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PAI-1 Transcriptional Regulation During the $G_0 \rightarrow G_1$ Transition in Human Epidermal Keratinocytes

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Abstract Plasminogen activator inhibitor type-1 (PAI-1) is the major negative regulator of the plasmin-dependent pericellular proteolytic cascade. PAI-1 gene expression is normally growth state regulated but frequently elevated in chronic fibroproliferative and neoplastic diseases affecting both stromal restructuring and cellular migratory activities. Kinetic modeling of cell cycle transit in synchronized human keratinocytes (HaCaT cells) indicated that PAI-1 transcription occurred early after serum stimulation of quiescent (G_0) cells and prior to entry into a cycling G_1 condition. PAI-1 repression (in G_0) was associated with upstream stimulatory factor-1 (USF-1) occupancy of two consensus E box motifs (5′-CACGTG-3′) at the PE1 and PE2 domains in the PF1 region (nucleotides −794 to −532) of the PAI-1 promoter. Chromatin immunoprecipitation (ChIP) analysis established that the PE1 and PE2 site E boxes were occupied by USF-1 in quiescent cells and by USF-2 in serum-activated, PAI-1-expressing keratinocytes. This reciprocal and growth state-dependent residence of USF family members (USF-1 vs. USF-2) at PE1/PE2 region chromatin characterized the $G_0 \rightarrow G_1$ transition period and the transcriptional status of the PAI-1 gene. A consensus E box motif was required for USF/E box interactions, as a CG → AT substitution at the two central nucleotides inhibited formation of USF/probe complexes. The 5′ flanking sites (AAT or AGAC) in the PE2 segment were not necessary for USF binding. USF recognition of the PE1/PE2 region E box sites required phosphorylation with several potential involved residues, including T153, maping to the USF-specific region (USR). A T153A substitution in USF-1 did not repress serum-induced PAI-1 expression whereas the T153D mutant was an effective suppressor. As anticipated from the ChIP results, transfection of wild-type USF-2 failed to inhibit PAI-1 induction. Collectively, these data suggest that USF family members are important regulators of PAI-1 gene control during serum-stimulated recruitment of quiescent human epithelial cells into the growth cycle. J. Cell. Biochem. 99: 495–507, 2006.

Key words: PAI-1; USF1/2; cell cycle; transcription

Mitogenic stimulation of quiescent (G_0) cells initiates a temporal program of transcriptional activity required for G_0/G_1 transit and subsequent progression through the proliferative cycle [Muller et al., 1993; Sherr, 1994; Stein et al., 1996]. A significant fraction of expressed sequences encode elements important in cell cycle control, regulation of stromal proteolysis, and extracellular matrix (ECM) remodeling suggesting a close relationship between cell growth "activation" and the tissue repair transcriptome [Iyer et al., 1999; Qi and Higgins, 2003]. One such prominent mitogen-responsive gene encodes the serine protease inhibitor (SERPIN) plasminogen activator inhibitor type-1 (PAI-1) [Ryan et al., 1996]. PAI-1 complexes with both urokinase and tissue-type plasminogen activators (u/tPA) limiting pericellular plasmin generation to maintain, thereby, a supporting "scaffold" for cell migration and/or proliferation [Bajou et al., 1998, 2001]. PAI-1 also disrupts vitronectin–uPA receptor (uPAR) interactions, detaching cells

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that utilize this receptor as a matrix anchor and inhibiting αv integrin-mediated attachment to vitronectin by blocking accessibility to the RGD motif [Kjoller et al., 1997; Loskutoff et al., 1999; Deng et al., 2001]. uPAR-associated uPA/PAI-1 complexes, moreover, are endocytosed by LDL receptor-related protein (LRP) family members potentially altering LRP and/or uPAR signaling [Ossawski and Aguirre-Ghiso, 2000; Chapman and Wei, 2001; Kjoller and Hall, 2001; Chazaud et al., 2002; Jo et al., 2003; Degryse et al., 2004]. PAI-1 appears to function, therefore, within the global program of tissue remodeling/cell growth activation to coordinate cycles of cell-to-substrate adhesion/detachment satisfying the prerequisites for both G_1/S transition and effective cellular migration [Kjoller et al., 1997; Mignatti and Rifkin, 2000; Deng et al., 2001; Chazaud et al., 2002; Palmeri et al., 2002; Providence et al., 2002; Al-Fakhri et al., 2003; Czekay et al., 2003; Providence and Higgins, 2004]. While the importance of PAI-1 as a modulator of injury resolution and cellular motile traits is clear [e.g., Bajou et al., 2001; Degryse et al., 2004; Providence and Higgins, 2004], excessive PAI-1 synthesis can have deleterious consequences on wound healing resulting in an exuberant repair response with pronounced scarring and fibrosis [Higgins et al., 1999; Tuan et al., 2003].

PAI-1 transcription is rapid and transient following addition of serum to quiescent cells [Ryan et al., 1996; Mu et al., 1998], restricted to approximately early-to-mid G_1 and declines (likely due to E2F1-mediated suppression) prior to entrance into DNA synthetic phase [Koziczak et al., 2000, 2001; White et al., 2000]. Cell cycle-associated expression controls, thus, are superimposed on this growth state-dependent program of PAI-1 gene regulation following exit from G_0 [Mu et al., 1998; Boehm et al., 1999]. While the specific signals for PAI-1 promoter activation during the $G_0 \rightarrow G_1$ transition are unknown, a consensus E box motif (nucleotides −165 to −160) in the rat PAI-1 gene is footprinted in growing cells [Johnson et al., 1992]. This site is critical for induced PAI-1 expression during exit from G_0 since the dinucleotide substitution CACGTG → TCCGTG in a CAT construct driven by 764 bp of PAI-1 promoter sequences significantly attenuated serum-stimulated reporter activity [White et al., 2000]. Similar to the TATA-proximal rat PAI-1 E box, the CACGTG sequence within the −550 to −596 bp region of the human PAI-1 promoter was originally identified as an upstream stimulatory factor-1 (USF-1)-binding site [Riccio et al., 1992]. This E box is juxtaposed to three 5′ SMAD motifs (AGAC) that reside in the PE2 segment of the PAI-1 gene (5′-CCTAGACAGACAAAACCTAGACAATCACGTGGCTGG-3′). Recent in vitro findings suggested that the PE2 E box hexanucleotide and its adjoining AGAC sites are occupied by the basic helix-loop-helix/leucine zipper (bHLH-LZ) protein TFE3 and SMAD-3, respectively, in response to transforming growth factor-β1 (TGF-β1) stimulation [Hua et al., 1999]. Prominent response elements in the human and rat PAI-1 promoters map, in fact, to specific E box motifs (in the PE1, PE2, HRE-1, HRE-2 sites) and closely related sequences recognized by the bHLH-LZ transcription factors MYC, MAX, TFE3, USF-1, and USF-2 [Hua et al., 1999; White et al., 2000; Samoylenko et al., 2001; Allen et al., 2005]. The PAI-1 PE2 region E box may function, therefore, as a platform for recruitment of positive and negative transcriptional regulators depending on the stimulus type and/or cellular growth state [e.g., Samoylenko et al., 2001; Qi and Higgins, 2003].

USF proteins are critical elements in cell cycle transit and regulate the expression of certain tumor suppressor genes [Corre and Gailbert, 2005]. Identification of USF target genes has significant implications, therefore, in understanding the molecular basis of both normal and pathologic proliferative controls. This article details occupancy of the human PAI-1 promoter PE1-PE2 subdomain E boxes by USF-1 in quiescent human keratinocytes, a growth-arrest state characterized by PAI-1 gene repression [e.g., Ryan et al., 1996; Staiano-Coico et al., 1996]. Kinetic modeling of the $G_0 \rightarrow G_1$ transition period indicates that PAI-1 expression is an early event in HaCaT cell reentry into the growth cycle and is associated with a change in USF-1/USF-2 dimer occupancy of the PE1-PE2 region E box sites. Site-directed mutagenesis of the putative MAP kinase target Thr_{153} residue in the USF-specific region (USR) confirmed the importance of USF proteins in PAI-1 gene control.

MATERIALS AND METHODS

Cell Culture and Immunocytochemistry

Human (HaCaT) keratinocytes were grown in DMEM/10% FBS and maintained in serum-free

medium for 3 days to initiate quiescence arrest [Allen et al., 2005] prior to stimulation by readdition of FBS. Cold 0.1% Triton-X100/0.08 M HCl/0.15 M NaCl buffer-permeabilized cells were incubated with acridine orange (AO; 10 μg/ml in PBS containing 1 mM EDTA, 0.15 M NaCl, 0.2 M Na_2HPO_4, 0.1 M citric acid, pH 6.0) prior to analysis by multi-parameter flow cytometry [Staiano-Coico et al., 1986, 1989]. Under these conditions, DNA:dye interactions result in green fluorescence with maximum emission of 530 nm (F_{530}) whereas AO interaction with RNA yields red metachromasia at 640 nm (F_{640}). The intensities of these reactions are proportional to cellular DNA and RNA content, respectively, and the data used to map G_0, G_1, and S phase transitions as a function of time after serum-stimulated release from quiescence. Specificity of staining was evaluated by treatment of cells with RNase A or with DNase I. Measurements utilized either a Coulter Epics Elite cell analyzer or an Epics 752 cell sorter, each equipped with a 488-nm argon ion laser (Coulter Cytometry Corp., Hialeah, FL). Red and green fluorescence signals were optically separated and debris and cell clumps eliminated by electronically gating on the peak and integrated green fluorescence signals. A minimum of 15,000 events were collected per specimen. For immunocytochemistry, keratinocytes were fixed in 10% formalin for 15 min, permeabilized in 0.5% Triton X-100 in Ca^{2+}/Mg^{2+}-free PBS, washed, blocked in 1% BSA/PBS for 20 min, then incubated with monoclonal antibodies to PAI-1 (#3785, 1:100; American Diagnostica, Greenwich, CT) and rabbit anti-USF-1 (SC-229X, 1:500; Santa Cruz Biotechnology, San Cruz, CA) followed by AlexaFluor 568-goat anti-mouse IgG and AlexaFluor 488-goat anti-rabbit IgG (both diluted 1:250).

Electrophoretic Mobility Shift Assay (EMSA)

Cells were disrupted in cold hypotonic buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM EGTA, 0.5 mM PMSF, 0.6% NP-40), nuclei collected at 15,000*g* for 1 min, lysed on ice for 30 min (in 20 mM HEPES, pH 7.9, containing 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF) and nuclear extracts clarified at 15,000*g* for 5 min. ^{32}P-end-labeled double-stranded PAI-1 promoter PE1 and PE2 E box region deoxyoligonucleotides 5′-GAGAGAGTCTGGACACGTGGGGGGAGTCAGCCGTGTATCATCGGAGG-3′ [PE1 top strand], 5′-CCAAGTCCTAGACAGACAAAACCTAGACAATCACGTGGCTGGCTGC-3′ [PE2 top strand] were incubated with 2–10 μg nuclear extract protein at room temperature for 20 min followed by an additional 30 min incubation (where indicated) with antibodies to USF-1, USF-2, MAX, or TEF3 (Santa Cruz Biotechnology) and complexes separated on 4% polyacrylamide gels in TBE buffer.

Northern Blot Analysis and Real-Time RT-PCR

Cellular RNA was denatured by incubation at 55°C for 15 min in 1× MOPS, 6.5% formaldehyde, and 50% formamide, size fractionated on 1% agarose/formaldehyde gels using 1× MOPS, transferred with 10× SSC to positively charged nylon membranes and UV crosslinked. RNA blots were hybridized simultaneously with ^{32}P-labeled human PAI-1 and GAPD cDNA probes overnight at 42°C in 50% formamide, 2.5× Denhardt's solution, 1% SDS, 100 μg/ml sheared/denatured salmon sperm DNA, 5× SSC, 10% dextran sulfate and washed three times with 0.1× SSC/0.1× SDS for 15 min each at 42°C then at 55°C prior to exposure to film. For real-time RT-PCR, total RNA was isolated with Qiagen RNeasy mini-columns (Qiagen, Valencia, CA) and first strand cDNA synthesized by addition of MMLV RNase H^+ iScript reverse transcriptase (BioRad, Hercules, CA) to a mixture of 2–10 μg RNA and oligo(dT)/random primers. The cDNA was subject to real-time PCR quantification with an iQ SYBR Green supermix (BioRad) containing hot-start iTaq DNA polymerase, optimized buffer, dNTPs and fluorescein for well-factor collection on the iCycler iQ and MyiQ real-time PCR detection systems. Raw data of C_t values from at least three independent experiments for PAI-1 gene expression, after validation by Northern blotting, were normalized against GAPDH signal using Excel software. PCR primer sets included (underlined nucleotides indicate junction of neighboring exons): 5′-GTTCTGCCCAAGTTCTCC-3′ and 5′-GAGAGGCTCTTGGTCTGA-3′ (for PAI-1) and 5′-CAAGATCATCAGCAATGC-3′ and 5′-GTGGTCATGAGTCCTTCC-3′ (for GAPDH).

Chromatin Immunoprecipitation (ChIP)

Cells were incubated with 1% formaldehyde at room temperature for 10 min, scraped in disruption buffer (50 mM Tris, pH 8.1, 1% SDS,

10 mM EDTA) and lysed on ice for 5 min. Chromatin was sonicated to an average size of 500 bp, precleared with protein A agarose for 30 min on ice, incubated overnight with antibodies to USF-1, USF-2, acetylated histone 4, or RNA polymerase II (Santa Cruz Biotechnology) or non-immune IgG, complexes collected with protein A agarose and washed sequentially with 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1 buffer containing 100 mM NaCl/0.5 M NaCl (first wash) and 0.25 M LiCl (second wash). Following incubation at 65°C for 4 h and proteinase K digestion at 45°C for 2 h, DNA was isolated with Qiagen columns for PCR amplification using ^{32}P-dCTP as tracer and primer sets described in the figure legends.

USF Expression Vectors and USF-1 Mutagenesis

The Thr_{153} residue in the pCMV-USF-1 expression construct [Gailbert et al., 2001] encoding wild-type human USF-1 was replaced with alanine (T153A) or aspartic acid (T153D) by site-directed mutagenesis using the primers 5′-GCACTGCTGGGGCAGGCGGCCCCTCCT-GGCACTGG-3′ and 5′-GCACTGCTGGGGCAG-GCGGACCCTCCTGGCACTGG-3′, respectively. The USF-2 expression vector pCMV-USF-2a [Lefrancois-Martinez et al., 1995] was the gift of Dr. Axel Kahn. HEK-293 cells were transfected for 5 h with the corresponding plasmids using Lipofectamine Plus and FBS (10%) added for overnight incubation. The next day, the medium was replaced with serum-free DMEM and 3 days later cells stimulated by addition of FBS.

RESULTS

PAI-1 Expression Is Induced Early During the Serum-Stimulated $G_0 \rightarrow G_1$ Transition

HaCaT cells were maintained at near confluency in FBS-free DMEM for 3 days to initiate entry into a quiescent (G_0) substrate. Release from growth arrest by reintroduction of serum was monitored by multi-parameter flow cytometry using AO to discriminate G_0, G_1, and S phase keratinocytes on the basis of RNA/DNA fluorescence signal and the kinetics of cell cycle transit as a function of time (Fig. 1A) superimposed on the profile of PAI-1 mRNA induction (Fig. 1B). PAI-1 transcripts were low to undetectable in quiescent cells, peaked approximately 2 h after serum addition (during residence in G_0/early G_1; i.e., activated G_0) and markedly decreased in abundance by 6–8 h post-stimulation (mid-G_1) (Fig. 1B). PAI-1 mRNA declined to approximately quiescence levels prior to synchronous entry of serum-stimulated HaCaT cells into S phase which occurs >12 h [i.e., at 15 h; Sardet et al., 1995] after release from growth arrest (Fig. 1A). Staining of low-density quiescent and 7-day serum-stimulated HaCaT cultures with crystal violet (insert in Fig. 1A) confirmed that the 3-day proliferative arrest was not irreversible and that the serum-induced recruitment of quiescent HaCaT cells into S phase reflected a complete growth recovery.

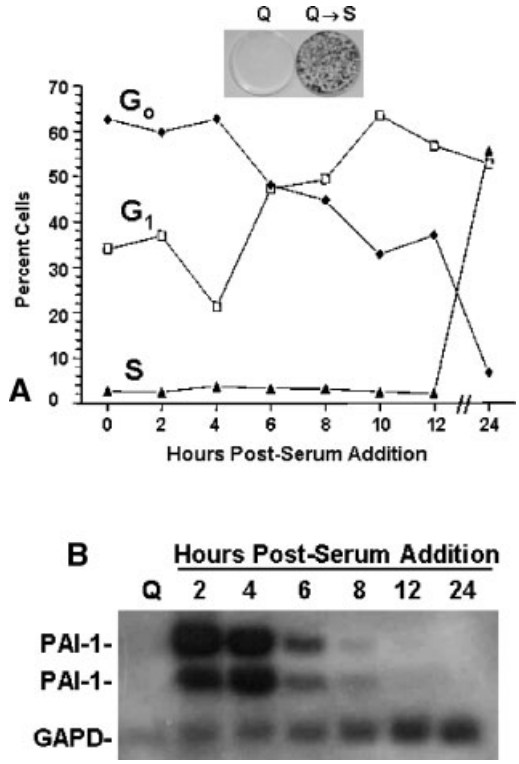

Fig. 1. Plasminogen activator inhibitor type-1 (PAI-1) mRNA transcripts are induced early after serum stimulation of quiescent HaCaT cells and prior to entry into a cycling G_1 state. G_0, G_1, and S phase transitions as a function of time after serum-stimulated release from quiescence were mapped by multi-parameter flow cytometry (RNA/DNA content) of acridine orange (AO)-stained keratinocytes (**A**). Graphed data represent the mean of six independent experiments. The standard deviation for the G_0/G_1 and S phase measurements was ≤9% and ≤18% of the mean values, respectively. PAI-1 transcripts (3.0 and 2.2 kb species) were induced early and maximally (within 2 h) after serum addition (**B**) with expression largely restricted to early $G_0 \rightarrow G_1$ phase (compare kinetic transitions (A) with northern analysis (**B**)). Crystal violet staining of low-density quiescent (Q) and 7-day serum-stimulated (Q → S) HaCaT cultures (insert in **A**) indicated that the synchronous serum-induced re-entry of quiescent HaCaT cells into the proliferative cycle (**A**) was accompanied by complete growth recovery.

USF Binds to the Proximal and Distal E Boxes in the PF1 Region of the Human PAI-1 Gene

An E box motif at nucleotides −160 to −165 upstream of the transcription start site in the rat PAI-1 gene is essential for serum-induced expression [White et al., 2000]. Two consensus E box elements (5′-CACGTG-3′), located within the PE1 and PE2 domains of the human PAI-1 gene, similarly map to the growth factor-responsive PF1 region (nucleotides −532 to −794) (Fig. 2A). The distal (i.e., PE1) E box is adjacent to the 4G/5G polymorphic site and a target for occupancy by USF-1/2 in human adipocytes

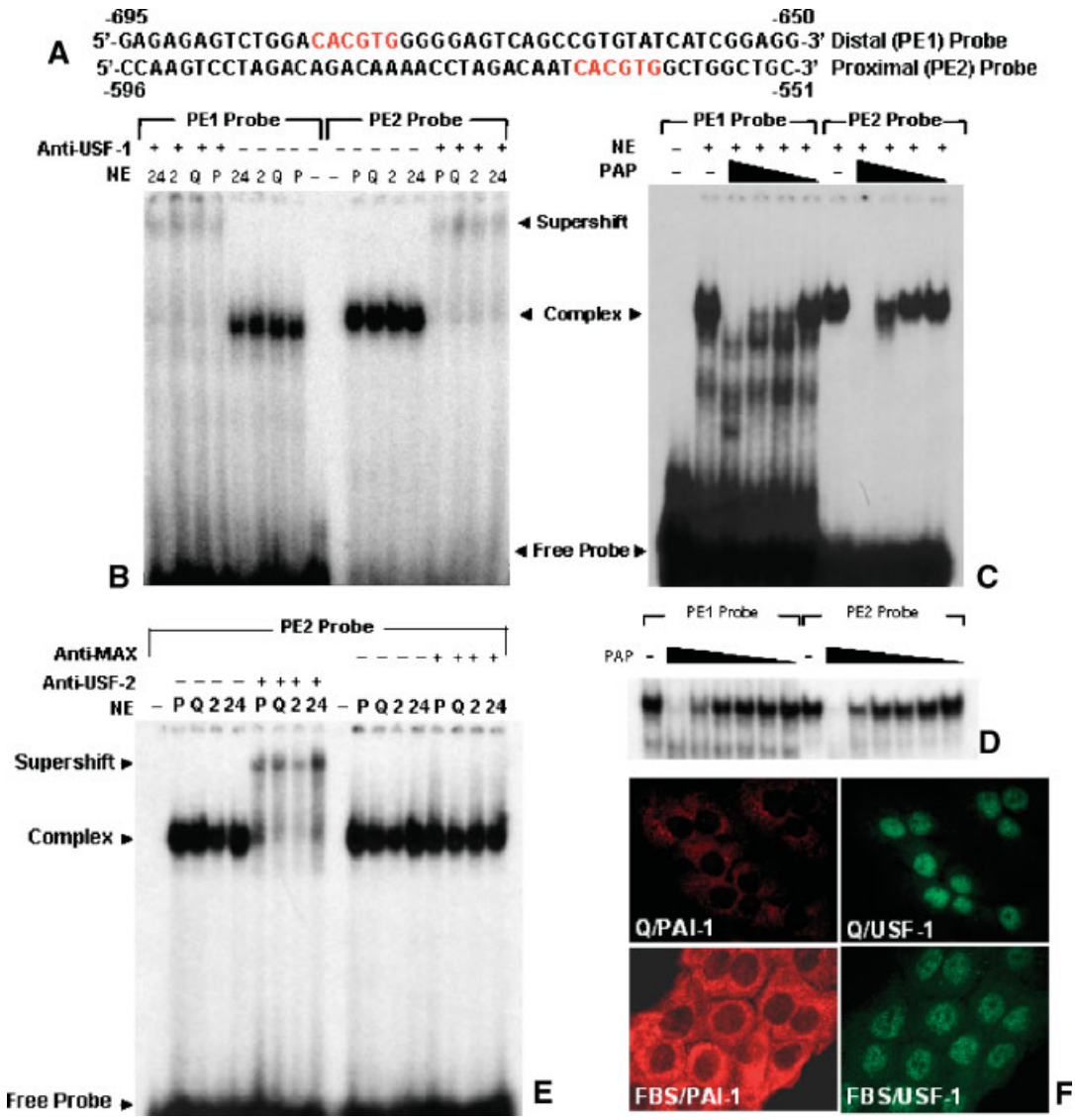

Fig. 2. Upstream stimulatory factor-1 (USF-1) binds to PE1 and PE2 region human PAI-1 E box DNA targets. Nuclear extracts (NE) from proliferating (P), quiescent (Q) and 2 as well as 24 h serum-stimulated (2,24) HaCaT cells were incubated with double-stranded ^{32}P-labeled PE1- or PE2-region constructs (**A**; only top strand shown for both probes). Antibodies (2 μg) were added (where indicted) and protein–probe complexes separated on non-denaturing polyacrylamide gels. (−) indicates absence of antibody or nuclear extract. Positions of the original protein–probe complex and the USF-1 antibody-induced supershift are indicated; it is also apparent that the USF-1 IgG effectively blocked USF-1/DNA interactions (**B**). Incubation of nuclear extracts from 2 h (**C**) and 24 h (**D**) serum-stimulated keratinocytes with increasing concentrations of potato acid phosphatase (PAP, ▬; 0.7, 0.07, 0.007, 0.0007 units/reaction (in **C**) with inclusion of two additional 10-fold serial dilutions (in **D**)) prior to probe addition progressively decreased complex formation. USF-2 also recognized the PE2 region probe as indicated by supershift of the protein/DNA complex by antibodies to USF-2. MAX antibodies, in contrast, failed to either supershift the formed complexes or block protein–DNA interactions (**E**). Nuclear USF-1 was evident in quiescent PAI-1-negative (Q) as well as FBS-stimulated PAI-1-positive HaCaT cells (**F**).

[Zietz et al., 2004]. The downstream E box, located in the PE2 region of the PF1 segment, is also a USF-1 binding element although differential occupancy by USF-1/TFE3 may depend on the specific stimulus, cell type, and/or cell cycle phase, as well as utilization of adjoining 5′ SMAD sites (AGAC) and/or the trinucleotide AAT "spacer" [Riccio et al., 1992; Hua et al., 1999; Allen et al., 2005]. Separate probes encompassing the PE1 and PE2 region E box sites were designed to assess if HaCaT nuclear factor/E box recognition activities were either cell cycle regulated and/or flanking sequence dependent (Fig. 2A). Nuclear USF-1 PE1/PE2 DNA target-binding ability was evident regardless of growth state (proliferating, quiescent, 2 or 24 h serum-stimulated cells) (Fig. 2B) and required phosphorylation since pretreatment with increasing concentrations of potato acid phosphatase (PAP) prior to probe addition effectively decreased complex formation (Fig. 2C,D). The closely related MYC family member USF-2 also recognized the PE1 (not shown) and PE2 (Fig. 2E) region probes but antibodies to other bHLH-LZ proteins with E box recognition activity including MAX (Fig. 2E) and TFE3 (not shown) failed to either produce supershifts or block complex formation. Construct binding by USF-1, as well as USF-1 nuclear localization, was independent of cell cycle stage and PAI-1 expression status (Figs. 1B and 2A,F). ChIP utilized primer sets to generate PE1 and PE2 region-specific PCR products from immunoprecipitated chromatin fragments (Fig. 3A), therefore, to evaluate USF subtype PE1/PE2 E box occupancy in vivo. USF-1 appeared to be present at both the PE1 and PE2 sites in quiescent keratinocytes (when the PAI-1 gene was transcriptionally repressed) (Fig. 3B) although motif residence by USF-1/USF-2 was clearly growth state dependent. The significantly reduced PE1/PE2 chromatin anti-USF-1 immunoreactivity at 2 h after serum stimulation contrasted with a marked increase in USF-2 binding (Fig. 3B). This reciprocal distribution of USF family member binding (USF-1 vs. USF-2) to PE1/PE2 chromatin reflects both increased binding of acetylated histone 4 to the initiation site of the PAI-1 promoter (Fig. 3C) and transcriptional activation of the PAI-1 gene (Fig. 1B).

Sequence Requirements for USF Occupancy of the PE2 Region E Box Motif

Since the PAI-1 gene is an in vivo USF target (Fig. 3B) and an intact consensus PE2 region E box motif is necessary for a maximal transcriptional response to growth factors [Allen et al., 2005], it was important to identify any additional sequence requirements for PE2 E box occupancy that might influence site residence. PE1 and PE2 probe recognition appeared dependent solely on an intact 5′-CACGTG-3′ motif since nuclear factor binding to individual PE1- and PE2-labeled probes (Fig. 4A) was successfully competed (regardless of growth state) by an unlabeled construct containing a consensus E box flanked by non-PAI-1 sequences (standard consensus 23-bp) whereas a mutant E box (5′-CAATTG-3′) "bait" failed to compete (Fig. 4B–E). Indeed, unlabeled CACGTG hexanucleotide-containing DNAs, regardless of the presence or absence of PAI-1-specific 5′ and 3′ flanking sequences, significantly decreased complex formation between labeled probe and HaCaT nuclear factors. It was important, however, to confirm these results using site-specific mutants within the context of native PAI-1 promoter sequences (e.g., the PE2 region backbone). A double-stranded 45-mer PE2 region deoxyoligonucleotide target (Fig. 5A) was ^{32}P-end-labeled and used in competitive mobility shift assays to assess the potential contributions of the SMAD-binding elements (SBEs), E box flanking nucleotides, the AAT trinucleotide spacer and the CACGTG motif to nuclear protein binding. Double-stranded PAI-1 PE2 E box deoxyoligonucleotides with all three SBEs mutated (AGAC → CTTG) or lacking the AAT spacer (Fig. 5A) successfully competed for protein binding with the labeled 45-bp PE2 DNA target (Fig. 5B), further minimizing the potential contribution of these PE2 region sequences to site occupancy. Most importantly, a PE2 deoxyoligonucleotide that differed from the consensus E box by a central CG → AT substitution (identical to the base change incorporated into the PAI-1 p806-Luc reporter that reduced growth factor-dependent expression [Allen et al., 2005] and to the dinucleotide replacement in the non-competing 23-bp non-PAI-1 flanking sequence target (Fig. 4B–E)) failed to compete with the PE2 region probe for factor binding while the same construct with an intact CACGTG motif was an effective competitor (Fig. 5B). The major protein/DNA interactions in the PE2 segment, therefore, appear to be E box dependent.

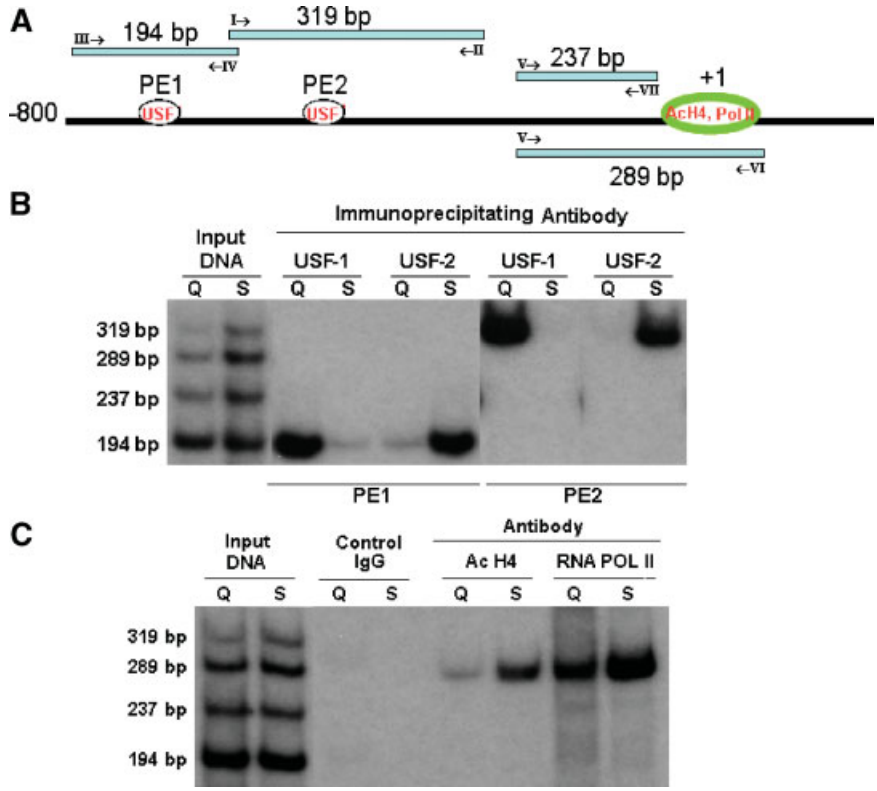

Fig. 3. USF-1 and USF-2 are differentially bound to the human PAI-1 promoter PE1 and PE2 E box sites in quiescent as compared to serum-stimulated keratinocytes. Chromatin was isolated from quiescent (Q) or 2 h FBS-stimulated (S) HaCaT cells. DNA fragments were precipitated with antibodies to USF-1, USF-2, RNA polymerase II or acetylated histone 4; reactions using non-immune IgG served as controls. PCR products (body-labeled using ^{32}P-dCTP) were amplified with primers (**A**) spanning the PE1 (194 bp) or PE2 (319 bp) regions, the transcription initiation site (298 bp) or a control sequence (237 bp) which has neither transcription initiation or TATAAA element sequences. Primer sets were as follows: 319 bp: PE2 E box, primers: (I) 5′-GGGAAAGACCAAGAGTCC-3′ and (II) 5′-ACTGTCTGC-CATGCCGGG-3′ 194 bp: PE1 E box, primers: (III) 5′-CTGGTCCCGTTCAGCCACC-3′ and (IV) 5′-ACTTGGGCCCAA-CAGAGG-3′ 289 bp: transcription initiation site primers: (V) 5′-CAGAAAGGTCAAGGGAGG-3′ and (VI) 5′-CCTGCAGC-CAAACACAGC-3′ 237 bp: specificity control primers: (V) 5′-CAGAAAGGTCAAGGGAGG-3′ and (VII) 5′-ATACCA-GATGTGGGCAGG-3′. Chromatin immunoprecipitation (ChIP) analysis indicated reciprocal occupancy of both PE1 and PE2 region chromation with USF-1 and USF-2 in quiescent (Q) versus serum-stimulated (S) cells (**B**). Input DNA refers to the PCR-generated ladder produced following addition of all seven primer sets to DNA isolated from quiescent or stimulated HaCaT cells. Primers for the PE1 and PE2 sites generate the 194 and 319 bp products, respectively (**B**). Substitution of control non-immune IgG and use of antibodies to acetylated histone 4 (AcH4) and RNA polymerase II (Pol II) confirmed that the 194 and 319 bp ChIP PCR products were specific to the immunoprecipitates developed with USF-1/2 antibodies and associated with chromatin remodeling events consistent with PAI-1 transcriptional activation (**C**). Differential amplification of the 289 bp as compared to the 237 bp fragment using precipitating antibodies to RNA polymerase II clearly indicates polymerase occupancy of the TATAAA element (nucleotides −28 to −22 in the human PAI-1 gene). Three independent ChIP analyses were performed with identical results.

Mutation of the Thr$_{153}$ in USF-1 Regulates Serum-Induced PAI-1 Expression

The ability of USF-1/2 to bind both the PE1 and PE2 region PAI-1 E box probes in vitro was growth state independent and (at least for USF-1) required phosphorylation (Fig. 2B–D). A similar phosphorylation dependency for USF-2 recognition of a PE2 construct was confirmed by deoxyoligonucleotide pull-down assay (not shown). It appears that nuclear phospho-USF-1/2 proteins are ubiquitous throughout the cell cycle and capable of target probe recognition although isoform residence on PE1/PE2 region chromatin in vivo is dynamic (Fig. 3B). MAP kinases, under certain circumstances, can use USF proteins as substrates [Gailbert et al., 2001] since targeting the ERK pathway with a dominant-negative MEK construct or the pharmacologic inhibitor PD98059 prevented p21^{ras}-induction of the USF-activated HOXB4 promoter [Giannola et al., 2000]. ERK-type kinases

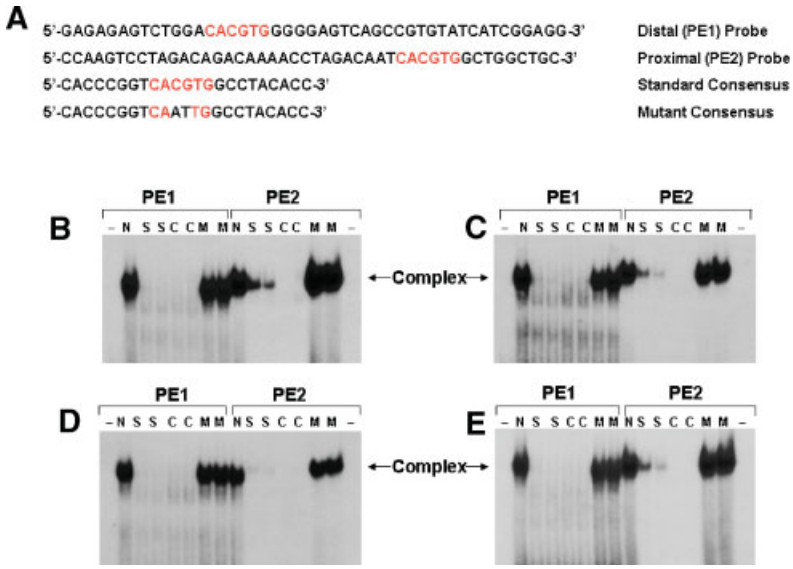

Fig. 4. Nuclear protein(s) capable of binding to both the PE1 and PE2 human PAI-1 promoter E box probes are present regardless of growth state and require a central CG dinucleotide motif for site occupancy. Electrophoretic mobility shift assay (EMSA) utilized end-labeled double-stranded PE1 and PE2 region probes (**A**; only top strand indicated for each) and HaCaT cell nuclear extracts from exponentially growing (**B**), 3-day serum-starved (**C**), or 2 (**D**) and 24 (**E**) h FBS-stimulated cultures. In (**B–E**), complexes developed with the PE1 and PE2 E box constructs (**A**) are indicated. Competing DNA was used in 100- and 500-fold molar excess (duplicate reactions shown). –, no nuclear extract; N, nuclear extract plus ^{32}P-labeled PE1 or PE2 probe without competitor. Extracts were also pre-incubated with the following DNAs prior to probe addition; S, unlabeled self (PE1 or PE2) competitor; C, unlabeled standard consensus E box deoxyoligonucleotide with non-PAI-1 flanking sequences (5′-CACCCGGTCACGTGGCCTACACC-3′); M, unlabeled E box mutant (CG → AT) deoxyoligonucleotide (5′-CACCCGGTCAATTGGCCTACACC-3) with non-PAI-1 flanking sequences.

may phosphorylate USF at residues required for DNA motif occupancy and/or modulate USF-1-dependent PAI-1 repression in quiescent cells. Several potential MAP kinase phosphorylation residues, including Thr_{153}, map to the USR of USF-1. Mutation of Thr_{153} to alanine (T153A) in the USR of USF-1 inhibited phosphorylation by specific MAP kinases (e.g., p38α) or the mixed lineage kinase MLK, suggesting that the Thr_{153} residue is, in fact, a phosphorylation target [Gailbert et al., 2001]. This mutation, as well as the aspartic acid (phospho-miminic) substitution T153D, was incorporated into the full-length USF-1 expression constructs for transfection into HEK-293 cells. The T153D mutant effectively suppressed serum-induced PAI-1 expression (by both Northern blotting as well as real-time RT-PCR analyses), whereas the T153A mutation was unable to repress PAI-1 gene activation in response to serum stimulation (Fig. 6). Transfection of a wild-type USF-2 expression vector did not alter the magnitude of serum-induced PAI-1 expression (as expected from the ChIP data) since transcription is already likely maximal 2 h after FBS addition.

DISCUSSION

While the CACGTG "core" is a target for occupancy by at least seven members of the bHLH-LZ transcription factor family (USF-1, USF-2, c-MYC, MAX, TFE3, TFEB, TFII-I), USF proteins have a preference for C or T at the −4 position in the presence of $MgCl_2$ [Bendall and Molloy, 1994]. Indeed, the human PAI-1 gene has a T at the −4 site of the PE2 region E box as well as a purine at +4 and −5 and a pyrimidine at +5 ($A_{-5}T_{-4}C_{-3}A_{-2}C_{-1}G_{+1}T_{+2}G_{+2}G_{+3}G_{+4}C_{+5}$), all of which facilitate USF binding [Bendall and Molloy, 1994]. ChIP assessment of the E box site in the PE2 region of the human PAI-1 gene, moreover, indicated a dynamic occupancy by USF subtypes (USF-1 vs. USF-2) as a function of growth state. This motif was clearly a platform for USF-1 binding in quiescent cells consistent with E box target probe analysis by EMSA. Early after serum-induced commitment to G_1 entry, however, PE1/PE2 region chromatin exhibited significantly diminished USF-1 immunoreactivity while fragment analysis using antibodies to acetylated histone 4 confirmed that the PAI-1 promoter underwent

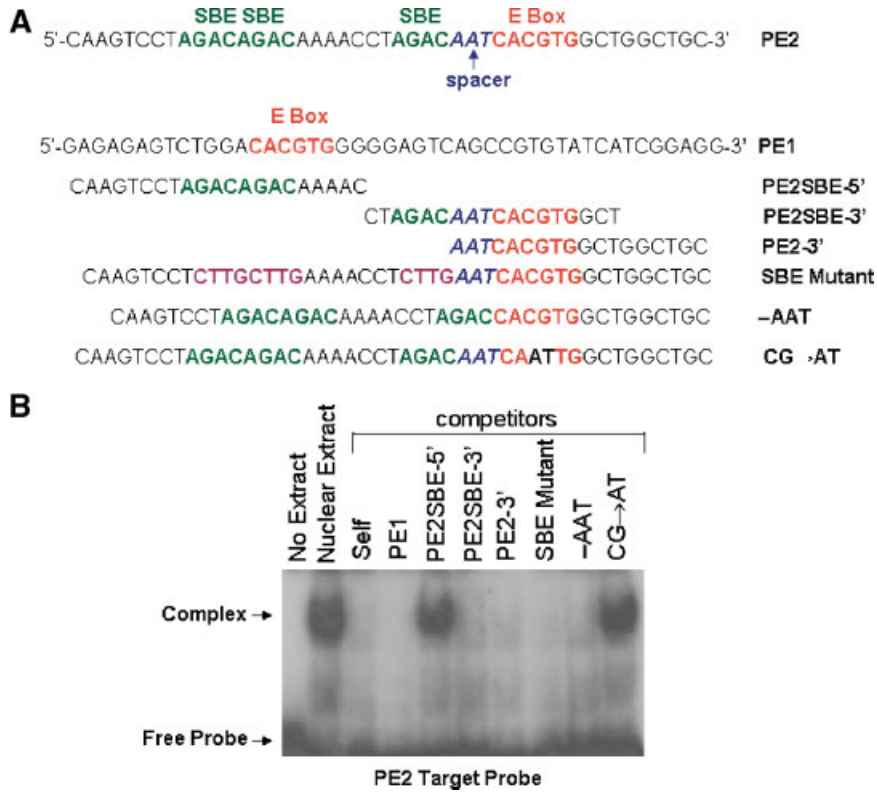

Fig. 5. PE2 region sequence requirements for probe binding. PE2 PAI-1 promoter sequence illustrating the position of the three SMAD-binding elements (SBE), the trinucleotide (AAT) spacer, and the E box motif; specific truncated and mutated sequences are highlighted (**A**). Nuclear extracts were incubated with a double-stranded ^{32}P-labeled PE2 DNA (**A**; only top strand indicated) in the presence or absence of a 100-fold molar excess of the indicated unlabeled competing DNA constructs and reaction products separated on non-denaturing 5% acrylamide gels (**B**).

remodeling events typical of transcriptional activation. An exchange of PE2 E box USF-1 homodimers with USF-2 homo- or USF-1/USF-2 heterodimers, moreover, closely correlated with PAI-1 gene activation. This switch may well determine the transcriptional status of the PAI-1 gene in quiescent versus cycling cells [Ghosh et al., 1997; Qi and Higgins, 2003]. Dimer replacement at the critical PE2 E box motif and induced PAI-1 expression occurs early after cellular "activation" (i.e., prior to the kinetically defined $G_0 \rightarrow G_1$ transition) and appears to be followed (in mid-to-late G_1) by binding of the PAI-1 repressor E2F1 to an adjoining 3′ GC-rich region in the PAI-1 promoter [Koziczak et al., 2000, 2001]. Such cell cycle-dependent changes in the ChIP profile, as well as the necessity for intact E box sites in induced PAI-1 expression [Hua et al., 1999; White et al., 2000], suggest that the PE2 site E box may have multiple functions depending on cellular growth state. Site occupancy and transcriptional activity, furthermore, require conservation of the PE2 core E box structure as the CACGTG → CACACGGA and TCCGTG dinucleotide substitutions (in the rat gene) [White et al., 2000] and a CACGTG → CAATTG or TCCGTG replacement (in the human gene), with retention of PAI-1 flanking sequences, resulted in loss of both competitive binding and growth factor-dependent reporter activity [Allen et al., 2005]. The CACGTG → TCCGTG mutation is particularly relevant since bHLH proteins with E box recognition activity have a conserved glutamate important for interaction with the first two nucleotides (CA) in the E box motif [Fisher and Goding, 1992]. These data are also consistent with the known hexanucleotide preference (CACGTG or CACATG) of USF proteins [Littlewood and Evan, 1995; Ismail et al., 1999; Samoylenko et al., 2001] and additionally suggest that USF family members with PAI-1 PE2 site E box occupancy potential (i.e., USF-1) are constitutively present (and active) regardless of

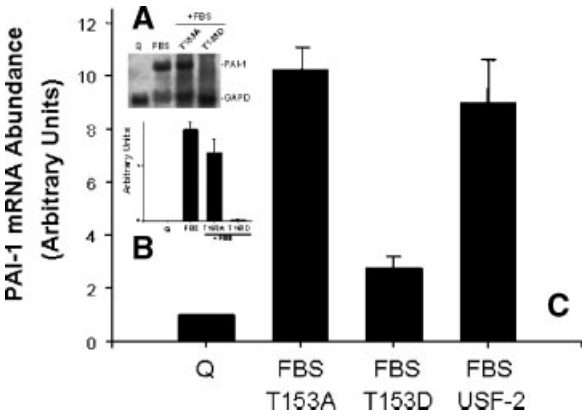

Fig. 6. Residue Thr_{153} regulates USF-1 transcriptional activity. HEK293 cells were transfected with expression vectors encoding the T153A or T153D USF-1 mutants as well as with a WT USF-2 construct. Cells were serum-deprived then allowed to remain quiescent (Q) or FBS stimulated. Similar to human keratinocytes (e.g., Fig. 1B), addition of serum to quiescent HEK293 cells (Q) resulted in a rapid induction of PAI-1 mRNA transcripts as determined by Northern blotting (**A**, **B**) or quantitative RT-PCR (**C**). Panel (**A**) is an example of the northern analyses from which the panel (**B**) densitometry was derived. Cultures transfected with the T153D USF-1 mutant (but not the T153A construct) effectively suppressed PAI-1 expression in response to serum. Data plotted (B) indicate PAI-1 mRNA abundance in arbitrary densitometric units (mean ± SD of triplicate experiments). Effects of the T153A and T153D mutants on serum-induced PAI-1 mRNA expression were confirmed by three independent RT-PCR assessments (**C**). Transfection of the wild-type USF-2 expression vector, as expected, did not decrease PAI-1 transcripts in serum-stimulated cultures.

cellular growth state. Expression control, therefore, is distinct from simple motif binding ability. Successful PAI-1 probe competition by a CACGTG "core" flanked by non-PAI-1 sequences (but with retention of T at −4 and a purine at +4) and the failure of specific E box mutants to similarly compete (or to produce band shifts when used as targets) further indicate that a consensus hexanucleotide E box at the PE2 site in the PAI-1 gene is both necessary and sufficient for USF binding. This contrasts with the highly cooperative constraints for E box recognition by other bHLH-LZ proteins (e.g., TFE3, MAX) that utilize accessory factors (e.g., SMADs) and their respective recognition sequences for optimal motif residence on the PAI-1 promoter [Hua et al., 1999; Grinberg and Kerppola, 2003]. Depending on the relative abundance of E box-binding factors in individual cell types (e.g., USF vs. TFE3), the promoter context and specific flanking nucleotides [e.g., Szentirmay et al., 2003], therefore, proteins that dock at adjoining sites may also be required.

USF-1 and TFE3 are phosphorylated at consensus MAP kinase target residues [Gailbert et al., 2001; Weibaecher et al., 2001] initiating a conformational switch that exposes the DNA-binding domain [Cheung et al., 1999]. Other growth-related kinases may also use USF-1, and the related factors MYC and MAX, as a substrate [Cheung et al., 1999; Lee et al., 2002]. Indeed, although p38 appears to phosphorylate USF-1 with subsequent modulation of its transcriptional ability [Gailbert et al., 2001], the significant reduction in growth factor-induced PAI-1 transcripts by pre-treatment with the MEK inhibitor PD98059 suggests that USF proteins may also be targets of activated ERKs [Kutz et al., 2001]. While targeting mechanisms vary, accessory factors such as JLP (c-Jun NH_2-terminal kinase-associated leucine zipper protein) tether JNK and p38 within a multi-kinase complex with MYC and MAX to activate specific signaling pathways [Lee et al., 2002]. Basally phosphorylated, transcriptionally suppressive, USF-1 may occupy the PAI-1 E box in quiescent cells. The important target residues are unknown but the present findings suggest that Thr_{153} may be a likely candidate. USF-1 is, in fact, a relatively weak *trans*-activator and USF-1 homodimer-dependent gene repression may be similar to that of MAX homodimers [Carter et al., 1997]. DNA-anchored USF-1 could also complex with translocated MAP kinases (via kinase docking sites located within or closely juxtaposed to the USR) [e.g., Gailbert et al., 2001] resulting in the hyper-phosphorylation of USF-1 (at secondary residues) potentially signaling release of E box-resident USF-1 prior to G_1 entry. Dephosphorylation of USF-1 upon serum stimulation, followed by loss of motif occupancy and subsequent transcriptional activation, cannot be ruled out although the ability of USF-1 to bind probe targets appears equivalent throughout the growth cycle. USF-1 activity may be further modified by either a recruited co-activator [Qyang et al., 1999; Xing et al., 2002] (e.g., USF-2) or direct replacement of USF-1 with USF-2 homodimers. In an analogous mechanism, the HPV-16 oncoprotein E6 activates telomerase reverse transcriptase (TERT) transcription by c-MYC induction and release of USF-dependent repression at the −34 to −29 E box site [McMurry and McCance, 2003]. These

findings are consistent with emerging concepts that USF-1 transcriptional effects are context dependent [Luo and Sawadogo, 1996; Carter et al., 1997; Qyang et al., 1999] and that USF-1 may function as a "basal repressor" of PAI-1 (or TERT) expression occupying E box sites to inhibit access of strong transcriptional activators that recognize the CACGTG motif (i.e., MYC, USF-2).

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