Oncogenic Activation of the Human *Pygopus2* Promoter by E74-Like Factor-1

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Abstract

Pygopus is a component of the T-cell factor/β-catenin transcriptional complex essential for activation of Wnt target genes and is also required for cell regulation in the absence of Wnt signaling. Human Pygopus2 (hPygo2) is overexpressed in a high proportion of breast and epithelial ovarian malignant tumors and is required for the growth of several cell lines derived from these carcinomas. The mechanisms regulating *hPygo2* gene activation, however, are unknown. Here, we have determined *cis*- and *trans*-interacting factors responsible for *hPygo2* expression in cancer. The minimal region required for a maximal 109-fold activation of the *hPygo2* promoter in MCF-7 breast cancer cells is 48 bp upstream of the start of transcription. Within 25 bp of the transcriptional start, there are two overlapping tandem Ets transcription factor–binding sites, which are critical for *hPygo2* promoter activity. *In vitro* DNA pull-down assays and proteomic analyses identified the Ets family members Elk-1 and E74-like factor-1 (Elf-1) as potential *hPygo2* promoter binding factors, whereas *in vivo* chromatin immunoprecipitation assays verified that only Elf-1 specifically bound to the *hPygo2* promoter in MCF-7 cells. Modulation of *elf-1* in MCF-7 cells by silencing via RNA interference or overexpression caused a corresponding decrease or increase, respectively, in *hPygo2* promoter activity. Overexpression of Elf-1 in HeLa cells, in which Elf-1 is expressed at a lower level than in MCF-7 cells, caused a 4-fold increase in endogenous *hPygo2* mRNA levels. These results provide new evidence that Elf-1 is involved in transcriptional activation of *hPygo2.* Like hPygo2, previous studies implicated Elf-1 in breast and ovarian cancer and our present findings suggest that the oncogenic requirement of *hPygo2* is fulfilled, in part, by Elf-1. (Mol Cancer Res 2008;6(2):259–66)

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Introduction

Deregulation of canonical Wnt signaling and misexpression of core Wnt pathway components occur frequently and are causal in the initiation and progression of cancer (1). Wnt ligand-stimulated activation of the canonical pathway results in translocation of the β-catenin transcriptional complex from the cytoplasm to the nucleus where it associates with, and activates, T-cell factor/lymphoid enhancer-binding factor-1–dependent transcription. Recent evidence has indicated that although nuclear sequestration of β-catenin involves the adaptor proteins B-cell lymphoma 9/Legless and Pygopus (2), once formed, the T-cell factor/β-catenin complex specifically requires these adaptors for target gene transcription (3, 4). In addition to its function in canonical Wnt transcription, Pygopus has also been implicated in Wnt-independent roles both in cancer (5) and in development (6, 7).

Because of its diverse roles in cell regulation, disruption of Pygopus function is proposed to be a strategy to target malignancy (2, 8). Consistent with this hypothesis, we determined recently that one of the two Pygopus family members, human Pygopus2 (hPygo2), is expressed in the majority of a sample of breast and epithelial ovarian primary carcinomas. Antisense oligonucleotides, used to silence endogenous *hPygo2*, also caused growth suppression of cell lines derived from both of these malignancies (5, 9). These observations strongly suggested that hPygo2 has an important involvement in cancer, but the mechanisms governing oncogenic activation of the *hPygo2* gene are unknown.

In this article, we present evidence characterizing the elements that regulate *hPygo2* in malignancy. Our data indicate that the minimal promoter of *hPygo2* binds nuclear proteins present in MCF-7 breast cancer cells that include members of the Ets family of transcription factors. E74-like factor-1 (Elf-1) in particular is present at the *hPygo2* minimal promoter in MCF-7 chromatin, and *hPygo2* promoter activity responds proportionately to the availability of Elf-1 in cells. Elf-1 is involved in breast (10, 11) and ovarian (12) carcinomas and is regulated by the retinoblastoma tumor suppressor (13). Together with these previous findings, our results suggest that Elf-1 is required for oncogenic activation of the *hPygo2* promoter.

Results

Identification of the hPygo2 Minimal Promoter

To study the regulation of *hPygo2*, a 1,568-bp fragment of genomic DNA spanning a region of *hPygo2* that is 1,494 nucleotide bases upstream and 74 bases downstream of the

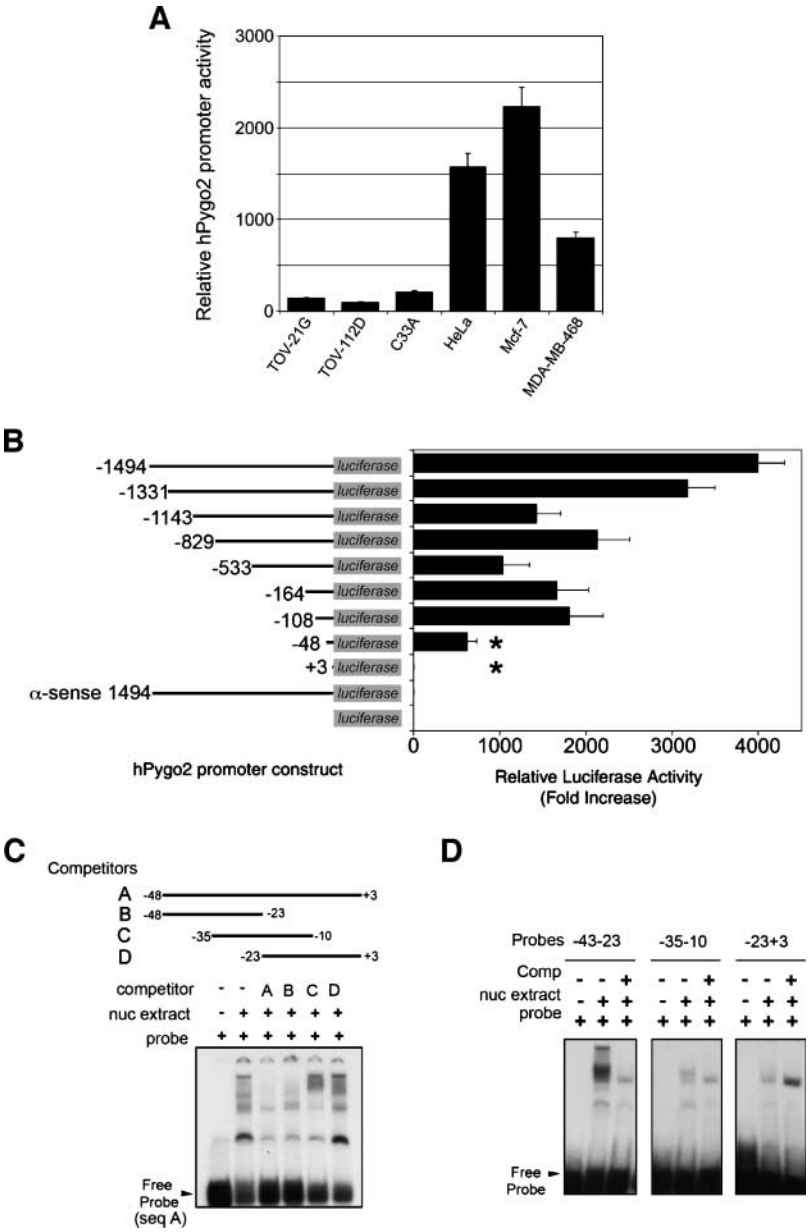

FIGURE 1. Identification of the hPygo2 minimal promoter region. **A.** Ovarian (TOV-21G and TOV-112D), cervical (C33A and HeLa), and breast (MCF-7 and MDA-MB-468) cancer cell lines were transiently transfected with a *luciferase* reporter under the control of the region corresponding to the 5′-regulatory region of the *hPygo2* gene [pGL3(−1494)]. Reporter activation was determined relative to the level of cotransfected β-galactosidase used to control for transfection efficiency. Columns, average of three independent experiments done in triplicate; bars, SD. **B.** Localization of the minimal promoter of *hPygo2* by 5′ deletion analysis. Columns, average of two independent experiments done in triplicate; bars, SD. *, largest fold decrease between two successive deletion constructs (109.5-fold). **C.** EMSAs were done using MCF-7 nuclear extract and a ^{32}P end-labeled probe corresponding to the *hPygo2* minimal promoter (bases −48 to +3). Protein complexes were competed using an excess of unlabeled oligonucleotide corresponding to the full-length probe (**A**) or three overlapping fragments of equal length (**B-D**). EMSAs were done using three labeled, overlapping oligonucleotides from **C** as probes. An excess of each corresponding unlabeled probe was used as a specific competitor.

putative start of transcription was inserted into the promoterless vector pGL3-basic to control expression of the *luciferase* reporter. This reporter plasmid, named pGL3(−1494) was cotransfected with a plasmid expressing β-galactosidase to control for transfection efficiency (pRSV-βGal) into two ovarian (TOV-21G and TOV-112D), two cervical (C33A and HeLa), and two breast (MCF-7 and MDA-MB-468) cancer cell lines. The ovarian and breast cell lines were specifically chosen because we previously showed that *hPygo2* is overexpressed in these malignant lines in comparison with normal epithelial cells (5, 9). We considered that these cell lines would be reasonable choices to screen for a model to study oncogenic up-regulation of *hPygo2*. The relative activity of the hPygo2 5′-regulatory sequence was compared between the cell lines by measuring luciferase activity, normalized to levels of β-galactosidase activity (Fig. 1A). MCF-7 breast cancer cells showed the highest *hPygo2* promoter activity and were thus selected for further analysis.

To isolate the minimal promoter, *luciferase* reporter plasmids containing successive 5′-deletions of pGL3(−1494) were transiently transfected into MCF-7 cells (Fig. 1B). As a negative control, a reporter plasmid containing antisense pGL3(−1494) was also transfected and showed no significant reporter activation above background levels of pGL3-basic. Small reductions in promoter activity were observed when regions spanning nucleotides −1,494 to −108 and nucleotides −108 to −48 were deleted, resulting in a 2.2-fold and a 2.9-fold decrease, respectively. Deletion of the region spanning nucleotides −48 to +3 caused a considerable decrease in promoter activity by 109.5-fold, therefore indicating that

the *hPygo2* minimal promoter was likely located within nucleotides −48 to +3.

To more precisely define the minimal promoter and analyze the proteins that bind to it, electrophoretic mobility shift assays (EMSA) were done using MCF-7 nuclear extract (Fig. 1C). Several bands appeared when we added extract, all of which disappeared on cold competition, suggesting that several proteins or protein complexes retarded the mobility of the full −48 to +3 sequence (−48+3, oligo A). Cold competition assays were done using three overlapping oligonucleotides spanning the putative minimal proximal promoter: nucleotides −48 to −23 (−48−23, oligo B), nucleotides −35 to −10 (−35−10, oligo C), and nucleotides −23 to +3 (−23+3, oligo D). The (−48−23) oligonucleotide, but not the (−23+3) oligonucleotide, competed with the full-length (−48+3) probe, indicating that the proteins bound to the 5′ or distal extent of the region spanning nucleotides −48 to +3. Conversely, the three overlapping fragments were labeled and used as probes to complement the cold competition assays shown in Fig. 1C. As expected, proteins bound strongly to the 25 bp region (−48−23), which corresponded to the most distal half of the minimal promoter, whereas weak binding was observed using the (−35−10) and (−23+3) probes (Fig. 1D). These results indicated that several nuclear proteins from MCF-7 cells bound within nucleotides −48 to −23 of the *hPygo2* promoter, suggesting that this short sequence binds proteins that likely play a role in the activation of the *hPygo2* gene.

Ets Transcription Factors Bind the Minimal Promoter

Several potential consensus transcription factor–binding sites within the (−48+3) region of the *hPygo2* minimal promoter were predicted by *in silico* sequence analysis using two independent internet search engines (TFSearch and Transcription Element Search System). The most frequently predicted motifs were those that bind the Ets family of transcription factors. In particular, both Ets-1 and Elk-1 motifs were detected among two tandem, overlapping Ets sites located within the (−48−23) region (Fig. 2A). Of lower probability were overlapping sequences for Aml-1 sites also detected within the (−48−23) region, as well as SP1, GATA, and AP-2/4 sites detected within the (−23+3) region.

Because Ets transcription factors had the highest probability of binding, we determined whether a previously defined oligonucleotide encoding an Ets-specific binding consensus sequence [E74 Ets-binding site (E74B); ref. 14] could also bind proteins from MCF-7 extracts that bound to the minimal promoter region (−48−23). Labeled E74B effectively bound proteins (Fig. 2B, *lane 2*) that were competed off using an excess of unlabeled E74B (Fig. 2B, *lane 3*). The proteins were also competed off with an excess of unlabeled (−48−23)

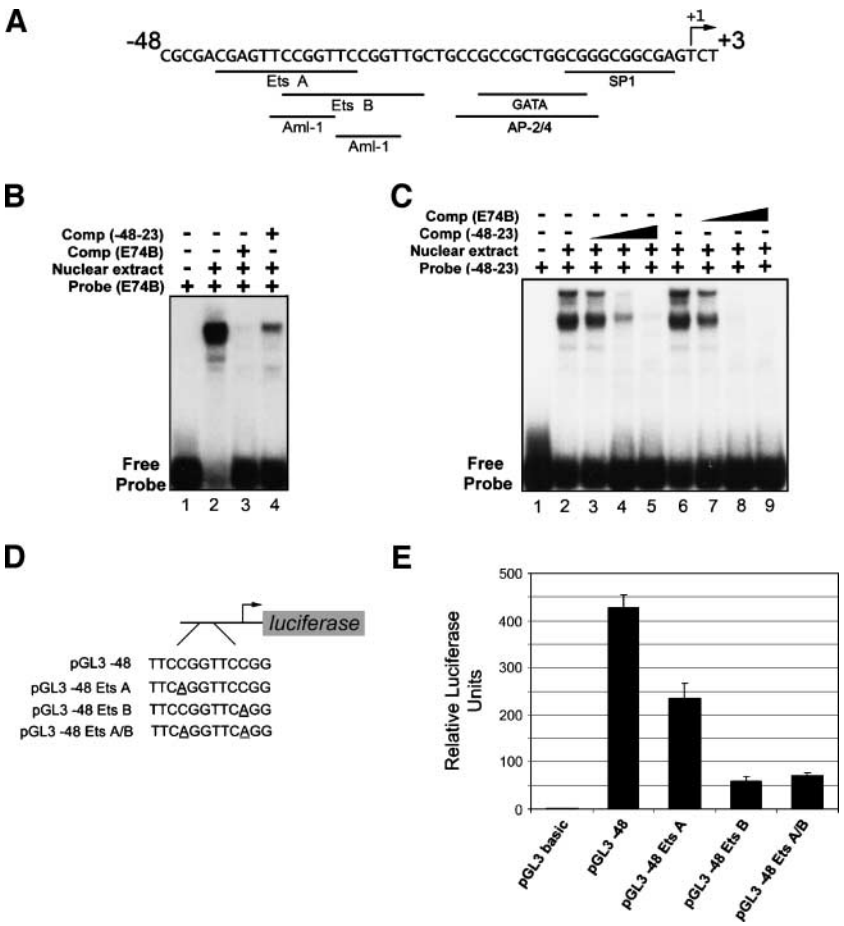

FIGURE 2. The hPygo2 minimal promoter region contains functional Ets-binding sites. **A.** Nucleotide sequence of the minimal *hPygo2* promoter region. The arrow at position +1 indicates the putative start of transcription. Underlined, potential transcription factor–binding sites. **B.** The minimal *hPygo2* promoter (−48−23) competes with complexes that bind to an Ets-specific oligonucleotide (*E74B*) probe. EMSAs were done using MCF-7 nuclear extract and ^{32}P end-labeled E74B. Specific protein complexes were competed using excess unlabeled E74B or hPygo2 (−48−23) oligonucleotide. **C.** E74B competes with complexes that bind to the *hPygo2* minimal promoter. MCF-7 nuclear extract was incubated with a ^{32}P-labeled *hPygo2* promoter (−48−23) probe. Specific protein complexes were competed using increasing amounts (0.25×, 2.5×, and 25×) of unlabeled (−48−23) oligonucleotide or E74B. **D.** Mutation of the core GGAA/TTCC region of Ets-binding sites in the *hPygo2* promoter. **E.** MCF-7 cells were transfected with wild-type *hPygo2* minimal promoter region (pGL3-48) or with Ets-mutated *hPygo2* minimal promoter constructs illustrated in **D**, and relative promoter activity, normalized to levels of cotransfected β-galactosidase, was measured for each mutation. Columns, average of three independent experiments done in triplicate; bars, SD.

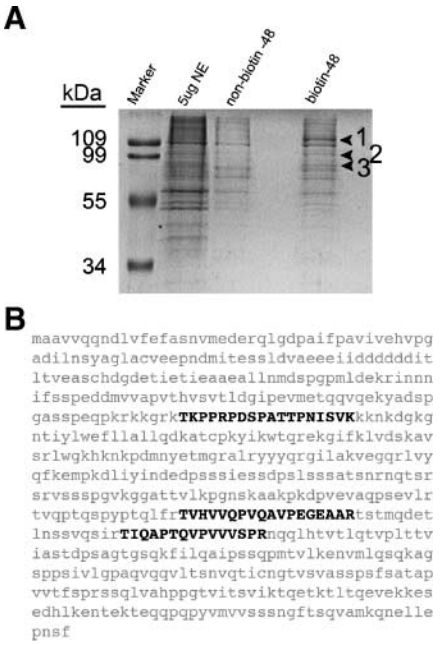

FIGURE 3. Identification of Elf-1 as a potential binding factor of the hPygo2 promoter by proteomics. **A.** Proteins from DNA pull-down assays using MCF-7 nuclear extract (*NE*) and biotinylated (*biotin-48*) and nonbiotinylated (*non-biotin-48*) *hPygo2* minimal promoter (−48+3) were resolved by SDS-PAGE. Three bands (*1, 2*, and *3*) specific to the biotin-48 sample were prepared for tandem mass spectrometry. **B.** Elf-1 sequence with peptide residue sequences derived from band 2 (*bold face*).

oligonucleotide (Fig. 2B, *lane 4*), indicating that the minimal promoter bound to similar factors as the E74B probe. Likewise, proteins bound to the −48−23 oligonucleotide probe (Fig. 2C, *lanes 2* and *6*) were competed off using increasing amounts of unlabeled (−48−23) probe (Fig. 2C, *lanes 3-5*) as well as excess unlabeled E74B (Fig. 2C, *lanes 7-9*). These results suggested that at least some proteins in MCF-7 cells related to the Ets family of transcription factors most likely bound to the *hPygo2* minimal promoter.

To determine the requirement of the Ets consensus on the *hPygo2* promoter, mutations within the Ets consensus sequences (Ets A and Ets B) of the *hPygo2* reporter pGL3-48 were assessed for their ability to affect promoter activity (Fig. 2D). Mutations to the core wild-type Ets consensus sites were introduced, resulting in TTCC to TTCA base-pair substitutions, either individually (pGL3-48 Ets A or pGL3-48 Ets B) or in combination (pGL3-48 Ets A/B). MCF-7 cells were then transiently cotransfected with pRSV-βGal and equal amounts of each vector encoding wild-type and mutated sequences. Reporter activation was determined, normalized to β-galactosidase levels to control for transfection efficiency. Mutation of the first and second Ets sequences (pGL3-48 Ets A and pGL3-48 Ets B) resulted in 1.8- and 7.1-fold reduction of reporter activation, respectively, when compared with the wild-type sequence (Fig. 2E). Mutation of both Ets sites (pGL3-48 Ets A/B) caused a 6.1-fold reduction, which was not significantly different when compared with the pGL3-48 Ets B mutant. These results indicated that both Ets-binding sites are required for promoter activation but that the more proximal Ets B site is likely more important for mediating transcription.

Identification of Ets Proteins That Bind to the hPygo2 Promoter

We next analyzed the proteins in MCF-7 nuclear extracts that bound to the *hPygo2* promoter using DNA pull-down assays. Complementary 5′-biotinylated −48 to +3 promoter sequences (biotin-48) were annealed and incubated with nuclear extract. Specific proteins bound to the biotinylated probe were separated by SDS-PAGE and compared with those processed using a nonbiotinylated probe. Unique proteins present in the biotinylated sample were processed for tandem mass spectroscopy. Proteins represented by bands 1 [poly(ADP-ribosyl)-transferase] and 3 (ATP-dependent DNA helicase II) in Fig. 3A were common DNA-binding factors found in unrelated complexes (15) and were discarded. Analysis of peptides derived from band 2, however (Table 1), indicated that they were identical to sequences found in the Ets family member Elf-1 (Fig. 3B).

Proteins resolved in DNA pull-down assays were identified by immunoblotting to verify that Ets proteins bound to the *hPygo2* promoter. Both Elf-1 and, at a lower affinity, Elk-1, but not Ets-1, bound specifically to the biotinylated probe, confirming our *in silico* and proteomic analyses (Fig. 4A). Most importantly, chromatin immunoprecipitation assays showed *in vivo* binding of Elf-1 to the *hPygo2* promoter in MCF-7 cells. Primers specific for the promoter region of *hPygo2* amplified a specific product over background in chromatin precipitated by Elf-1, but not Elk-1 antibodies (Fig. 4B). As a negative control, primers specific for *hPygo2* exonic sequence showed a low and equal level of background amplification. As a positive control for Elk-1, primers complementary to the *early growth response-1* promoter bound by Elk-1 (16) revealed that Elk-1 was present at the promoter of *early growth response-1* but not *hPygo2* (Fig. 4B). The specific presence of Elf-1 at the *hPygo2* promoter *in vivo* suggested that it is involved in cellular regulation of *hPygo2*. Immunoblot analysis done using nuclear extracts of the cell lines used in Fig. 1 indicated that there was a strong correlation between endogenous expression of Elf-1 and hPygo2 protein (Fig. 4C). The highest levels of Elf-1 and hPygo2 were in the nuclei of MCF-7 cells, which also showed the highest levels of hPygo2 promoter activation (Fig. 1A). These results suggest that *in vivo* availability of Elf-1 in nuclei is important for *hPygo2* activation and expression.

Activation of hPygo2 by Elf-1

To further assess the involvement of Elf-1 in *hPygo2* promoter activation, we determined the effect of its loss and gain of function. To establish loss of function, MCF-7 cells

Table 1. Proteomic Analysis of DNA Pull-Down Products

Peptide Sequence	Precursor Ions *m/z*	Monoisotopic Molecular Mass	
		Observed	Calculated
TIQAPTQVPVVVSPR	796.47	1,590.93	1,590.91
TVHVVQPVQAVPEGEAAR	629.70	1,886.07	1,886.00
TKPPRPDSPATTPNISVK	636.04	1,905.09	1,905.03

were cotransfected with small inhibitory RNAs (siRNA) and equal amounts of pRSV-βGal and the pGL3-48 reporter plasmid 24 h later. *Luciferase* activation, normalized to β-galactosidase activity, was then determined relative to that of mock-transfected cells. We used one nonspecific control siRNA and two *elf-1*–specific siRNAs. Immunoblotting indicated that both of the Elf-1 siRNAs that repressed reporter activity also reduced the level of Elf-1 protein >50% compared with the mock-transfected and nonspecific siRNA-transfected negative controls (Fig. 5A). The nonspecific control did not significantly alter endogenous levels of reporter activation, whereas both *elf-1*–specific sequences caused a significant ($P < 0.01$, $n = 9$ per treatment) 25% to 35% reduction in reporter activity (Fig. 5B). Although the less-than-complete reduction in silencing may account for the modest, yet significant, reduction of promoter activity, these results nonetheless indicate that decreasing the availability of Elf-1 protein in MCF-7 cells had a negative effect on the activity of the *hPygo2* promoter.

Gain-of-function experiments were done by overexpression of Elf-1. MCF-7 cells were cotransfected with the minimal *hPygo2* reporter plasmid (pGL3-48) along with increasing amounts of elf-1 cDNA, which resulted in a corresponding increase in Elf-1 protein levels (Fig. 5C). Increasing amounts of Elf-1 caused a dose-dependent increase in relative *hPygo2* promoter activity (Fig. 5D), indicating that increased availability of Elf-1 in cells had a positive effect on the *hPygo2* promoter.

In addition to modulating the expression of the hPygo2 minimal reporter, we determined the effect of Elf-1 overexpression on endogenous *hPygo2* gene expression. Elf-1 was transiently transfected into HeLa cells, which normally show lower levels of endogenous Elf-1 expression than MCF-7 cells (Fig. 4C). Relative *hPygo2* gene expression was then determined by quantitative real-time PCR analysis. In at least three independent experiments, transfection of 0.25 to 0.5 μg of an expression plasmid that encoded Elf-1 (pCS2+/*elf-1*) caused endogenous levels of *hPygo2* mRNA to increase >4-fold over control-transfected levels (Fig. 5E). This result confirmed that increased availability of Elf-1 has a positive effect on endogenous *hPygo2* gene expression.

These loss-of-function and gain-of-function experiments, in combination with our promoter analyses, provide positive evidence that Elf-1 is involved in the activation of the *hPygo2* promoter in cancer cells.

Discussion

Elf-1 is a transcription factor required for regulation of lymphoid-specific genes (17, 18). Elf-1 has also been implicated in the regulation of genes involved in breast cancer cell growth and differentiation, such as BRCA2 and ErbB2 (HER2/neu; refs. 10, 11). We previously showed that *hPygo2* is overexpressed in, and required for, growth of breast cancer cell lines (9) and we have now determined that Elf-1 is likely involved in controlling its expression. Our results therefore provide evidence to suggest that *hPygo2* is part of the repertoire of genes targeted by Elf-1 that are required for breast cancer growth regulation.

Ets transcription factors bind DNA through the core sequence GGAA, whereas the surrounding sequence is specific for each individual Ets factor (19). As suggested, the partial effects on promoter activity in our *elf-1* silencing experiments could be due to incomplete knockdown of Elf-1. It is also possible that the binding of multiple proteins or a complex of proteins to the *hPygo2* promoter (Fig. 1C and D) that may compensate for loss of Elf-1 would account for the weaker-than-expected phenotype (Fig. 5A). For instance, the *in vitro* binding of the ternary transcription factor Elk-1 to the *hPygo2* promoter suggests that other Ets family members might bind to

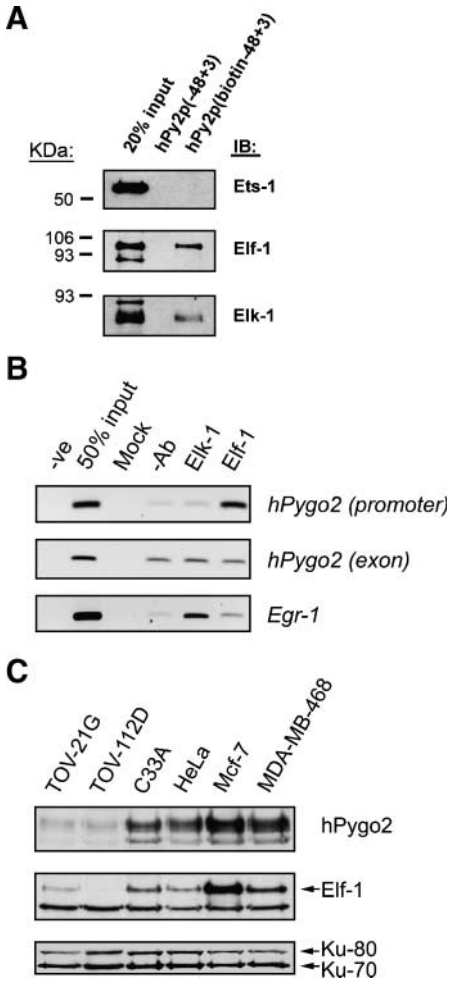

FIGURE 4. Binding of Ets proteins to the hPygo2 minimal promoter. **A.** Confirmation of specific Ets factor binding from MCF-7 nuclear extracts to the *hPygo2* minimal promoter by DNA pull-down assays using MCF-7 nuclear extract incubated with biotinylated [*hPy2p(biotin−48+3)*] and nonbiotinylated [*hPy2p(−48+3)*] *hPygo2* minimal promoter sequence. Proteins resolved by SDS-PAGE were immunoblotted (*IB*) using antibodies specific to Ets-1, Elf-1, and Elk-1. Left, sizes of molecular weight markers. **B.** Chromatin immunoprecipitation assays indicate that Elf-1 is present at the *hPygo2* promoter in MCF-7 cells. Primers used were specific for *hPygo2* promoter and *early growth response-1* (*Egr-1*) as a positive control for Elk-1. Primers spanning an exonic region of *hPygo2* were used to display background levels of nonspecific amplification. -ve, negative PCR control; 50% input, positive PCR control; Mock, negative control without chromatin, -Ab, negative control containing chromatin but no antibody; Elk-1 and Elf-1, chromatin immunoprecipitated using Elk-1 and Elf-1 antibodies, respectively. **C.** Correlation of hPygo2 and Elf-1 protein levels in cancer cell lines. For a nuclear loading control, immunoblot analysis was used to detect the presence of Ku-70/80. Each assay was done at least twice in two independent experiments.

the promoter of *hPygo2*. It is therefore possible that, under varying cellular contexts, Elk-1 might act competitively or cooperatively with other factors, such as Elf-1, to determine the transcriptional status of *hPygo2*.

The regulation of Elf-1 function is complex and occurs at many different levels. It exists as an 80-kDa form in the cytoplasm and, on modification by phosphorylation and glycosylation, moves into the nucleus as a 98-kDa form (20). Our DNA pull-down experiments indicated that a 98-kDa form of Elf-1 specifically binds to the minimal hPygo2 promoter region. Furthermore, overexpression of Elf-1 in MCF-7 cells resulted in higher levels of the 98-kDa form that correlated with activation at the *hPygo2* promoter. These observations suggest that Elf-1 posttranslational modification may be required for its binding to *hPygo2*.

Finally, regulation of the transactivation function of Elf-1 can occur by its specific binding to the underphosphorylated form of the retinoblastoma tumor suppressor (13). Thus, control of *hPygo2* expression via Elf-1 may be regulated coordinately with the cell cycle. This hypothesis would suggest that canonical Wnt-dependent events that stimulate cell cycle progression would include activation *hPygo2*, a key component of canonical Wnt signal transduction itself, therefore suggesting the possibility of autoactivation of Wnt-dependent signaling components in cancer.

Materials and Methods

Cell Lines

TOV-21G and TOV-112D ovarian cancer cells, C33A and HeLa cervical cancer cells, and MCF-7 and MDA-MB-468 breast cancer cells were obtained from the American Type Culture Collection and cultured as per the American Type Culture Collection recommended medium supplemented with 10% fetal bovine serum.

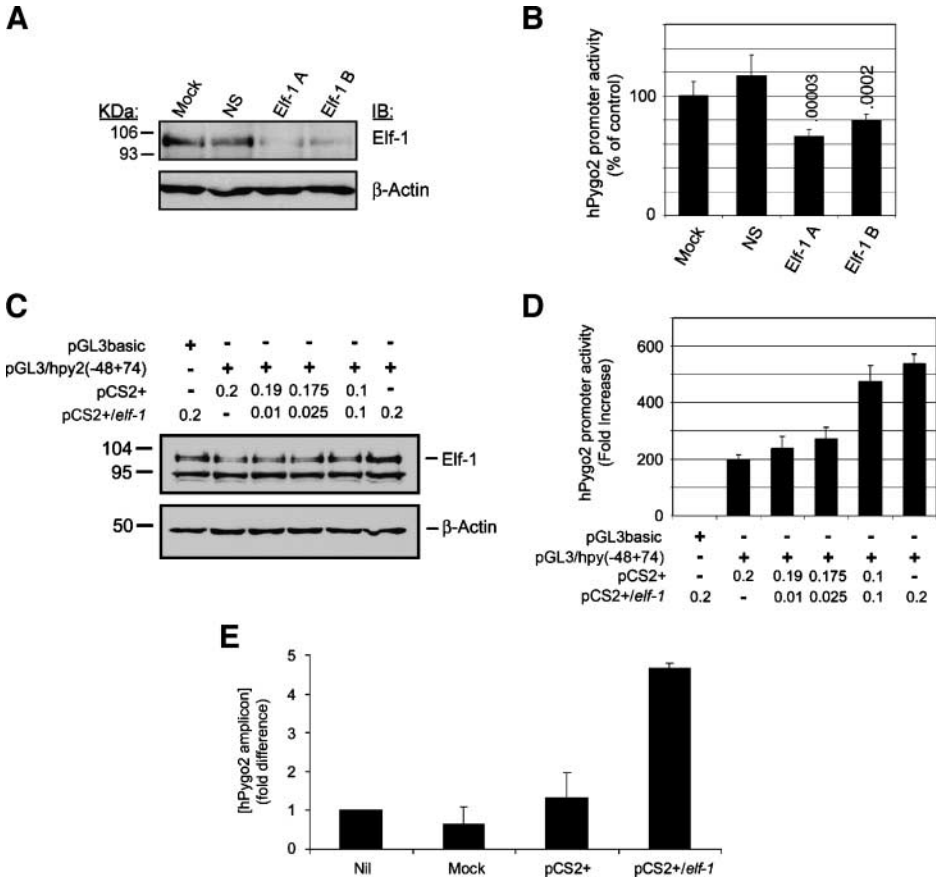

FIGURE 5. Modulation of hPygo2 expression by Elf-1. **A.** Silencing of *elf-1* by siRNA. A representative immunoblot of Elf-1 in protein extracted from MCF-7 cells transfected with nonspecific control (*NS*) and two specific Elf-1 (**A** and **B**) siRNAs using equal amounts of each sample as determined by levels of β-actin. **B.** Silencing *elf-1* in MCF-7 cells by RNA interference. MCF-7 cells were treated with three siRNAs, including one nonspecific control and two specific for Elf-1 (**A** and **B**). Twenty-four hours later, the cells were transiently cotransfected with pGL3-48 and pRSV-βGal. Promoter activity, normalized to transfection efficiency, was expressed relative to the mock-transfected sample. *elf-1*–specific siRNAs significantly reduced the levels of reporter activation when compared with the nonspecific control siRNA; numbers over the *elf-1* data indicate calculated *P* values using the Student's *t* test ($n = 9$). **C.** Overexpression of Elf-1 in MCF-7 cells with increasing proportion of transfected Elf-1 cDNA using β-actin as a loading control. **D.** Transient transfection of Elf-1 results in an increase of *hPygo2* promoter activity. MCF-7 cells were cotransfected with pGL3-48 and increasing proportions of an expression vector encoding Elf-1 (*pCS2+/elf-1*). Promoter activity for each sample was calculated relative to that of the empty vector. Columns, mean of three experiments done in triplicate; bars, SD. **E.** Overexpression of Elf-1 in HeLa cells causes an increase in *hPygo2* mRNA levels. Relative *hPygo2* mRNA levels were determined by quantitative real-time PCR in HeLa cells transiently transfected with an expression vector encoding human Elf-1 (*pCS2+/elf-1*). Controls included untransfected cells (*Nil*), cells treated with transfection reagents only (*Mock*), and cells transfected with empty vector (*CS2+*). Changes in *hPygo2* mRNA levels normalized to that of *β-actin* mRNA were calculated using the $2^{-\Delta\Delta Ct}$ method (23) and expressed on the *Y* axis as fold difference from untransfected cells. Columns, mean ($n = 3$); bars, SD.

Table 2. Primers Used in the Construction of hPygo2 Promoter Constructs

Construct	Forward Primer	Reverse Primer
pGL3(−1494)/(as-1494)	5′-CGGTTTGGGTTGGGATAACAGAGG	5′-CCGAGCTGCAGCAACCACAAAGTG
pGL3(−1331)	5′-GTATATACATTTCATAAGGCCTGCC	5′-CCGAGCTGCAGCAACCACAAAGTG
pGL3(−1143)	5′-GCATTGAGACTATAAAGCAGTAGAC	5′-CCGAGCTGCAGCAACCACAAAGTG
pGL3(−829)	5′-GGTCTCTTACCGCGAGACTGAG	5′-CCGAGCTGCAGCAACCACAAAGTG
pGL3(−533)	5′-CTTCTGGGAAGCCTATTGTGTACGCC	5′-CCGAGCTGCAGCAACCACAAAGTG
pGL3(−164)	5′-CCTTTCCACTCGCGTGGTGCCGTG	5′-CCGAGCTGCAGCAACCACAAAGTG
pGL3(−108)	5′-CAGGCGTAGCGTCTCGTCCGGTC	5′-CCGAGCTGCAGCAACCACAAAGTG
pGL3(−48)	5′-CGCGACGAGTTCCGGTTCCG	5′-CCGAGCTGCAGCAACCACAAAGTG
pGL3(−48) Ets A	5′-GATCTCGCGACGAGTTCAGGTTCCGGTTGCTGCC	5′-GGCAGCAACCGGAACCTGAACTCGTCGCGAGATC
pGL3(−48) Ets B	5′-GCGACGGAGTTCCGGTTCAGGTTGCTGCCGCCG	5′-CGGCGGCAGCAACCTGAACCGGAACTCGTCGC
pGL3(−48) Ets A+B	5′-GATCTCGCGACGAGTTCAGGTTCAGGTTGCTGCCGCCG	5′-CGGCGGCAGCAACCTGAACCTGAACTCGTCGCGAGATC

Plasmid Construction

PCR primers used in the construction of cDNAs and mutant regulatory sequences are listed in Table 2. The *hPygo2* 5′-regulatory region was amplified from Hs-574 genomic DNA and inserted into the *Hin*dIII site in the promoterless vector pGL3-basic in both sense and antisense orientations [pGL3(−1494)]. *hPygo2* promoter 5′-deletion constructs, except for pGL3 hPy2p +3, were PCR amplified and inserted into the *Hin*dIII and *Bgl*2 sites of pGL3. pGL3 hPy2p +3 was constructed by annealing complementary oligonucleotides corresponding to +3 to +74 bases of the *hPygo2* promoter inserted into *Hin*dIII and *Bgl*2 sites of pGL3-basic. The *hPygo2* promoter Ets-binding site mutants were generated with the QuikChange Site-Directed Mutagenesis kit (Stratagene). Elf-1 cDNA was PCR amplified (forward, 5′-ATGGCTGCTGTTGTCCAACAGAACG; reverse, 5′-CTAAAAAGAGTTGGGTTCCAGCAG) from human testis cDNA (BD Biosciences) and inserted into the *Xho*I site of the mammalian expression vector pCS2+. All plasmid constructs were sequenced to verify the correct sequences.

In silico *Analysis of the hPygo2 Promoter*

The *hPygo2* promoter sequence was analyzed with PROSCAN[3] for promoter sites and TATA box prediction. TFSearch,[4] Transcription Element Search System,[5] and PROSCAN were used in the prediction of transcription factor–binding sites.

Transfections and Luciferase Assays

MCF-7 cells were seeded in triplicate 18 h before transfection at a density of 1.5×10^5 per well in 12-well plates. Reporter plasmids were cotransfected with a plasmid expressing β-galactosidase (pRSV-βgal; Promega) using Lipofectamine with Plus Reagent (Invitrogen). Elf-1 siRNA (Santa Cruz Biotechnology) and nonspecific siRNA (Upstate) were transfected using Oligofectamine (Invitrogen). Luciferase assays were done using luciferase assay reagent (Promega), using a Monolight 2010 Luminometer. Relative luciferase unit values were normalized to β-galactosidase activity and expressed as fold change over the value obtained with empty vector. Where necessary, the Student's *t* test was used to compare differences between results. siRNAs were transfected to a final concentration of 100 nmol/L using Oligofectamine as per the manufacturers' protocol. Elf-1 A siRNA was purchased from Santa Cruz Biotechnology. Nonspecific and Elf-1 B siRNAs were purchased from Dharmacon. Elf-1 B siRNA (target sequence, 5′-GAAAGAGAACACTGAGAAA) was designed using siDESIGN Center siRNA design tool software (Dharmacon).

Antibodies

Anti–β-actin (Sigma) and Ku-70/80 (Chemicon) antibodies were used to confirm equal loading/transfer of total protein and nuclear protein, respectively. Anti–Elf-1, Elk-1, and Ets-1 antibodies were purchased from Santa Cruz Biotechnology. Anti-hPygo2 antisera were developed and used as described (5).

DNA-Binding Assays

EMSAs were done with [^{32}P]dATP end-labeled double-stranded oligonucleotides corresponding to either *hPygo2* promoter [−48+3, 5′-CGCGACGAGTTCCGGTTCCGGTTGCTGCCGCCGCTGGCGGGCGGCGAGTC (forward) and 5′-GACTCGCCGCCCGCCAGCGGCGGCAGCAACCGGAACCGGAACTCGTCGCG (reverse); −48−23, 5′-CGCGACGAGTTCCGGTTCCGGTTGC (forward) and 5′-GCAACCGGAACCGGAACTCGTCGCG (reverse); −35−10, 5′-GGTTCCGGTTGCTGCCGCCGCTGGC (forward) and 5′-GCCAGCGGCGGCAGCAACCGGAACC (reverse); −23+3, 5′-TGCCGCCGCTGGCGGGCGGCGAGTC (forward) and 5′-GACTCGCCGCCCGCCAGCGGCGGCA (reverse)] or the *Drosophila* E74B (14). EMSAs were done as described (21). For competitions, 0.25, 2.5, and 25 times molar excess of unlabeled probe was added to each reaction.

DNA pull-down assays were done using 5′-biotinylated double-stranded annealed oligonucleotides corresponding to the *hPygo2* promoter −48 to +3 (−48+3) region or nonbiotinylated (−48+3) as a negative control. Nuclear extracts were prepared using the NE-PER (Pierce) extraction kit. Probe (1 μg) was incubated with 50 μg of MCF-7 nuclear extract in the same buffer as used for EMSA for 30 min at

[3] http://bimas.cit.nih.gov/molbio/proscan
[4] http://www.cbrc.jp/research/db/TFSEARCH.html
[5] http://www.cbil.upenn.edu/tess

room temperature. Streptavidin-agarose beads (Pierce) were blocked in 5% skim milk in wash buffer containing 20 mmol/L Tris (pH 7.5), 1 mmol/L EDTA (pH 8.0), 1 mmol/L DTT, 50 mmol/L NaCl, and 10% glycerol. Blocked beads were then washed briefly with wash buffer and added to each reaction and incubated for a further 30 min at 4°C and then washed in wash buffer and 1× PBS. Bound proteins were then resolved by SDS-PAGE, transferred to nitrocellulose, and probed as described (5, 9).

Tandem Mass Spectrometry

Nuclear extract (375 μg) was incubated with 5 μg probe according to the DNA pull-down protocol outlined above. Bound proteins were resolved by SDS-PAGE and gels were stained with Coomassie Brilliant Blue. Excised bands were digested using the Trypsin Profile IGD kit (Sigma). Peptides were extracted in 0.1% trifluoroacetic acid and 50% acetonitrile, dried, and analyzed by a Dionex UltiMate 3000 liquid chromatography separation system interfaced with a QSTAR XL tandem mass spectrometer (Applied Biosystems). Peptides were identified using Analyst (Applied Biosystems) and MASCOT[6] software. Results were expressed as observed isotopic mass, calculated from the mass-to-charge ratio for each peptide (*m/z*), and compared with the predicted mass shown in Table 2.

Chromatin Immunoprecipitation Assays

Chromatin immunoprecipitation assays were done essentially as described (22). Samples were subjected to immunoprecipitation using anti–Elk-1 or anti–Elf-1 antibodies overnight at 4°C. PCR was done using 5 μL of DNA and primers corresponding to the hPygo2 promoter region [pGL3(−108) forward and reverse; Table 2], hPygo2 exonic sequence (forward, 5′-CTACAGAGATCCAGGAAACC-3′; reverse, 5′-CCTCTGGCCAGAAACCTTTAAC-3′), and the early growth response-1 promoter (16).

Quantitative Real-time PCR Analysis

HeLa cells were seeded at a density of 1.5×10^5 per well in six-well plates. Total RNA was extracted 24 h after transfection and cDNA was prepared as described (9). Relative gene expression was assayed by real-time reverse transcription-PCR (ABI Prism 7000 Sequence Detection System) using SYBR Green (Applied Biosystems) at an annealing temperature of 60°C for 15 s and elongation at 72°C for 30 s. Oligonucleotide primers specific for hPygo2 (forward, 5′-GTCCCCCACTCCATGGCCGCCTCG-3′; reverse, 5′-TCGCTTCTTTTCTGGACTCTTC-3′) and β-actin (21) were synthesized commercially (Invitrogen).

[6] http://www.matrixscience.com

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