TATA box and can also bind TBP. Furthermore, although the N194A mutation significantly reduces the DNA binding activity of the PRH homeodomain, this mutation has no effect on the ability of PRH137–277 to bind TBP. The ability of PRH_{HDM}137–277 to bind TBP might be another explanation for the transcriptional repression observed when high amounts of the GAL4-PRH_{HDM} expression plasmid are cotransfected with the TK promoter (Fig. 5D).

Mapping the PRH Repression Domains—The N194A mutation abolishes the DNA binding activity of the PRH homeodomain and significantly reduces the ability of PRH to repress transcription from the TK promoter. However, this mutation does not affect the ability of PRH to repress transcription when tethered upstream of the TK promoter by the GAL4 DNA-binding domain. This suggests that when bound upstream of

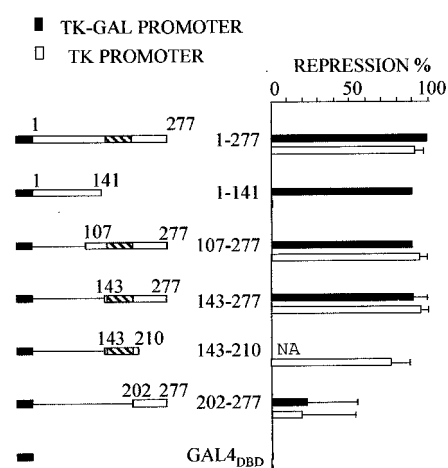


FIG. 6. PRH contains at least two independent transcriptional repression domains. A series of GAL4-PRH fusion proteins were assayed for their ability to repress transcription from the pTK reporter (solid bars) and the pTK-GAL reporter (empty bars). BM2 cells were transiently cotransfected with 1 μ g of each GAL4-PRH expression plasmid, 5 μ g of the each reporter plasmid, and 10 μ g of pSV-*lacZ*. The difference between TK promoter activity in the presence of the GAL4 DBD alone and TK promoter activity in the presence of full-length PRH (PRH1–277) fused to the GAL4 DBD was taken to represent 100% repression. All other details are as described in Fig. 1B. NA, not assayed.

the promoter, PRH might repress transcription via protein-protein interactions with TBP and/or via homeodomain-independent mechanisms. To delineate the regions of PRH that function as repression domains, a number of deletion mutants of PRH were assayed for their ability to repress transcription in transient transfection experiments. The GAL4-PRH expression plasmid described above was cotransfected into BM2 cells along with either the TK or TK-GAL reporter plasmids. The addition of 1 μ g of the GAL4-PRH expression plasmid brings about 100% repression of the TK-GAL promoter, and 95% repression of the TK promoter (Fig. 6, first line). Because 1 μ g of GAL4-PRH is sufficient to give almost complete repression of both the TK and TK-GAL promoters, this amount of expressor plasmid was chosen for all subsequent transfections. A series of deletion mutants of PRH were placed in frame with the GAL4 DBD in pMLV-GAL147plink. As can be seen from the data (Fig. 6, second line), the N-terminal 141 amino acids of PRH (which excludes the PRH homeodomain) are sufficient to bring about full repression of the TK-GAL promoter. However, this fusion protein does not repress the TK promoter. Thus, PRH contains a proline-rich N-terminal repression domain that functions when tethered to a promoter either via the PRH homeodomain or via the GAL4 DBD. The C-terminal 134 amino acids of PRH (aa 143–277), which includes the PRH homeodomain, brings about full repression of both TK reporters (Fig. 6, fourth line), and a further deletion of amino acids 210–277 shows that the PRH homeodomain alone is sufficient to repress transcription from the TK promoter (Fig. 6, fifth line). In contrast, the C-terminal amino acids from 202–277 only weakly repress transcription (Fig. 6, sixth line). These data confirm that the PRH homeodomain is responsible for the repression of the TK promoter seen in the absence of upstream PRH binding sites. The C-terminal acidic region of PRH might contribute to repression by the homeodomain (Fig. 6, compare fourth and fifth lines); however, the increased repression seen in the presence of this region might be a consequence of increased protein expression and/or stability.

Deletion Analysis of the Proline-rich Repression Domain—The proline-rich N-terminal 141 amino acids of PRH function as an independent transferable repression domain. To investi-

gate this repression domain, a series of deletion fragments from the PRH N terminus were fused in frame with the GAL4 DBD. The proline-rich domain was deleted in ~20-amino acid intervals from the N terminus to amino acid 107. Because we were unable to detect protein expression levels for any of these constructs in BM2 cells (data not shown), we transiently transfected these constructs into QT6 cells, a quail fibroblast cell line. We first established that the PRH N terminus can repress transcription in quail fibroblasts and is detectable by Western experiments (Fig. 7, A, *first line*, and C, *first track*). This suggests that the proline-rich repression domain is functional in these cells and that there are no essential species-specific or cell-type requirements for the activity of this domain. Interestingly none of the deletions that remove N-terminal amino acids greatly affect repression (Fig. 7A). Although the GAL4-PRH 81–141 and GAL4-PRH 107–141 constructs appear to repress somewhat less well than the GAL4-PRH 1–141 construct, the former are expressed at virtually undetectable levels (Fig. 7C, *fourth* and *fifth tracks*, compared with *first track*). However, both GAL4-PRH 81–141 and GAL4-PRH 107–141 still bring about at least 50% repression of the TK-GAL promoter (Fig. 7A, *fourth* and *fifth lines*). Thus, the 34 amino acids from 107 to 141 can function as a repression domain.

Fig. 7B shows the effect of deletions into the C terminus of the proline-rich repression domain. Deletion of the C-terminal 16 amino acids to create GAL4-PRH 1–125 has a minor effect on repression (Fig. 7B, *second line*). A further deletion of 20 amino acids to produce GAL4-PRH 1–105 completely abolishes repression (Fig. 7B, *third line*). However, although the GAL4-PRH 1–105 construct completely fails to repress transcription, it is expressed in QT6 fibroblasts at a much lower level than GAL4-PRH 1–125 (Fig. 7C). Although these data suggest that amino acids 105–141 are essential for repression, two further deletion constructs indicate that this is not the case. PRH derivatives GAL4-PRH 28–141 and GAL4-PRH 28–105 both strongly repress transcription and are both strongly expressed in these cells (Fig. 7B, *fourth* and *fifth lines*). Thus the 77 amino acids from 28 to 105 can also function as a repression domain. One possible explanation for the lack of repression by GAL4-PRH 1–105 is that although this construct is expressed, (albeit at low level), it may be misfolded and either no longer interacts or interacts aberrantly with the transcription apparatus. Taken together with the experiments described in Fig. 7A, these data suggest that the N-terminal repression domain is composed of at least two elements that can independently bring about repression. The first element lies within the region 28–105 of PRH, and the second element lies within the region 107–141. Thus, the PRH proline-rich repression domain is not composed of a single discrete region that is essential for transcriptional repression but may instead be composed of multiple regions that are independently capable of transcriptional repression.

DISCUSSION

Using a series of truncated proteins, we have shown that PRH contains two independently acting transcription repression domains. One repression domain consists of the PRH homeodomain, whereas the other consists of the proline-rich N-terminal region of the protein. The homeodomain proteins Eve and Msx-1 can bind directly to TBP, and these interactions are probably important for transcriptional repression (25, 26, 45). Although PRH is similar to Eve in that it can also bind directly to both TBP and DNA, the mechanism of repression appears to be different. Mutations in the Eve homeodomain that block binding to DNA have no effect on the binding of this protein to TBP and do not prevent Eve from repressing transcription (45). In direct contrast, a mutation in the PRH homeo-

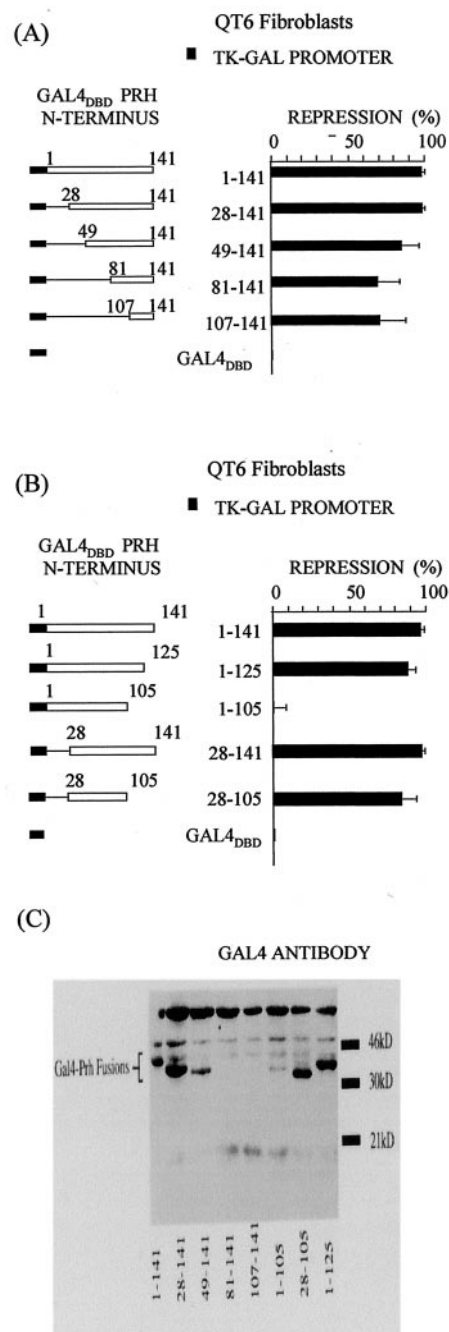


FIG. 7. Fine mapping of the PRH N-terminal repression domain. A, defining the N-terminal boundary of the proline-rich repression domain. The series of GAL4-PRH fusion proteins shown in the figure were assayed for their ability to repress transcription from the pTK-GAL reporter. QT6 cells were transiently cotransfected with 1 μ g of each GAL4-PRH expression plasmid, 5 μ g of pTK-GAL, and 5 μ g of pSV-lacZ. The percentage repression was determined as described in Fig. 6. B, defining the C-terminal boundary of the proline-rich repression domain. The series of GAL4-PRH fusion proteins shown in the figure were assayed for their ability to repress transcription from the pTK-GAL reporter exactly as described in A. C, a Western blot of the GAL4-PRH N-terminal fusion proteins after transient transfection into QT6 cells. Proteins were probed with a monoclonal antibody against the GAL4 DBD, and specific antibody binding was detected by ECL. The bracket marks the positions of the fusion proteins. The other bands are endogenous proteins that cross-react with the antibody.

domain that blocks binding to DNA has no effect on the binding of this protein to TBP but significantly reduces transcriptional repression. These data suggest that PRH might repress transcription by binding to the TATA box and sterically hinder the

binding of TBP. This mechanism of repression has been observed previously *in vitro* for the En protein (20). However, this is the first strong evidence to suggest that a homeodomain protein can regulate transcription in intact cells using this passive repression mechanism. Several observations support this conclusion. First, repression by the full-length PRH protein does not require the presence of PRH binding sites upstream of the target promoter. Second, PRH can bind to an oligonucleotide carrying the TK TATA box *in vitro*. Third, PRH is capable of repressing transcription from TATA box containing promoters but is very inefficient at repressing transcription from a TATA-less promoter. Fourth, the PRH homeodomain alone is able to repress transcription *in vivo*. Interestingly, although En and PRH seem to use a similar mechanism for the repression of transcription, PRH unlike En, can interact directly with TBP. To determine whether TBP and PRH can form a tripartite complex with DNA, we carried out electrophoretic mobility shift assay with both proteins. However, TBP was unable to bind to the TK TATA box under conditions that allowed the strong binding of PRH. Thus, the functional significance of the TBP-PRH interaction could not be assessed using this method. The possibility remains, therefore, that in addition to the passive repression mechanism observed at this promoter, under some circumstances the PRH homeodomain might also repress by direct repression of the basal transcription complex by interaction with TBP.

Proline-rich transcription repression domains have been identified in a number of homeodomain proteins including Eve, Msx-1, and Evx-1 (35, 46, 47). The Eve proline-rich repression domain is involved in mediating protein-protein interactions that result in the cooperative binding of Eve to DNA (21). However, it is not yet clear how proline-rich domains bring about repression. The proline-rich PRH N-terminal domain represses transcription when tethered upstream of a promoter both in the hematopoietic BM2 cell line and also in QT6 fibroblasts. Deletions within the PRH N terminus showed that there are at least two regions within the PRH N terminus that are capable of significantly repressing transcription. Region 28–105 of PRH is both strongly expressed in QT6 fibroblasts and shows strong repression activity. Region 107–141 of PRH, although not detectable in Western experiments, also displays significant repression activity. Thus, we infer that there are at least two nonoverlapping regions within this domain that can function independently. Han and Manley (48) have shown that a proline-leucine-rich peptide only 27 amino acids in length is capable of acting as a potent repressor in transient transfection assays; they suggest that a key feature of a repression domain is that it is relatively unstructured and hydrophobic. In keeping with this view, region 28–105 of PRH contains a large proportion of alanine (14%), proline (23%), and leucine (6%) residues. However, the 34-amino acid region (107–141) contains only 11% proline residues and 11% leucine residues. Studies on the Wilm's tumor (WT) protein repression domain have shown that proline residues outside the WT minimal repression domain are important for repression. These proline residues may aid the accessibility of key amino acids from the WT minimal repression domain with interacting proteins (49). Similarly, it is possible that the proline and leucine residues from region 107–141 in PRH may allow other amino acids within this region to be more accessible to any interacting proteins.

There are two sequences within the PRH N terminus that might mediate interactions with other transcription factors. The first sequence LLWSPF (amino acids 131–136 in avian PRH and 124–129 in human PRH) is located 7 amino acids upstream of the PRH homeodomain and is within the region

107–141. Hexapeptide sequences that loosely match this motif are located upstream of the homeodomain in several members of the HOX family of transcription factors, and HOX proteins use these sequences to contact members of the PBC family of homeodomain proteins (50). The PBC proteins modulate transcription of the HOX proteins by altering either their binding specificity (4) or their transcriptional regulatory properties (5, 6). Similar tryptophan containing sequences (WRPY and WRPW) are also found in proteins that recruit the *Drosophila* corepressor protein Groucho (51, 52). The second sequence TPFYIEDILGR (amino acids 33–43 in avian PRH and 30–40 in human PRH) is present in region 28–105. This sequence strongly resembles the eh1 motif found in En that mediates the interaction of En with Groucho (34). Deletions in PRH that remove either this putative eh1 motif or the LLWSPF sequence do not block transcriptional repression. One possibility is that the PRH N terminus interacts with multiple proteins or that any interacting proteins might make several contacts with the PRH N terminus and that the removal of any one contact might only partially block the interaction. Certainly in the case of the interaction of TLE1, the human equivalent of Groucho, with the AML1 protein, several regions of AML1 are important for the interaction (40).

In summary, the PRH homeodomain can passively repress transcription by binding to TATA box sequences. However, the proline-rich repression domain of PRH also represses transcription and may do so by interacting with basal transcription factors or by altering chromatin structure in conjunction with corepressors. We have shown previously that the proline-rich domain of PRH plays a role in the control of cell growth and differentiation in the hematopoietic compartment (16). Future experiments may allow us to determine which of these repression mechanisms are important for the function of PRH *in vivo*.

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