A Highly Active Homeobox Gene Promoter Regulated by Ets and Sp1 Family Members in Normal Granulosa Cells and Diverse Tumor Cell Types

Manjeet K. Rao, Sourindra Maiti, Honnavara N. Ananthaswamy and Miles F. Wilkinson

From the Department of Immunology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

Running Title: Pem Homeobox Gene Regulation

Key words: homeobox; Pem; promoter; Ras; Ets; Sp1; Sp3

*Corresponding author. Mailing address: Department of Immunology, Box 180, University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030. Phone: (713) 794-5526. Fax: (713) 745-0846. E-mail: mwilkins@mdanderson.org
One mechanism by which normal cells become converted to tumor cells involves the aberrant transcriptional activation of genes that are normally silent. Here, we characterize a promoter that normally exhibits highly tissue- and stage-specific expression but displays ubiquitous expression when cells become immortalized or malignant, regardless of their lineage or tissue origin. This promoter normally drives the expression of the *Pem* homeobox gene in specific cell types in ovary and placenta but is aberrantly expressed in lymphomas, neuroblastomas, retinoblastomas, carcinomas, and sarcomas. By deletion analysis we identified a region between nt -80 and -104 that was absolutely critical for the expression from this distal *Pem* promoter (*Pem* _Pd). Site-specific mutagenesis and transfection studies revealed that this region contains two consensus Ets sites and a single Sp1 site that were necessary for *Pem* _Pd_ expression. Gel-shift analysis showed that Ets and Sp1 family members bound to these sites. Transfection studies demonstrated that the Ets family members Elf1 and Gabp and the Sp1 family members Sp1 and Sp3 transactivated the *Pem* _Pd_. Surprisingly, we found that Sp3 was a more potent activator of the *Pem* _Pd_ than was Sp1; this is unusual, as Sp3 is either a weak activator or a repressor of most other promoters. Activation by either Elf1 or Gabp required an intact Sp1 family member-binding site, suggesting that Ets and Sp1 family members cooperate to activate *Pem* _Pd_ transcription. Expression from the *Pem* _Pd_ (either transiently transfected or endogenous) depended on the ras pathway, which could explain both its Ets- and Sp1-dependent expression in normal cells and its aberrant expression in tumor cells, in which _ras_ protooncogenes are frequently mutated. We suggest that the *Pem* _Pd_ may
be a useful model system to understand the molecular mechanism by which a tissue-specific promoter can be corrupted in tumor cells.
The malignant and metastatic characteristics of cancer cells derive in part from the deregulation of genes whose normal role is to control the division, differentiation, and migration of embryonic cells during development (1). One important class of genes that regulates both developmental and tumorigenic events is the homeobox gene family. This gene family encodes a large group of transcription factors that each contain a 60 amino-acid DNA-binding motif termed a homeodomain (2,3).

Although much is known about the function of homeodomain transcription factors, little is known about the regulation of the genes that encode them, particularly in response to signals in tumor cells. One homeobox gene of interest in this regard is Pem, which we originally cloned by subtraction hybridization from a T-cell lymphoma clone (4,5). Pem is the founding member of the recently defined PEPP homeobox subfamily, a small group of homeobox genes on the X chromosome that are all normally expressed in reproductive tissues (6-8). Pem is expressed in parietal and visceral endodermal cells, where it appears to regulate the development of cells that eventually form portions of the placenta (5,9,10). In neonatal and adult mice and rats, Pem is specifically expressed in ovary, testis, and epididymis (5,10-12). In the ovary, Pem expression is restricted to granulosa cells of preovulatory follicles. In the testis, Pem is specifically expressed in Sertoli cells at stages VI-VIII of the seminiferous epithelial cycle. In striking contrast to its tissue- and stage-specific expression in normal tissues, Pem is aberrantly expressed in a wide range of tumor cell types regardless of their origin (5). It is not known whether Pem's ubiquitous expression in tumors reflects a causal role for Pem in promoting tumor cell growth and metastasis or whether instead Pem expression is a consequence of
immortalization and malignant conversion. In support of the former, it was recently shown that Pem physically interacts with the tumor suppressor protein menin (13) and is a potent tumor antigen (14).

We previously showed that Pem transcripts are derived from two promoters that are independently regulated in a tissue-specific manner (15). The proximal promoter (Pem Pp) is expressed exclusively in male reproductive tissues, whereas the distal promoter (Pem Pd) is preferentially transcribed in the female reproductive tissues ovary and placenta (7,15). In the present investigation, we determined which promoter is responsible for Pem's ubiquitous expression in tumor cells. We found that the Pem Pd was responsible for this aberrant expression. We then defined the cis- elements upstream of the Pem Pd transcription start site that were necessary and sufficient for expression in both tumor cells and normal granulosa cells. We identified specific Ets and Sp1 transcription factor family members that bound to these cis-elements and controlled the expression of Pem in a ras-dependent manner. Our data suggest that the Pem Pd is normally a tissue-specific promoter that is aberrantly expressed in diverse tumor cell types because it is activated by ubiquitously expressed transcription factors that are regulated in normal cells but constitutively activated in tumor cells.
EXPERIMENTAL PROCEDURES

Cells, Chemicals, and Biochemicals - The SL12.1, SL12.3, SL12.4, N4TG1, M12, Ras/RAT-1, PS-1, S194/5, and 10T\textsuperscript{1/2} cell lines were maintained in tissue-culture plates containing Dulbecco’s modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum and 50 mg of both penicillin and streptomycin per ml. *Drosophila melanogaster* SL2 Schneider cells were kindly provided by Dr. Mitzi Kuroda (Baylor College of Medicine, Houston, TX) and were maintained in tissue-culture flasks containing Schneider’s Drosophila Medium (Gibco BRL) supplemented with 10% fetal bovine serum and 50 mg of both penicillin and streptomycin per ml. All cell culture reagents were obtained from Gibco BRL. Antibodies for Sp1 (Sp1 SC-420), Sp3 (Sp3 D-20) and Elf 1 (C-20) were obtained from Santa Cruz Biotechnology. Rabbit polyclonal antibodies, directed against recombinant GABP\textalpha and GABP\textbeta1 (16), were gifts from Dr. Thomas Brown (Pfizer Central Research, Groton, CT).

Granulosa Cell Culture - Immature female rats (55-60 g., 23 days old) were injected with 1.5 mg of 17\textbeta-estradiol per day and then kept for 3 days before granulosa cells were harvested. The rats were treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals. All protocols were approved by the Institutional Committee on Animal Care of M. D. Anderson Cancer Center. Ovarian granulosa cells were isolated as described previously (17). Briefly, the ovaries were punctured multiple times with a 22-gauge needle to isolate granulosa cells. The granulosa cells were pooled and treated with 20 \mu g trypsin per ml for 1 min, and then 300 \mu g of soybean trypsin inhibitor per ml...
and 160 µg of Dnase I per ml were added to remove necrotic cells. The cells were washed twice and cultured in DMEM:F12 medium at 37°C in 95% air and 5% CO₂ for 16 h.

**Plasmids** - A 1.3-kb Spe1-Sal1 rat genomic Pem fragment containing exon 1 and upstream sequences (15) was cloned into the eukaryotic expression vector pRL-Null (Promega Co.) to generate the -304 construct (Pem-128). This construct was then used as a polymerase chain reaction (PCR) template to generate deletion construct -112 (Pem-147), -104 (Pem-170), -94 (Pem-167), and -73 (Pem-148), which were each made by using a T3 polymerase antisense oligonucleotide (oligo) in combination with the sense oligos MDA-298, MDA-396, MDA-392, and MDA-319, respectively (Table I). All of these sense primers contained a NdeI site except for the primer used to generate Pem-148, which contained an XhoI site instead. Substitution mutations were generated with the Quick Change site-directed mutagenesis kit (Stratagene Inc.) according to the manufacturer's instructions and then confirmed by DNA sequencing. The site-specific mutagenized constructs -112/S1 (Pem-171), -112/E1 (Pem-172) and -112/E2 (Pem-175) were made by using the sense oligos MDA-397, MDA-399, and MDA-419, in combination with the antisense oligos MDA-398, MDA-400, and MDA-420, respectively. To generate the -81 to -104/Δc-fos construct (Pem-179), a single copy of sequences nt -104 to -81 was cloned into the Sall-HindIII site of the plasmid Δ56FosdE-luciferase (kindly provided by Craig A. Hauser, Burnham Institute, La Jolla, CA), which contains a minimal c-fos promoter. The *Drosophila* expression plasmids pPac-Sp1 and pPac-Sp3 were kindly provided by GuntramSuske (Institut for Molekularbiologie and Tumorfurchung, Marburg, Germany). The *Drosophila* expression plasmids pPac-Elf1
and pPac-Uo were a gift from Philip A. Marsden (University of Toronto, Toronto, Canada). The *Drosophila* expression plasmids pPac-*Gabp* α and β were kindly provided by Thomas Brown (Pfizer Inc.). pCMV-DNSp3, which encodes the dominant negative (DN) form of Sp3 in pCDNA3.1/His C, was generously provided by Yoshihiro Sowa and Toshiyuki Sakai (Kyoto Prefectural University of Medicine, Kyoto, Japan). DN-*Gabp* α and β expression plasmids were provided by Seroz Thierry (Neurobiologie Moleculaire, Institut Pasteur, France). The DN-*ras* (17N) and activated Ha-*ras* (61L) expression plasmids were kindly provided by Craig A. Hauser (Burnham Institute, La Jolla, CA).

**Transient Transfection Assays** - For transfections, DNA concentrations were independently determined by using a fluorometer and analytical gel electrophoresis. Before transfection, all mammalian cell lines were replated in 6-well plates at a density of \(~10^6\) cells per well in 0.8 ml of serum-free medium. Plasmid DNA was suspended in 100 µl of serum-free medium, mixed with 100 µl of serum-free medium premixed with 15 µl of Lipofectamine (Gibco BRL), and then incubated for 40 min at room temperature. The DNA-lipid complex was then added to the wells and incubated at 37°C in a CO₂ incubator for 12 to 14 h. The cells were then replenished with fresh medium containing serum and incubated for 48 h. Stably transfected SL12.4 and 10T½ stable cell lines were generated by providing 2 mg/ml G418 every other day until only antibiotic-resistant cells remained alive (2 to 3 weeks).

*D. melanogaster* Schneider cells and primary ovarian granulosa cells were transfected were by using the calcium phosphate method (18). Briefly, plasmid DNA diluted in 2X HBS buffer {4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes)-buffered saline (280 mM NaCl, 1.5 mM Na₂HPO₄, and 50 mM Hepes [pH 7.2])} was precipitated by
dropwise addition of 250 mM CaCl$_2$. After incubation for 30 min, 300 µl of the DNA precipitate was added dropwise to each well and then the cells were incubated for 6 to 8 h at 26°C (for the Schneider cells) or at 37°C (for the granulosa cells). The incubation medium was then carefully aspirated and replaced with fresh media, and then the Schneider and granulosa cells were incubated for 48 and 6 h, respectively.

Cell lysates for luciferase assay were prepared by centrifuging the cells at 1000 rpm (750 rcf) for 2 min and then 1X lysis buffer (Promega Inc.) was added to the cell pellet. Luciferase activity was measured by the dual-luciferase reporter assay system (Promega Inc.) according to the manufacturer's suggestion. For all transient transfection experiments, Renilla luciferase activity from the $Pem$ PD was normalized relative to firefly luciferase activity expressed from a thymidine kinase promoter-driven construct (pGL2TK). Expression from plasmids containing $Pem$ sequences was compared with the empty control vectors pRL-Null, pPac, or Δc-fos.

**RNA Isolation and Northern Blot Analysis** - Total cellular RNA was isolated as described previously by guanidinium isothiocynate lysis and centrifugation over a 5.7 M CsCl cushion (4). The $Pem$ cDNA probe used for northern blot analysis was prepared as described previously (5). A cyclophilin cDNA probe (19) was used as a loading control.

**Ribonuclease Protection Analysis** - The RNA was analyzed by ribonuclease protection analysis (RPA), performed as described previously (11). The $[^{32}P]$ UTP-labeled mouse $Pem$ ribonuclease protection analysis probe was prepared by in vitro transcription, as previously described ($Pem$ probe E; (15). The β-actin RPA probe was prepared in the same manner; it contained nt 135 to 169 of human β-actin exon 3 (Gene bank accession
number X00351). A set of RNA size markers generated from the century ladder template (Ambion, Inc.) was included in all gels.

*Nuclear Extract Preparation and Electrophoretic Mobility Shift Assay (EMSA)* - Crude nuclear extracts used for EMSAs were prepared as follows. SL12.4 cells (~$10^7$) were collected by centrifuging the cells at 700 rpm (~400 rcf) for 3 min. The cells were then washed with ice-cold Tris-saline, resuspended in 400 µl of hypotonic buffer A (10 mM Hepes [pH 7.9], 10 mM KCl, 0.1 mM EDTA [pH 8.0], 0.1 mM ethylene glycol bis-N,N,N',N'-tetraacetic acid [EGTA] [pH 8.0], 1 mM dithiothreitol [DTT] and 0.5 mM phenylmethylsulfonyl fluoride [PMSF]), and incubated on ice for 15 minutes. The cells were then centrifuged at 2000 rpm (3100 rcf) for 30 s and resuspended in 400 µl of buffer B (buffer A plus 0.15% Nonidet P-40) on ice for 15 minutes. Nuclear proteins were extracted in 100 µl of hypertonic buffer C (20 mM Hepes [pH 7.9], 400 mM NaCl, 1 mM EDTA [pH 8.0], 1 mM EGTA [pH 8.0], 1 mM DTT, and 1 mM PMSF) at 4°C on a rocking platform.

EMSAs were performed by using a $^32$P-labeled blunt-end double-stranded probe generated by annealing two complementary oligonucleotides (oligos) (MDA-473 and MDA-474). The probe ($5 \times 10^4$ cpm) was incubated for 30 min at room temperature in 20 µl of binding buffer (10 mM Tris [pH7.9], 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 5% glycerol, and 1 µg of poly dI:dC) containing 2 µg of nuclear extract. For antibody supershift and blocking assays the reaction mixture was preincubated with monoclonal antibodies or polyclonal antisera (2 µg) at room temperature for 20 min before addition of the 5′ $^32$P-labeled blunt-ended double-stranded oligonucleotide. The DNA-protein
complexes were resolved on 4.5%-5% non-denaturing polyacrylamide gels at 150 V for 3 to 4 h at 4°C.
RESULTS

Diverse Tumor Cells Express Pem Transcripts from the Pem Pd - We previously showed that despite its cell-type specific expression in normal cells, Pem is ubiquitously expressed in a variety of tumors cell lines regardless of their lineage (5). Here we first determined whether Pem is expressed in tumor cells from its distal promoter (Pem Pd) or its proximal promoter (Pem Pp). We investigated this issue in six tumor cell lines derived from different tissues and cell lineages, all of which expressed Pem mRNA, as assessed by Northern-blot analysis (Fig. 1a). To determine promoter usage, RPA was performed with a probe that distinguishes between Pd- and Pp-derived Pem transcripts (15). As shown in Fig. 1b, all cell lines protected a band of ~80 nt that corresponded to the Pem Pd transcript. In contrast, none of the cell lines expressed the Pem Pp transcript, which is normally expressed in testes (Fig. 1b).

A 32-nt Element is Necessary and Sufficient to Permit Pem Pd Transcription - We next determined the sequences important for Pem Pd transcription. Promoter activity was assessed by measuring luciferase levels in transiently transfected SL12.4 T-cell lymphoma cells. We first tested a construct containing 304 nt of sequence upstream of the exon 1-intron 1 junction. As shown in Fig. 2a, this -304 construct expressed high levels of luciferase activity (90-to-110 fold more than empty vector), indicating that it contained sequences sufficient for high level Pem Pd transcription.

Deletion analysis was then performed to define functionally important cis-elements in this 304-nt region. All deletion constructs tested had a common 3’ end that included the transcription start sites previously defined between 22 and 40 nt upstream of the exon 1-
intron 1 junction (15). Deletion of sequences from nt -304 to nt -104 did not appreciably reduce promoter activity (Fig. 2a). In contrast, the -94 construct had much lower activity (about ten-fold less) than the -104 construct, indicating that sequences between nt -104 and nt -94 were critical for maximal Pem Pd transcription. Even less luciferase activity was detected from the -73 construct (60-fold less than the -104 construct). We therefore conclude that the region between nt -73 and nt -104 is indispensable for promoter activity.

Inspection of this 32-nt region revealed two putative Ets protein-binding sites (E1 and E2 in Fig. 2b) and one Sp1 protein-binding site (S1 in Fig. 2b). To determine whether these putative binding sites were necessary for Pem gene transcription, we mutated them and tested transcriptional activity. As shown in Fig. 2b, mutation of either the E1 or S1 site in the -112 Pem construct strongly decreased promoter activity. Mutation of both sites led to a further decrease in promoter activity, suggesting the importance of both the Sp1 and the E1 Ets site for transcription. Mutation of the other Ets site (E2) led to a less drastic but still significant decrease in promoter activity (Fig. 2b).

Next we determined whether the region containing the Ets and Sp1 binding sites is sufficient to drive transcription. As shown in Fig. 2c, a single copy of a sequence containing these three sites (nt -81 to -104) was sufficient to drive transcription from a heterologous minimal c-fos promoter (four-fold to six-fold over basal level). This confirmed the functional importance of these Ets and Sp1 family binding sites, and thus we elected to call this region the Pem Pd promoter element.

To determine the specificity of this Pem Pd promoter element, we transfected the minimal -112 Pem construct containing this element into the immature T-cell lymphoma
cell clones SL12.1 and SL12.3, both of which express little or no Pem transcripts from the endogenous Pem gene (Fig. 3a). We found that the SL12.1 cell clone expressed luciferase activity that was indistinguishable from background levels, and the SL12.3 cell clone expressed trace levels of luciferase activity that was much lower than that from the SL12.4 cell clone (Fig. 3b).

Identification of Proteins Interacting with the Pem Pd Element - Having shown that the Sp1 and Ets family binding sites upstream of the Pem Pd transcription start site are necessary for Pem Pd transcription, it was imperative to identify the proteins interacting with these sites. Incubation of a double-stranded 26-mer oligo probe containing this region with SL12.4 nuclear extracts and analysis by EMSA revealed at least 4 specific protein-DNA complexes (Fig. 4a, complexes 1, 2, 3, and 4). An unlabelled oligo with a mutation in the Sp1-binding site (oligo M1) competed for bands 3 and 4 but not band 1 and 2, and thus band 1 and 2 represented proteins binding to the Sp1 site. Likewise, an oligo with mutations in the Ets-binding site (M2) competed with bands 1 and 2 only, indicating that complexes 3 and 4 contained proteins binding to the Ets site. An oligo with both the Sp1 and Ets (E1) sites (M3) had no effect on complex formation, demonstrating that these two sites are responsible for the four complexes.

To identify specific proteins that bind to the Pem Pd element, we used antibodies against Sp1 and Ets family members. Monoclonal antibodies against Sp1 and Sp3 supershifted complexes 1 and 2, respectively (Fig. 4b), consistent with our mutant-oligo competition experiments that demonstrated that complex 1 and 2 contain proteins binding to the Sp1-family site in the Pem Pd element (Fig. 4a). To identify the transcription factors that bound to the Ets-binding site, we used a battery of antibodies against different
Ets family members. We found that polyclonal antibodies against Elf1 and Gabp (α and β) inhibited the generation of complexes 3 and 4, respectively (Fig. 4c). In contrast, addition of monoclonal or polyclonal antibodies directed against various other Ets family members, including Ets1, Ets2, Erg1, and Elk1, failed to modify the nucleoprotein complexes formed with the Pem Pd probe (data not shown). We conclude that the Ets family members Elf1 and Gabp and the Sp1 family members Sp1 and Sp3 bind to the Pem Pd promoter element.

Transactivation of the Pem Pd by Sp1, Sp3, Elf1, and Gabp - To determine the functional importance of Sp1 and Ets family members in Pem transcription, we performed a series of transient transfection experiments with the D. melanogaster Schneider SL12 cell line, which lacks Sp1 family members (20,21). As shown in Fig. 5a, transfection of increasing amounts of Sp1 expression plasmid (25 to 100 ng) resulted in a concentration-dependent increase (5- to 20-fold) in functional promoter activity. Similarly, Sp3 also transactivated the Pem Pd in a dose-dependent manner. In fact, Sp3 was a more potent activator (20- to 800-fold) than Sp1 was. Sp1 and Sp3 together exerted an additive effect in transactivating the Pem Pd (Fig. 5a). Elf1 and Gabp also stimulated luciferase activity in a concentration-dependent manner (Fig. 5b). Transfection of Elf1 and Gabp simultaneously resulted in an additive increase in promoter activity (Fig 5b). Transfection of all four transcription factor plasmids (Elf1, Gabp, Sp1, and Sp3) also resulted in additive stimulation of promoter activity.

To determine which of the three DNA-binding sites for these different transcription factors were necessary for transactivation, we performed cotransfection experiments with mutant Pem Pd constructs. In these experiments, we used the Sp3 expression plasmid
rather than the \( Sp1 \) expression plasmid, as \( Sp3 \) had more potent transactivation activity than \( Sp1 \). Results from cotransfection of \( Sp3 \) expression plasmid with a \( Pem Pd \) construct containing a mutation at the \( Sp1 \) site (-112/S1) demonstrated that transactivation by \( Sp3 \) required its cognate binding site (Fig. 6). Surprisingly, \( Sp3 \) transactivation also required the Ets-binding sites, as mutation of either the E1 or E2 sites prevented the induction of \( Pem Pd \) transcription in response to \( Sp3 \) (Fig. 6). Thus it appeared that one or more Schneider cell factors must bind to the Ets-binding sites for \( Sp3 \)-mediated transactivation to occur. Similarly, Elf1 and Gabp transactivation required not only the Ets-binding sites but also the adjacent \( Sp1 \) site (Fig. 6). This implies that Schneider cells express an endogenous \( Sp1 \) site-binding factor (though not an \( Sp1 \) family member) that participate with mammalian Ets factors to drive \( Pem Pd \) transcription.

These results suggest that Ets and \( Sp1 \) family members functionally cooperate to activate \( Pem Pd \) transcription (see Discussion).

To assess the functional importance of these transcription factors for \( Pem \) transcription in mammalian cells, we transiently transfected plasmids encoding DN mutants into the SL12.4 T-cell clone. As shown in Fig. 7a, transfection of DN-\( Gabp \) (\( \alpha \) and \( \beta \)) expression plasmids caused a dose-dependent repression of \( Pem Pd \) promoter activity (Fig. 7a). A DN-\( Sp3 \) expression plasmid also strongly inhibited \( Pem Pd \) transcriptional activity in a dose-dependent manner (Fig. 7b). Because the encoded DN-\( Sp3 \) protein lacks a transcription activation domain but has a DNA-binding domain, it probably inhibits the activity of all \( Sp1 \) family members, as they all have the same DNA-binding specificity (21). Thus, the inhibition by this DN protein shows that one or more \( Sp1 \) family members are required for \( Pem \) transcription in T cells. We conclude that the Ets factor
Gabp and at least one Sp1 family member is critical for *Pem* transcription in SL12.4 cells.

Although these transient transfection experiments indicated the importance of Sp1 and Ets family members for expression from our minimal *Pem Pd* expression plasmid, they did not address whether the endogenous *Pem* gene is similarly regulated. To assess this point, we stably transfected SL12.4 cells with the DN*-Sp3* expression plasmid and examined mRNA expression from the endogenous *Pem* gene in these cells. As shown in Fig. 7c, stable transfection of DN*-Sp3* decreased endogenous *Pem* mRNA levels, indicating that the endogenous *Pem* promoter requires Sp3 and/or other Sp1 family members for activity.

**Ets and Sp1 Transcription Factors Drive Aberrant Transcription of the Pem Pd in Diverse Tumor Cell Types** - To determine whether the *cis*- and *trans*-acting factors responsible for *Pem Pd* transcription in the SL12.4 T-lymphoma cell clone also regulate *Pem*’s expression in other tumor cell types, we examined *Pem Pd* regulation in five other tumor cell lines. These cell lines exhibited a range of *Pem Pd* transcription rates (Fig. 8a). The neuroblastoma cell line N4TG1 and the prostate cancer cell line PS-1 had a high *Pem Pd* transcription rate, which was from 150 to 200 fold above that of the empty control vector. The *ras*-transformed fibroblast cell line RAT-1 had a transcription rate 30 fold above that of the control vector. The B-myeloma cell lines S194/5 and M12 had comparatively low transcription rates that were 5-6 fold above that of the empty control vector.

We found that four of the five tumor cell lines required all three transcription-factor binding sites (S1, E1, and E2) in the *Pem Pd* element for optimal transcription (Fig. 8a).
The one exception was the M12 cell line, which appeared to only require the S1 and E2 sites, as it did not exhibit a statistically significant (P>0.05) decrease in luciferase activity when the E1 site was mutated. Transfection experiments with the DN-\(\text{Gabp}\) and DN-\(\text{Sp3}\) plasmids showed that the Ets factor Gabp and one or more Sp1 family members were necessary for maximal \(\text{Pem Pd}\) transcription in all the tumor cell lines (Fig. 8b). We conclude that \(\text{Pem Pd}\) transcription in a wide range of tumor cell types depends on Ets and Sp1 family members.

\textit{Ras is Essential for Endogenous Pem Expression} - Because the ras signaling pathway is known to positively regulate Ets and Sp1 family members (see Discussion), we examined the role of \(\text{ras}\) in \(\text{Pem}\) transcription. We found that transient transfection of a DN-\(\text{ras}\) expression plasmid almost completely abrogated \(\text{Pem Pd}\) transcriptional activity from the \(-112\ \text{Pem}\) construct in SL12.4 cells, indicating that indeed \(\text{ras}\) does activate the \(\text{Pem Pd}\) (Fig. 9a). To determine whether \(\text{ras}\) also positively regulates \(\text{Pem Pd}\) transcription from the endogenous \(\text{Pem}\) gene, we took two approaches. First we stably transfected SL12.4 cells with the DN-\(\text{ras}\) plasmid and selected cell clones that expressed the plasmid. We found that DN-\(\text{ras}\)-positive cell clones expressed lower levels of endogenous \(\text{Pem}\) mRNA (up to \(~3\)-fold) than did untransfected cells (Fig. 9b) or stably transfected cells that expressed little or no DN-\(\text{ras}\) (data not shown). Second, to determine whether \(\text{ras}\) expression was sufficient to activate endogenous \(\text{Pem}\) gene expression, we stably transfected an activated Ha-\(\text{ras}\) expression plasmid into a \(\text{Pem}\)-negative cell line, \(10T^1/2\). We found that constitutively active Ha-\(\text{ras}\) strongly induced gene expression from the endogenous \(\text{Pem}\) gene in \(10T^1/2\) cells (Fig. 9b).
Lastly, we determined whether non-malignant cells require the same regulatory element for Pem transcription as do malignant cells. We therefore investigated Pem Pd transcription in primary ovarian granulosa cells, as mice granulosa cells normally express Pem (22). As shown in Fig. 10, we found that primary granulosa cells expressed high levels of luciferase from the Pem -112 construct (almost 100-fold over that from the empty vector). Mutation of Ets- and Sp1-binding sites in the -112 construct drastically reduced luciferase expression, suggesting that as in tumor cells, granulosa cells require these binding sites for transcription. Cotransfection of the DN-ras construct dramatically reduced expression. We conclude that, like tumor cells, normal granulosa cells express the Pem Pd in a ras-dependent manner that involves Ets and Sp1 family members.
DISCUSSION

We have characterized a promoter active in normal ovarian granulosa cells, placental trophoblast cells, and tumor cells from a variety of lineages (Figs. 1, 8, and 10 herein; reference (15)). We showed that this *Pem Pd* was transcriptionally activated in response to the cell type-specific Ets transcription factors Gabp and Elf1, as well as the ubiquitous zinc-finger transcription factors Sp1 and Sp3 (Figs. 4, 5). Three closely spaced binding sites for these factors between nt -80 and -104 were sufficient for *Pem Pd* transcription (Figs. 2c, 5a). We found that *Pem Pd* transcription required the ras pathway (Fig. 9), which is known to activate both Ets and Sp1 family transcription factors and is constitutively activated in a large proportion of tumors of different origins (23-26).

That Ets transcription factors are essential for *Pem* transcription in diverse tumor cell types is consistent with the fact that many Ets family members play a role in tumor cell growth, invasion, and metastasis. For example, the founding member of the Ets gene family, the v-ets retroviral gene, was originally identified as an oncogene based on its ability to transform cells (27,28). Since then, at least 10 of the ~ 30 Ets domain-containing genes have been shown to play a role in cancer, including ETS1, ETS2, FLI1, TEL, ERG, and PSE (29). Our finding that Gabp was required for *Pem* transcription in every tumor cell line tested (Figs. 7a, 8) indicates that this Ets transcription factor is also important in activating gene transcription in tumor cells. However, whether Gabp is an oncogene that causes cancer remains to be determined.

Ets family members are also critical for the proper control of cellular proliferation and differentiation of normal cells (30). There is a wealth of knowledge about the role of Ets factors in some biological systems but less is known regarding their role in the female
reproductive tract, where Pem is expressed. In *D. melanogaster*, Elg, the fly orthologue of Gabp, is essential for normal egg development, as null mutations in Elg cause tiny-egg syndrome (31). In mammals, Gabp positively regulates the expression of the *FBP* gene in placenta (32) and thus this Ets factor may be important for normal placental development and function. Part of Gabp's function in placenta may be to allow Pem expression, as we previously showed that Pem is strongly expressed in this tissue (5,15) and we demonstrated here that Gabp positively regulated *Pem* transcription in *D. melanogaster* Schneider cells and several mammalian cell lines (Figs. 6, 7, 8). Gabp may also positively regulate Pem expression in ovarian granulosa cells, as Pem is expressed in ovarian granulosa cells *in vivo* (10,22), and we found that its expression in these cells depends on the presence of an Ets-factor binding site (Fig. 10).

Our demonstration that Sp1 family members are essential for *Pem* transcription is not surprising given the fact that this set of transcription factors participates in the regulation of a wide variety of genes expressed in both normal and malignant cells (33-36). Sp1-regulated genes include those expressed in granulosa and trophoblast cells, the type of cells that normally express Pem (32,37). Sp1 is at a wide range of levels in different tissues (35,38), which could partly explain Pem's unique expression pattern in different tissues. For example, the high expression of Sp1 in normal trophoblasts (38) and some types of tumor cells (38,39) could, in part, explain Pem's high expression in these cell types (5,10).

Surprisingly, we found that Sp1 was a less potent activator of *Pem* than was the related family member Sp3 (Fig. 5a). This response contrasts with most other genes, which are more strongly transcriptionally activated in response to Sp1 than to Sp3 (40-42). In fact,
rather than acting as an activator, Sp3 functions as a repressor for most gene promoters, including many which are activated by Sp1 (21,43-47). In contrast, we found that Sp3 had an additive affect on Sp1-mediated transactivation of the *Pem Pd*. We speculate that Sp3 is a potent transcriptional activator of *Pem* because the *Pem Pd* regulatory element possesses only a single Sp1 family-binding site (Fig. 2b). This notion is based on studies conducted on other gene promoters that demonstrated that a single Sp1 family-binding site supports Sp3-mediated transcriptional activation, whereas multiple Sp1 family-binding sites support Sp3-mediated repression (48,49). This differential response has been suggested to result from the inability of a single Sp3 repressor domain to overcome Sp3’s glutamine-rich activating region (48,50,51). Another factor that can dictate the transcriptional response to Sp3 is the type of cell in which it acts. For example, Sp3 activates the *CD11d* gene in myeloid cells but represses this same gene in B and T cells (21,52-54). However, cell type cannot be the only determinant governing positive *Pem Pd* regulation by Sp3, as we found that the *Pem Pd* was induced by Sp3 in a wide range of cell types, including ones that engender negative regulation for other genes in response to Sp3 (21,36,37,55,56). Thus, we believe that intrinsic features of the *Pem Pd*, rather than cell type, dictates its strong induction in response to Sp3.

Our data support the notion that the *Pem Pd* is regulated by Ets and Sp1 family members acting in a cooperative manner. First, the close proximity of their binding sites in the *Pem Pd* regulatory element (Fig. 2b, 4a) suggests that Ets and Sp1 family members physically interact. Second, both Ets sites and the single Sp1 site were required for maximal *Pem* transcription in several different cell types, including normal granulosa cells and tumor cells from a variety of cell lineages (Figs. 6-8, 10). Third, any single
mammalian Ets or Sp1 family member transactivated the *Pem Pd* less well than a combination of them. An additive transcriptional response to Ets and Sp1 family members was observed in Schneider cells, which we believe is an underestimate, as our results suggested that there are endogenous *D. melanogaster* factors that bind to the Ets and Sp1 binding sites and therefore elevate transcription levels in response to any single mammalian transcription factor (Fig. 5). That Ets and Sp1 family members cooperate to activate *Pem Pd* transcription is supported by several other studies showing that most Ets family members cooperate with other transcription factors, including Sp1 family members, to strengthen their transactivation potential (26). For example, the Ets factor Gabp has been shown to cooperate with Sp1 to activate at least three genes, including the *FBP* gene in placenta (32,57,58). Elf1 forms a complex with Sp1 or Sp3 to activate the transcription of the MRG1 and stem cell leukemia tal-1 genes (59,60).

That Sp1 and Ets family members and ras are ubiquitously expressed in many tumor cell types, may partly explain why *Pem* is ubiquitously expressed in tumor cells. In addition, we showed that more than one member of each family is capable of triggering *Pem* transcription, thereby further increasing the range of tumor cell types that could express *Pem*. For example, some cell types that express low levels of Sp1 express high levels of Sp3 (49), both of which we found activated the *Pem Pd* (Fig. 5). Another probable reason for *Pem* expression in a wide variety of tumor cells is that ras, which we demonstrated is required for *Pem* transcription (Fig. 9), is constitutively activated in approximately 50% of human tumors as a result of mutation (23,61). Ras induces the phosphorylation and consequent activation of Ets family members (23), and thus ras may drive *Pem* expression in tumor cells as result of its ability to stimulate the activity of Ets.
transcription factors. Consistent with this notion, *Pem* is widely expressed in T-cell tumors but it is not expressed in normal T cells (5), perhaps because Elf1, which is present in both normal and malignant T cells, is only constitutively activated when T cells become transformed (62). Ras proteins have also been reported to activate gene transcription via Sp1 family members (25,39,63), which could be another mechanism by which *Pem* is transcriptionally activated in response to the ras pathway.

Why is *Pem* ubiquitously expressed in a wide variety of tumor cells but in a cell type- and stage-specific manner in normal cells? First, despite the ubiquitous expression of Sp1 and Sp3, they are known to display complex and intricate interactions with other transcription factors (35,38,58,64) that may confine their ability to activate the *Pem* Pd in normal cells and expand this ability in tumor cells. Second, we found that only some members of the Ets family can activate *Pem* transcription and thus the normally restricted expression pattern of individual Ets transcription factors will confine *Pem* expression to certain cell types. Third, and perhaps most importantly, we speculate that even if a cell contains a particular Ets factor required for *Pem* transcription, in most cases the Ets factor will not be phosphorylated and hence will not be capable of activating *Pem* transcription. This follows from the fact that extracellular stimuli that activate ras and downstream mitogen-activated protein kinase (MAPK) pathways are known to cause the phosphorylation of many Ets factors, an event that appears to be necessary for Ets factors to regulate the transcription of specific target genes (65,66). Consistent with this notion, *Pem* is weakly expressed in quiescent liver macrophages but is dramatically induced when these cells are treated with lipopolysaccharide, a known activator of ras and MAPK pathways in macrophages (22,53). Quiescent macrophages constitutively express all the
transcription factors known to activate *Pem* transcription (Elf1, Gabp, Sp1, and Sp3) (46,47,54,67,68) and thus the induction of *Pem* in these cells probably results from the activation of one or more of these factors by phosphorylation rather than an induction in their levels.

Our discovery that the *Pem* homeobox gene is regulated by a tissue- and stage-specific promoter that is aberrantly expressed in tumors raises several questions. First, what are the signaling pathways that normally trigger *Pem* transcription in response to Ets and Sp1 family factors? The Ets factor Gabp is known to be activated by the c-Jun-N-terminal kinase (JNK) and extracellular-signal-regulated kinase (ERK) MAPK pathways (69,70), and thus it will be of interest to determine whether these pathways signal *Pem* transcription in normal granulosa and trophoblast cells. Second, what receptor-ligand interactions trigger *Pem* transcription in normal cells? Virtually nothing is known about either the receptors or ligands that activate Ets transcription factors in the female reproductive cells that express *Pem*. Third, what is the precise mechanism by which tumor cells bypass *Pem*'s normal regulatory constraints to constitutively express this homeobox transcription factor? A full understanding of the regulatory networks that control the *Pem* homeobox gene promoter that we have defined in this report may be useful towards elucidating the transcriptional perturbations that occur when normal cells become malignant.
REFERENCES

1. Ford, H. L. (1998) Cell Biol Int 22, 397-400
2. Caldas, C., and Aparicio, S. (1999) Cancer Metastasis Rev 18, 313-329
3. Castronovo, V., Kusaka, M., Chariot, A., Gielen, J., and Sobel, M. (1994)
   Biochem Pharmacol 47, 137-143
4. Wilkinson, M., Doskow, J., and Lindsey, S. (1991) Nucleic Acids Res. 19, 679
5. Wilkinson, M. F., Kleeman, J., Richards, J., and MacLeod, C. L. (1990) Dev.
   Biol. 141, 451-455
6. Maiti, S., Meistrich, M. L., Wilson, G., Shetty, G., Marcelli, M., McPhaul, M. J.,
   Morris, P. L., and Wilkinson, M. F. (2001) Endocrinology 142, 1567-1577
7. Rao, M., and Wilkinson, M. F. (2002) in The Epididymis: From Molecules to
   Clinical Practice (Robaire, B., and Hinton, B. T., eds), pp. 269-283, Kluwer
   Academic/Plenum Publishers, New York
8. Sutton, K. A., and Wilkinson, M. F. (1997) J. Mol. Evol. 45, 579-588
9. Fan, Y., Melhem, M. F., and Chaillet, J. R. (1999) Dev Biol 210, 481-496
10. Lin, T. P., Labosky, P. A., Grabel, L. B., Kozak, C. A., Pitman, J. L., Kleeman, J.,
    and MacLeod, C. L. (1994) Dev. Biol. 166, 170-179
11. Lindsey, J. S., and Wilkinson, M. F. (1996) Dev. Biol. 179, 471-484
12. Lindsey, J. S., and Wilkinson, M. F. (1996) Biol. Reprod. 55, 975-983
13. Lemmens, I. H., Forsberg, L., Pannett, A. A., Meyen, E., Piehl, F., Turner, J. J., Van de Ven, W. J., Thakker, R. V., Larsson, C., and Kas, K. (2001) *Biochem Biophys Res Commun* **286**, 426-431

14. Ono, T., Sato, S., Kimura, N., Tanaka, M., Shibuya, A., Old, L. J., and Nakayama, E. (2000) *Int J Cancer* **88**, 845-851

15. Maiti, S., Doskow, J., Li, S., Nhim, R. P., Lindsey, J. S., and Wilkinson, M. F. (1996) *J. Biol. Chem.* **271**, 17536-17546

16. Brown, T. A., and McKnight, S. L. (1992) *Genes Dev* **6**, 2502-2512

17. Morris, J. K., and Richards, J. S. (1996) *J Biol Chem* **271**, 16633-16643

18. Chen, C. A., and Okayama, H. (1988) *Biotechniques* **6**, 632-638

19. Danielson, P. E., Forss-Petter, S., Brow, M. A., Calavetta, L., Douglass, J., Milner, R. J., and Sutcliffe, J. G. (1988) *DNA* **7**, 261-267

20. Courey, A. J., and Tjian, R. (1988) *Cell* **55**, 887-898

21. Hagen, G., Muller, S., Beato, M., and Suske, G. (1994) *Embo J* **13**, 3843-3851

22. Pitman, J. L., Lin, T. P., Kleeman, J. E., Erickson, G. F., and MacLeod, C. L. (1998) *Dev. Biol.* **202**, 196-214

23. Galang, C. K., Der, C. J., and Hauser, C. A. (1994) *Oncogene* **9**, 2913-2921

24. Halfon, M. S., Carmena, A., Gisselbrecht, S., Sackerson, C. M., Jimenez, F., Baylies, M. K., and Michelson, A. M. (2000) *Cell* **103**, 63-74

25. Kivinen, L., Tsubari, M., Haapajarvi, T., Datto, M. B., Wang, X. F., and Laiho, M. (1999) *Oncogene* **18**, 6252-6261

26. Wasylyk, B., Hahn, S. L., and Giovane, A. (1993) *Eur J Biochem* **211**, 7-18

27. Janknecht, R., and Nordheim, A. (1993) *Biochim Biophys Acta* **1155**, 346-356
28. Leprince, D., Gegonne, A., Coll, J., de Taisne, C., Schneeberger, A., Lagrou, C., and Stehelin, D. (1983) *Nature* **306**, 395-397
29. Dittmer, J., and Nordheim, A. (1998) *Biochim Biophys Acta* **1377**, F1-11
30. Sementchenko, V. I., and Watson, D. K. (2000) *Oncogene* **19**, 6533-6548
31. Schulz, R. A., The, S. M., Hogue, D. A., Galewsky, S., and Guo, Q. (1993) *Proc Natl Acad Sci U S A* **90**, 10076-10080
32. Sadasivan, E., Cedeno, M. M., and Rothenberg, S. P. (1994) *J Biol Chem* **269**, 4725-4735
33. Kumar, A. P., and Butler, A. P. (1999) *Cancer Lett* **137**, 159-165
34. Ping, D., Boekhoudt, G., Zhang, F., Morris, A., Philipsen, S., Warren, S. T., and Boss, J. M. (2000) *J Biol Chem* **275**, 1708-1714
35. Rieber, M., and Strasberg Rieber, M. (1999) *Int J Cancer* **83**, 359-364
36. Schanke, J. T., Durning, M., Johnson, K. J., Bennett, L. K., and Golos, T. G. (1998) *Mol Endocrinol* **12**, 405-417
37. Alliston, T. N., Maiyar, A. C., Buse, P., Firestone, G. L., and Richards, J. S. (1997) *Mol Endocrinol* **11**, 1934-1949
38. Saffer, J. D., Jackson, S. P., and Annarella, M. B. (1991) *Mol Cell Biol* **11**, 2189-2199
39. Zheng, X. L., Matsubara, S., Diao, C., Hollenberg, M. D., and Wong, N. C. (2001) *J Biol Chem* **276**, 13822-13829
40. McEwen, D. G., and Ornitz, D. M. (1998) *J Biol Chem* **273**, 5349-5357
41. Ritchie, S., Boyd, F. M., Wong, J., and Bonham, K. (2000) *J Biol Chem* **275**, 847-854
42. Tone, M., Powell, M. J., Tone, Y., Thompson, S. A., and Waldmann, H. (2000) *J Immunol* **165**, 286-291

43. Discher, D. J., Bishopric, N. H., Wu, X., Peterson, C. A., and Webster, K. A. (1998) *J Biol Chem* **273**, 26087-26093

44. Krikun, G., Schatz, F., Mackman, N., Guller, S., Demopoulos, R., and Lockwood, C. J. (2000) *Mol Endocrinol* **14**, 393-400

45. Kwon, H. S., Kim, M. S., Edenberg, H. J., and Hur, M. W. (1999) *J Biol Chem* **274**, 20-28

46. Noti, J. D., Johnson, A. K., and Dillon, J. D. (2000) *J Biol Chem* **275**, 8959-8969

47. Tomaras, G. D., Foster, D. A., Burrer, C. M., and Taffet, S. M. (1999) *J Leukoc Biol* **66**, 183-193

48. Majello, B., De Luca, P., and Lania, L. (1997) *J Biol Chem* **272**, 4021-4026

49. Rajakumar, R. A., Thamotharan, S., Menon, R. K., and Devaskar, S. U. (1998) *J Biol Chem* **273**, 27474-27483

50. Birnbaum, M. J., van Wijnen, A. J., Odgren, P. R., Last, T. J., Suske, G., Stein, G. S., and Stein, J. L. (1995) *Biochemistry* **34**, 16503-16508

51. Kennett, S. B., Udvadia, A. J., and Horowitz, J. M. (1997) *Nucleic Acids Res* **25**, 3110-3117

52. De Luca, P., Majello, B., and Lania, L. (1996) *J Biol Chem* **271**, 8533-8536

53. Rao, K. M. (2001) *J Leukoc Biol* **69**, 3-10

54. Noti, J. D. (1997) *J Biol Chem* **272**, 24038-24045

55. Hagen, G., Dennig, J., Preiss, A., Beato, M., and Suske, G. (1995) *J Biol Chem* **270**, 24989-24994
56. Majello, B., De Luca, P., Hagen, G., Suske, G., and Lania, L. (1994) *Nucleic Acids Res* **22**, 4914-4921

57. Rosmarin, A. G., Luo, M., Caprio, D. G., Shang, J., and Simkevich, C. P. (1998) *J Biol Chem* **273**, 13097-13103

58. Shirasaki, F., Makhluf, H. A., LeRoy, C., Watson, D. K., and Trojanowska, M. (1999) *Oncogene* **18**, 7755-7764

59. Bockamp, E. O., Fordham, J. L., Gottgens, B., Murrell, A. M., Sanchez, M. J., and Green, A. R. (1998) *J Biol Chem* **273**, 29032-29042

60. Han, B., Liu, N., Yang, X., Sun, H. B., and Yang, Y. C. (2001) *J Biol Chem* **276**, 7937-7942

61. Zachos, G., and Spandidos, D. A. (1997) *Crit Rev Oncol Hematol* **26**, 65-75

62. Bassuk, A. G., Barton, K. P., Anandappa, R. T., Lu, M. M., and Leiden, J. M. (1998) *Mol Med* **4**, 392-401

63. Spencer, J. A., and Misra, R. P. (1999) *Oncogene* **18**, 7319-7327

64. Tajima, A., Miyamoto, Y., Kadowaki, H., and Hayashi, M. (2000) *Biochim Biophys Acta* **1492**, 377-384

65. Yordy, J. S., and Muise-Helmericks, R. C. (2000) *Oncogene* **19**, 6503-6513

66. Wasylyk, B., Hagman, J., and Gutierrez-Hartmann, A. (1998) *Trends Biochem Sci* **23**, 213-216

67. Serio, K. J., Hodulik, C. R., and Bigby, T. D. (2000) *Am J Respir Cell Mol Biol* **23**, 234-240
68. Tsai, E. Y., Falvo, J. V., Tsytsykova, A. V., Barczak, A. K., Reimold, A. M., Glimcher, L. H., Fenton, M. J., Gordon, D. C., Dunn, I. F., and Goldfeld, A. E. (2000) *Mol Cell Biol* 20, 6084-6094

69. Avots, A., Hoffmeyer, A., Flory, E., Cimanis, A., Rapp, U. R., and Serfling, E. (1997) *Mol Cell Biol* 17, 4381-4389

70. Hoffmeyer, A., Avots, A., Flory, E., Weber, C. K., Serfling, E., and Rapp, U. R. (1998) *J Biol Chem* 273, 10112-10119
| Name    | Orientation | Sequence                                      |
|---------|-------------|-----------------------------------------------|
| MDA-298 | Sense       | AGGCCCATATGCTCAGTG                            |
| MDA-396 | Sense       | TGCTCATATGAAGGAAGA                            |
| MDA-392 | Sense       | GAAGGCATATGCGGGACTT                          |
| MDA-319 | Sense       | GATCTCTCGAGGAAAGAG                           |
| MDA-397 | Sense       | GTGGAAGGAATAGGATTTACTCCGGATC                 |
| MDA-398 | Antisense   | GATCCGGAAGTAAATCTATTCTCTTCAC                 |
| MDA-399 | Sense       | AATAGGCCGGGACCATGGATCTCTGGA                  |
| MDA-400 | Antisense   | TCCAGAGATCCATGCCTCCCGCCTATT                  |
| MDA-401 | Sense       | AGGAATAGGATTTACGCATGGATCTCTGGA              |
| MDA-402 | Antisense   | TCCAGAGATCCATGCCTAAATCTATTTCCT              |
| MDA-419 | Sense       | GCTCAGTGTCCCTTAATAGCGGGGACTTC               |
| MDA-420 | Antisense   | GAGTCCCGCTATTAAAGGACACTGAGC                 |
| MDA-473 | Sense       | TCGACAATAGGCGGGACTTCCGGATC                  |
| MDA-474 | Antisense   | AGCTTGATCCGGAAGTCCCCGCTATTG                |
| T3      | Antisense   | CTCTGGGAATGGCAGGG                           |
FIGURE LEGENDS

FIG. 1. **The Pem Pd is transcriptionally active in tumor cell lines.**  A, Northern-blot analysis of total cellular RNA (10 µg) isolated from the following tumor cell lines: SL12.4 (T-cell lymphoma), N4TG1 (neuroblastoma), M12 (B-cell myeloma), PS-1 (transformed prostate smooth muscle), S194/5 (B-cell lymphoma) and Ras / RAT-1 (ras-transformed fibroblast). Similar loading and transfer of RNA were verified by hybridization with a probe for cyclophilin (cyclo).  B, RPA of total cellular RNA (10 µg) from testes and the cell lines shown, annealed with the Pem and β-actin probes. The length of the probe protected was ~80 nt and ~140 nt for Pem Pd and Pp mRNA, respectively, and ~40 nt for β-actin mRNA.

FIG. 2. **Ets and Sp1 consensus sites are critical for Pem Pd transcriptional activity.**  A-C, The Pem gene constructs indicated were transiently transfected (1 µg) into SL12.4 cells and then luciferase (luc) activity was measured. The values shown are Renilla luciferase activity expressed from the Pem constructs, normalized against firefly luciferase activity from the pGL2TK internal control vector (± standard error [S.E.]). Panel A shows expression from constructs containing the deletions indicated; the numbers refer to the nts upstream of the exon 1-intron 1 junction (the first black box is exon 1). Shown are the average values (relative to luciferase expression from the -304 construct, which was arbitrarily given a value of 100) from four independent transfection experiments performed in triplicate. Panel B shows the two putative Ets family protein-binding sites (E1 and E2) and the putative Sp1 family protein-binding site (S1) that when
mutated (indicated by small letters) abrogate Pem Pd transcription. Shown are the average values (relative to luciferase expression from the –112 construct, which was arbitrarily given a value of 100) from six independent transfection experiments performed in triplicate. Panel C shows that a single copy of the Pem promoter element sequence containing the S1, E1, and E2 binding sites (-81 nt to -112 nt) suffices for transcription from a heterologous minimal promoter (Δc-fos). Shown are the average values (relative to that from the empty vector [Δc-fos], which was given an arbitrary value of 1) from five independent transfection experiments performed in triplicate.

FIG. 3. The Pem Pd element drives transcription in a cell line-specific manner. A, Northern-blot analysis of total cellular RNA (10 µg) isolated from the immature T-cell lymphoma clones (SL12.1 and SL12.3) and a mature T-cell lymphoma clone (SL12.4). Similar loading and transfer of RNA were shown by hybridization with a probe for cyclophilin. B, The cell clones indicated were transiently transfected (1 µg) with the -112 Pem gene construct. Shown is the amount of Renilla luciferase activity from the -112 construct (± S.E.) relative to that from the empty vector (which was given an arbitrary value of 1) after normalization of both against firefly luciferase activity expressed from the internal control reporter plasmid. The data are from two independent transfection experiments performed in triplicate.

FIG. 4. Sp1 and Ets family transcription factors bind to the Pem Pd element. EMSA of SL12.4 nuclear protein extracts (1 µg) incubated with a 32P-labeled Pem Pd element probe (nt -81 to -104). A, Cold-competition experiment in which a 50-fold molar excess
of unlabeled double-stranded oligos with mutations in the Sp1 site (M1), the Ets site (M2) or both sites (M3) were added prior to incubation with hot probe.  

B, Complex 1 and 2 correspond to Sp1 and Sp3, respectively, as shown by supershift assay using anti-Sp1 (left panel) and anti-Sp3 (right panel) monoclonal antibodies (α). The arrows indicate the relative migration of the relevant protein-DNA complexes with and without antibody.  

C, Complex 3 and 4 correspond to Elf1 and Gabp, respectively, as shown by EMSAs performed by preincubating nuclear proteins with rabbit polyclonal antisera against Elf1 and Gabp (α+β). The arrows indicate the relevant complexes inhibited by antibody preincubation.

FIG. 5. Sp1, Sp3, Elf1, and Gabp transactivate Pem Pd transcription.  

A, B, Schneider cells cotransfected with the -112 Pem gene construct (0.5 µg) and the indicated amount of pPac-Sp1, pPac-Sp3, pPac-Elf1, pPac-Gabp (α and β combined), or combinations of these. Shown is average luciferase activity, calculated as described for Fig. 3B from six independent transfection experiments performed in triplicate, displayed in logarithmic scale (left) or arithmetic scale (right).

FIG. 6. Transactivation of the Pem Pd requires the Sp1/Sp3- and Ets-binding sites.  

Schneider cells were cotransfected with 0.5 µg of either the wild-type -112 construct (WT) or versions of it that had mutations in the Sp1 site (S1) or one of the two Ets sites (E1, E2) and 50 ng of pPac-Sp1, pPac-Sp3, pPac-Elf1, or pPac-Gabp (α and β). Shown is average luciferase activity, calculated as described for Fig. 3B from six independent transfection experiments performed in triplicate.
FIG. 7. **Ets and Sp1 family members are essential for Pem Pd transcription in SL12.4 T-lymphoma cells.** *A, B*, SL12.4 cells cotransfected with the -112 construct (1 µg) and the indicated amount of the DN-Gabp and DN-Sp3 constructs. Shown is average luciferase activity, calculated as described for Fig. 3B from six independent transfection experiments performed in triplicate. *C*, Northern-blot analysis of total cellular RNA (10 µg) from untransfected SL12.4 cells (control) and SL12.4 cells stably transfected with the DN-Sp3 expression plasmid. Similar loading and transfer of RNA were verified by hybridization with a probe for cyclophilin (cyclo).

FIG. 8. **Ets and Sp1 family members are required for Pem Pd transcription in diverse tumor cells from different tissues.** *A*, Tumor cell lines transfected with 1 µg of either the wild-type -112 construct (WT) or versions of it that had mutations in the Sp1 site (S1) or one of the two Ets sites (E1 and E2). Shown is average luciferase activity, calculated as described for Fig. 3B from three independent transfection experiments performed in triplicate. *B*, Tumor cell lines cotransfected with the Pem -112 construct (1 µg) and the indicated amount of the DN-Sp3, DN-Gabp, or empty control construct (2 µg). Shown is average luciferase activity, calculated as described for Fig. 3B from three independent transfection experiments performed in triplicate.

FIG. 9. **Pem Pd transcription requires the ras signaling pathway.** *A*, SL12.4 cells cotransfected with the -112 construct (1 µg) and DN-ras (2 µg). Shown is average luciferase activity, calculated as described for Fig. 3B from two independent transfection
experiments performed in triplicate. B, Northern-blot analysis of total cellular RNA (10 µg) from the cells shown. Left panel: untransfected SL12.4 cells (control) and SL12.4 cells stably transfected with the DN-ras plasmid. Transfected cells that expressed little or no DN-ras mRNA expressed the same level of Pem mRNA as untransfected cells (data not shown). Right panel: untransfected 10T1/2 cells (control) and 10T1/2 cells stably transfected with an activated Ha-ras expression plasmid. 10T1/2 cells stably transfected with empty vector lacked Pem mRNA expression, just like the untransfected cells (data not shown). Similar loading and transfer of RNA were verified by hybridization with a probe for cyclophilin (cyclo).

FIG. 10. **Pem Pd transcription in primary granulosa cells requires Sp1/Sp3- and Ets-binding sites and the ras signalling pathway.** Primary granulosa cells transfected with 1 µg of either the wild-type -112 construct (WT) or versions of it that had mutations in the Sp1 (S1) or Ets (E1) sites. Also shown are granulosa cells cotransfected with the -112 construct (1 µg) and DN-ras (2 µg). Shown is average luciferase activity, calculated as described for Fig. 3B from two independent transfection experiments performed in triplicate.
Fig. 1

A.

| SL 12.4 | N4TG1 | M12 | PS-1 | RAT-1 | S194/5 |
|---------|-------|-----|------|-------|--------|
|         | Pem   |     | Cyclo|       |        |

B.

| tRNA  | SL 12.4 | N4TG1 | M12 | PS-1 | RAT-1 | S194/5 |
|-------|---------|-------|-----|------|-------|--------|
|       |         |       |     | Pp   | Pd    | Testis |
|       |         |       |     |      |       | bp     |

β-actin
A.

\[
\begin{array}{c}
-304 \\
-112 \\
-104 \\
-94 \\
-73 \\
\end{array}
\]

\(\text{luc}\)

\(\text{P}_d\)

B.

\[
\begin{array}{c}
-104 \\
GTGGAAGGAATAGGCGGGACTTCCG \\
\underline{\text{Ets}} \\
GTGGAAGGAATAGG\underline{\text{G}}\text{atttACTTCCG} \\
\underline{\text{Sp1}} \\
GTGGAAGGAATAGGC\underline{\text{G}}\text{GACgcatG} \\
\underline{\text{Ets}} \\
\text{-80} \\
-112 \\
\end{array}
\]

\[
\begin{array}{c}
\text{S1} \\
\text{S1} \\
\text{E1} \\
\text{E1} \\
\text{S1}+ \\
\text{E1} \\
\text{E2} \\
\text{E2} \\
\end{array}
\]

Rao et. al.  

**Fig. 2**

![Graph showing relative promoter activity](image-url)
Fig. 2

Rao et. al.

C.

![Bar chart showing the relative promoter activity of Δc-fos and -81 to -104/Δc-fos.](http://www.jbc.org/)
Fig. 3

A.

Pem -
Cyclo -

B.

Relative promoter activity

SL12.4
SL12.1
SL12.3
**Fig. 4**

A.

```
-104  Sp  Ets  -80
GTGGAAGGAATAGGCGGGACTTCCG
ATTT  GCAT
 M1   M2
     M3
```

|           | P_d Probe | M1 | M2 | M3 |
|-----------|-----------|----|----|----|
| P_d Probe | + + + + + | -  | +  | -  |
| M1        | - + - -   | -  | -  | -  |
| M2        | - - + -   | -  | -  | +  |
| M3        | - - - +   | -  | -  | -  |

![Image of a gel showing bands labeled 1, 2, 3, and 4]
B. Rao et. al. Fig. 4

|          | P_d Probe | +  | +  | P_d Probe | +  | +  |
|----------|-----------|----|----|-----------|----|----|
| α Sp1    | -         | +  |    | α Sp3     | -  | +  |

Supershift

Sp1

Sp3
C. Rao et. al. Fig. 4

|           | 1 | 2 | 3 | 4 |
|-----------|---|---|---|---|
| P_δ Probe| + | + | + |   |
| α Gabp   | - | - | + |   |
| α Elf1   | - | + | - |   |
Fig. 5

Rao et. al.

A.

Relative promoter activity

µg of plasmid

µg of plasmid
B.

Rao et. al.  Fig. 5

![Graph showing relative promoter activity](image-url)
Relative promoter activity

Fig. 6

Rao et. al.

-112 Construct
Transactivator plasmid

Sp3 Gabp Elf1
Fig. 7

A. Relative promoter activity

B. Relative promoter activity

C. Rao et. al.

Control  DN Sp3

Pem

Cyclo

SL12.4
A. Relative promoter activity

-112 Construct

|        | WT | S1 | E1 | E2 |
|--------|----|----|----|----|
| N4TG1  |    |    |    |    |
| S194/5 |    |    |    |    |
| RAT-1  |    |    |    |    |
| PS-1   |    |    |    |    |
| M12    |    |    |    |    |

Fig. 8A. Rao et al.
Relative promoter activity

-112
DN Gabp
DN Sp3

Rao et. al. Fig. 8
Rao et. al. Fig. 9

A. Relative promoter activity

B. [Images of gel analysis with bands labeled as 'Control', 'DN Ras', 'Cyclo', 'Pem', 'SL12.4', '10T1/2', 'DN Ras']
Rao et. al. Fig. 10

Relative promoter activity

Granulosa Cells

pRL-null  -112  -112S1  -112E1  -112 + DN Ras
A highly active homeobox gene promoter regulated by Ets and Sp1 family members in normal granulosa cells and diverse tumor cell types
Manjeet K. Rao, Sourindra Maiti, Honnavara N. Ananthaswamy and Miles F. Wilkinson

J. Biol. Chem. published online May 1, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M203374200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts