THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 286, NO. 20, pp. 17796–17808, May 20, 2011

Repression of Androgen Receptor Activity by HEYL, a Third Member of the Hairy/Enhancer-of-split-related Family of Notch Effectors*[S]

Received for publication, November 8, 2010, and in revised form, March 16, 2011 Published, JBC Papers in Press, March 17, 2011, DOI 10.1074/jbc.M110.198655

Derek N. Lavery[‡], M. Angeles Villaronga[§], Marjorie M. Walker[¶], Anup Patel[‖], Borja Belandia[§], and Charlotte L. Bevan[‡1]

From the [‡]Androgen Signalling Laboratory, Department of Surgery and Cancer, Imperial College London, London W12 0NN, United Kingdom, [¶]Centre for Pathology, Division of Medicine, Imperial College London, London W2 1NY, United Kingdom, [§]Departamento de Biología del Cáncer, Instituto de Investigaciones Biomédicas Alberto Sols, Consejo Superior de Investigaciones Científicas-Universidad Autónoma de Madrid, 28029 Madrid, Spain, and [‖]Department of Urology, St. Mary's Hospital at Imperial College Healthcare Trust, London W2 1NY, United Kingdom

The Hairy/Enhancer-of-split-related with YRPW-like motif (HEY) family of proteins are transcriptional repressors and downstream effectors of Notch signaling. We previously reported that HEY1 and HEY2 selectively repress androgen receptor (AR) signaling in mammalian cell lines and have shown that in human tissue HEY1 is excluded from the nuclei in prostate cancer but not benign prostatic hyperplasia. We have now characterized a third member of this family, HEYL, which is a more potent repressor of AR activity. HEYL interacted with and repressed AR activation function-1 domain and competitively inhibited SRC1e activation of AR transcriptional activity. Using a cell line inducibly expressing exogenous HEYL, we showed that HEYL represses endogenous AR-regulated genes and reduces androgen-dependent prostate cancer cell growth. Using a *trans*-repression assay, we identified both trichostatin-sensitive and -insensitive domains within HEYL; however, analysis of endogenous AR target genes suggested that HEYL represses AR activity through histone deacetylase I/II-independent mechanisms. Immunohistochemical analyses of tissue indicated that, in a fashion similar to that previously reported for HEY1, HEYL is excluded from the nuclei in prostate cancer but not adjacent benign tissue. This suggests that nuclear exclusion of HEY proteins may be an important step in the progression of prostate cancer.

Cancer of the prostate is the second most common cancer among men and fifth most common cancer in the world; it was estimated that 900,000 new cases would be diagnosed in 2010 (1). In the early stages, tumor growth is dependent upon androgens. Under both normal and disease conditions, the androgen receptor (AR),[2] a member of the nuclear receptor superfamily of transcription factors, mediates the effects of androgens, including testosterone and the more potent dihydrotestosterone (2). In response to dihydrotestosterone, the AR controls growth, maintenance, and differentiation of the prostate by regulating transcription of a variety of downstream genes. Treatment for non-organ-confined prostate cancer usually involves administration of luteinizing hormone-releasing hormone analogs, which abrogate the production of testicular androgens, and/or antiandrogens, which bind to and block the activity of the AR. In the majority of cases, patients initially respond well to such treatment, but after a median of 2 years, tumors progress toward the more aggressive "androgen-independent" stage of the disease (3). The postulated molecular mechanisms that drive the transition to androgen independence include mutation and amplification of the receptor, altered levels/distribution of coregulator proteins, and growth factor-activated pathways (2–4). At this stage of the disease, patients have a median survival of 12–18 months; thus, it is important to better understand the mechanisms that regulate AR activity and drive prostate cancer progression.

AR signaling is regulated at various stages and through multiple mechanisms, including ligand binding, receptor stability, N/C-terminal interactions, post-translational modifications, DNA binding, and recruitment of coregulators that directly modulate the transcriptional activity of the AR (2, 5–8). Coactivators, including the p160/SRC family and p300/CBP, have been shown to be recruited to the promoter and enhancer regions of AR-regulated genes, forming a transcriptional complex and potentiating AR activity largely via acetylation of histones and relaxation of chromatin structure (9, 10). Recently, a variety of AR corepressors, including Cyclin D1 (11, 12), silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) (13, 14), nuclear receptor corepressor (NCoR) (15),

* This work was supported by Cancer Research UK Grants C11509/A8570 and C11509/A12426 and Ministerio de Ciencia e Innovación Grants SAF2007-62642 and SAF2010-21013.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3.

[1] To whom correspondence should be addressed: Androgen Signalling Laboratory, Inst. of Reproductive and Developmental Biology, Imperial College London, Hammersmith Hospital, Du Cane Rd., London W12 0NN, UK. Tel.: 44-207-5941685; Fax: 44-208-3835830; E-mail: charlotte.bevan@imperial.ac.uk.

[2] The abbreviations used are: AR, androgen receptor; AF, activation function; bHLH, basic helix-loop-helix; CBP, cAMP-response element-binding protein (CREB)-binding protein; DRG1, developmentally regulated GTP binding protein 1; HDAC, histone deacetylase; HEY, Hairy/Enhancer-of-split-related with YRPW-like motif; KLK2, kallikrein-related peptidase 2; LBD, ligand-binding domain; NES, nuclear export sequence; NTD, N-terminal domain; NLS, nuclear localization sequence; PAS, per-arnt-sim; PSA, prostate-specific antigen; SRC, steroid receptor coactivator; TAU, transactivation unit; TSA, trichostatin A; qRT-PCR, quantitative RT-PCR; RPMI, Roswell Park Memorial Institute 1640; DBD, DNA-binding domain; NCoR, nuclear receptor corepressor.

death domain-associated protein (Daxx) (16), Amino-terminal Enhancer of split (AES) (17), and Prohibitin (18–20), have been characterized. Corepressors may act in a variety of ways to inhibit AR activity, including blocking the N/C termini interaction, sequestering the AR, competing with coactivators, inhibition of DNA binding, and/or recruitment of histone deacetylases (HDACs) (21).

HEY proteins (also known as Hairy-related transcription factor (HRT), cardiovascular helix-loop-helix factor (CHF), and Hairy/Enhancer-of-split-related repressor protein (HERP)) are downstream targets or effectors of the Notch pathway and "Class C" basic helix-loop-helix (bHLH) transcriptional repressors (22). The Notch pathway is a juxtacrine signaling pathway that regulates cell fate, stem cell renewal, and cellular differentiation. Furthermore, mutations in or deregulation of the Notch pathway have been implicated in a variety of cancers, including T-acute lymphoblastic leukemia, breast cancer, ovarian cancer, and prostate cancer (23, 24). There are three related HEY proteins, HEY1, HEY2, and HEYL. We previously identified and characterized HEY1 as an AR-selective corepressor that contains both HDAC-dependent and -independent repression domains (25). Patterns of expression in human prostate samples suggested that nuclear exclusion of HEY1 may be an important step in cancer formation and/or progression (25, 26). We now report that HEYL is a more potent AR corepressor, repressing AR activity in a variety of promoter and cell contexts, including prostate cell lines. We describe the relevant functional domains of HEYL and its expression in prostate tumor tissue, which suggests that HEYL, in a fashion similar to HEY1, is excluded from the nuclei of malignant prostate cells but not in normal/adjacent tissue. This mislocalization of HEY proteins appears to be a characteristic of prostate cancer and may be involved in progression of the disease.

EXPERIMENTAL PROCEDURES

Plasmids—The plasmids pSG5-FLAG-HEY1 (25), pSG5-FLAG-HEY2 (25), GST-NTD (25), GST-LBD (25), GST-HEY1 (25), pVP16-LEXA (27), pSVARo (28), Probasin-LUC (7), pSG5-ARΔLBD (29), pSG5-SRC1e (30), $LexA_{(7)}$-$Gal4_{(4)}$-LUC (25), TAT-GRE-E1B-LUC (29), ARΔ*τ*1 (also known as AR123) (31), and ARΔ*τ*5 (also known as AR131) (31) have been described previously. HEYL was amplified from MCF7 cDNA with primers (forward, 5′-GCA GCC TGC GGA ATT CAT GAA GCG A-3′; and reverse, 5′-GAA GGG GAT CCT CAG AAA GCC CC-3′), digested with EcoRI and BamHI, and cloned into pSG5 to create pSG5-HEYL. For localization studies, HEYL was amplified with primers (forward, 5′-CCT GCG AGA TCT ATG AAG CGA CCC-3′; and reverse, 5′-CGA AGG GTC GAC TCA GAA AGC C-3′), digested with BglII and SalI, and cloned into pEGFPC1 to create pEGFP-HEYL. Putative HEYL nuclear localization and export sequences (amino acids 36–59 and 125–160, respectively) were amplified with primers (HEYL NLS forward, 5′-GGG AGA TCT CCC AGC TCT TCG-3′; HEYL NLS reverse, 5′-CCC GGA TCC TCA ACT GTT GAT GCG-3′; HEYL NES forward, 5′-GGG AGA TCT CTC ACT GAG GTC ATC AGG-3′; and HEYL NES reverse, 5′-CCC GGA TCC TCA CTC CAT CTC GGC TGC-3′), digested with BglII and BamHI, and cloned into pEGFPC1 to create pEGFP-NLS and pEGFP-NES, respectively. Two subsequent rounds of site-directed mutagenesis (QuikChange, Stratagene) were used to create pEGFP-HEYLmutNLS using the primers Forward-mut1 (5′-CAG ATG CAA GCC AGG GCG GCA CGC AGA GGG ATC ATA GAG AAA CGG-3′), Reveresemut1 (5′-CCG TTT CTC TAT GAT CCC TCT GCG TGC CGC CCT GGC TTG CAT CTG-3′), forwardmut2 (5′-CAG ATG CAA GCC GCG GCG GCA GCC GCA GGG ATC ATA GAG AAA CGG-3′), and reversemut2 (5′-CCG TTT CTC TAT GAT CCC TGC GGC TGC CGC CGC GGC TTG CAT CTG). For the creation of LNCaP:HEYL stable cell lines, HEYL was amplified from pSG5-HEYL with primers (forward, 5′-GGG AAG CTT GAC ATG GAC GAC TAC AAG GAC GAC GAT GAC AAG GAC TAC AAG GAC GAC GAT GAC AAG ATG AAG CGA GCT CAC CCC GAG TAC AGC-3′; and reverse, 5′-CCC CTC GAG TCA GAA AGC CCC GAT TTC AGT GAT TTC-3′) that amplify the entire HEYL cDNA with the addition of 2xFLAG (DYKDDDDK) 5′ to the ATG start site. The purified PCR product was digested with HindIII and XhoI and cloned into these sites in pCDNA4TO (Invitrogen) to yield pCDNA4TO-FLAG-HEYL. FLAG-tagged HEY1 was cloned into pCDNA4TO as above with the following primers: forward, 5′-GGG AAG CTT GAC ATG GAC GAC TAC AAG GAC GAC GAT GAC AAG GAC TAC AAG GAC GAC GAT GAC AAG ATG AAG CGA GCT CAC CCC GAG TAC AGC-3′; and reverse, 5′-CCC CTC GAG TCA GAA AGC CTC GAT TTC AGT GAT TTC. To create Gal4-DBD-tagged HEY1, HEY1 was digested with EcoRI and BamHI and subcloned into pM-DNA-BD (Clontech) to create pM-HEY1. pM-HEYL was created by amplifying full-length *HEYL* from pEGFP-HEYL with the following primers: forwardF, 5′-GGG GAA TTC ATG AAG CGA CCC AAG G-3′; and reverseF, 5′-CCC GGA TCC TCA GAA AGC CCC G-3′. To create a pM construct containing HEYL domains, amino acids 1–110 were amplified using the "forwardF" primer from above and the following reverse primer: reverseN, 5′-CCC GGA TCC TCA TCG GGC ATC AAA GAA TCC-3′. Amino acids 110–165 were amplified with the following primers: forwardM, 5′-GGG GAA TTC CGA GCC CTG GCA GTT GAC TTC C-3′; and reverseM, 5′-CCC GGA TCC TCA GGG CGT GGG CGA AGG C-3′. Amino acids 165–328 were amplified using forwardC (5′-GGG GAA TTC TCC TCC CCAGGG CC-3′) and reverseF (described above). Amplified inserts were digested with EcoRI and BamHI and ligated into pM-DNA-BD. To create pCDNA3.1 domain constructs, amino acids 110–165 were amplified as above. Amino acids 110–165 were amplified with using forwardM2 (5′-GGG GGA TCC ATG CGA GCC CTG GCA GTT GAC TTC C-3′) and reverseM above. Amino acids 165–328 were amplified using forwardC2 (5′-GGGGGA TCC ATG ACG CCC ACT GGC CCT TTG G-3′) and reverseF above. All plasmids were confirmed by diagnostic digests and sequencing.

Antibodies and Reagents—For immunoblotting, primary antibodies were mouse anti-FLAG-M2 (1:5000; Sigma), mouse anti-AR-441 (1:2000; Dako), rabbit anti-GFP (1:5000; Abcam), mouse anti-HEYL (1:1000; Abnova), and mouse anti-*β*-actin (1:5000; Abcam). Secondary antibodies (goat anti-mouse HRP and goat anti-rabbit HRP) were from Dako and were used at a concentration of 1:2000. For immunohistochemistry, the pri-

mary antibodies used were mouse anti-FLAG-M2 (1:500; Sigma), mouse anti-HEYL (1:200), and rabbit anti-AR-N15 (1:500). The Alexa Fluor secondary antibodies used were goat anti-mouse (Alexa Fluor 488) and goat anti-rabbit (Alexa Fluor 595). The synthetic androgen mibolerone (PerkinElmer Life Sciences) was prepared in 100% ethanol at a stock concentration of 1 M. A working concentration of 10 μM was prepared, and mibolerone was added to cells at a final concentration of 10 nM. The HDAC inhibitors used were trichostatin A (Class I/II), valproic acid (Class I/IIa), and sodium butyrate (Class I/II) and were from Sigma. Trichostatin A was prepared in ethanol and used at a final concentration of 100 nM. The remaining inhibitors were prepared in distilled H_2O and used at a final concentration of 5 mM.

Chromatin Immunoprecipitation Assays—LNCaP cells were grown in 15-cm dishes in the presence or absence of 1 μM doxycycline for 48 h. Cells were subsequently washed, and medium was replaced with "starvation" medium (phenol red-free medium, 5% charcoal-stripped FCS) for a further 72 h in the absence or presence of doxycycline. Androgen signaling was activated by addition of 10 nM mibolerone for 2 h after which cells were fixed in 1% formaldehyde (Sigma) for 10 min at room temperature and washed three times with PBS. Cells were collected and lysed in Lysis Buffer (50 mM HEPES, pH 7.4, 1 mM EDTA, 0.5 mM EGTA, 140 mM NaCl, 10% (v/v) glycerol, 0.5% (v/v) Nonidet P-40, 0.25% (v/v) Triton-X100) containing 0.2 mM PMSF and protease inhibitors for 10 min at 4 °C. Nuclear fractions were collected by centrifugation and washed further in Wash Buffer (10 mM Tris, pH 8, 1 mM EDTA, 0.5 mM EGTA, 200 mM NaCl) containing 0.2 mM PMSF and protease inhibitors for 10 min at 4 °C. The centrifugation step was repeated, and the supernatant was discarded. The pellet was suspended in radioimmune precipitation assay buffer (10 mM Tris, pH 8, 1 mM EDTA, 0.5 mM EGTA, 140 mM NaCl, 5% (v/v) glycerol, 0.1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1% (v/v) Triton-X100) containing 0.2 mM PMSF and protease inhibitors equivalent to 200 μl/1 $\times$ 10^6 cells and sonicated to shear DNA to between 200 and 1000 bp in length. Cellular debris were pelleted by centrifugation at 13,000 rpm for 15 min at 4 °C. Lysate (the equivalent of 1 $\times$ 10^6 cells) was precleared with agarose beads (Dynabeads, Invitrogen) for 1 h at 4 °C. Precleared lysate was incubated with 1 μg of either IgG, anti-AR (N20, Dako), anti-FLAG (M2, Sigma), or anti-SRC1 (M-341X) overnight at 4 °C. The following day, immunocomplexes were sequestered with the addition of 20 μl of agarose beads (Dynabeads, Invitrogen) for 2 h at 4 °C. Beads were washed five times in radioimmune precipitation assay buffer to remove nonspecific binding. Proteinase K (200 μg/ml) was added in a buffer containing 50 mM Tris, pH 8, 1 mM EDTA, 100 mM NaCl, and 0.5% SDS for 2 h at 55 °C. Beads were collected by centrifugation, supernatants were removed, and cross-links were reversed overnight at 65 °C. DNA was purified by phenol/chloroform extraction and suspended in a suitable volume of distilled H_2O. Recruitment of AR, SRC1, and HEYL was assessed at the prostate-specific antigen (*PSA*) locus by quantitative RT-PCR using the following primer sets: *PSA* negative: Forward, 5′-TCC ACT CCA GCT CTA AGA TGG T-3′; and Reverse, 5′-CAG GTA AAC TCC AAG CAC AGT GA-3′; and *PSA* enhancer: Forward, 5′-TGA CAG TAA ACA AAT CTG TTG TAA GAG ACA-3′; and reverse, 5′-AGC AGG CAT CCT TGC AAG AT-3′. Data are presented as percent occupancy for each primer set relative to input (set as 100%).

HEYL Depletion Assays—HEYL levels were depleted using an On-Target Dharmacon siRNA pool (L-008690-00-0005, ThermoScientific) as reported previously (25, 27). Briefly, MCF7 cells were plated in serum-rich medium and grown to 70% confluence before being washed several times in starvation medium and transfected with siRNA. After 48 h, cells were transfected with plasmids TAT-GRE-E1B (1 μg) and PDM-LACZ-β-GAL (250 ng) using FuGENE (Roche Applied Science). After a further 24 h, cells were treated with 10 nM mibolerone or vehicle for 24 h. Cells were harvested and assessed for luciferase and galactosidase activities (see below) and *DRG1* levels using quantitative RT-PCR.

Generating Doxycycline-inducible Cell Line Expressing HEYL (LNCaP:HEYL)—An LNCaP/TR2 stable cell line (20) was transfected with pCDNA4TO-FLAG-HEYL using FuGENE 6 and grown in doxycycline-free medium in the presence of blasticidin (10 μg/ml) and Zeocin (0.3 mg/ml). Medium was changed every 2–3 days, and distinct colonies were selected and expanded after 2–3 weeks. After expansion, individual clones were assessed for HEYL expression at the RNA and protein levels in response to a range of doxycycline concentrations (0–1000 nM) and for effects of doxycycline on growth and AR-regulated gene expression (supplemental Fig. S1).

Cell Culture and Transfection—LNCaP and BPH-1 cells were maintained at 37 °C at 5% CO_2 in Roswell Park Memorial Institute 1640 (RPMI; Sigma) medium supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% (v/v) fetal calf serum. PC3wtAR cells were maintained as above but with the addition of Geneticin (4 μg/ml; Invitrogen). LNCaP:HEYL cells were maintained as above but with the addition of blasticidin and Zeocin at concentrations of 10 μg/ml and 0.3 mg/ml, respectively. RWPE-1 cells were maintained in keratinocyte serum-free medium supplemented with bovine pituitary extract and EGF (Invitrogen). COS-1 and MCF7 cells were maintained at 37 °C at 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% (v/v) fetal calf serum. For HEYL overexpression experiments, cells were plated out in 24-well dishes in starvation medium. Twenty-four hours later, cells were transfected as follows. COS-1 cells were transfected using calcium phosphate with 100 ng of PDM-LACZ-β-GAL, 50 ng of AR expression vector (pSVAR, pSG5-AR, or pSG5-ARΔLBD), 0–200 ng of pSG5-FLAG-HEYL, 0 or 200 ng of pSG5-SRC1e, and 1 μg of reporter (TAT-GRE-E1A-LUC or PROBASIN-PROM-LUC) per well. The following day, cells were washed with starvation medium and then incubated with ligand for 24 h before being lysed in passive lysis buffer (Promega). Luciferase and β-galactosidase activities were measured using the LucLite Plus (PerkinElmer Life Sciences) and GalactoLite Plus (Tropix) kits, respectively, as recommended by the manufacturers. PC3wtAR and MCF7 cells were transfected as above but with FuGENE 6. LNCaP cells were transfected as above but with FuGENE HD. For lipid-based transfections, the final concentrations of plas-

mids (per well) were as follows: 50 ng of PDM-LACZ-β-GAL, 0–200 ng of pSG5-FLAG-HEYL, and 250 ng of reporter plasmid. In all cases, total DNA per well was standardized using empty vector where appropriate.

trans-Repression Assays—*trans*-Repression assays were carried out essentially as reported previously (25, 27).

Cell Proliferation Assays—Sulforhodamine B was used to assess proliferation of cells as described previously (19).

Quantitative RT-PCR Analyses—LNCaP:HEYL cells were plated in 6-well plates in RPMI medium at 60–70% confluence in the presence or absence of 1 μM doxycycline for 72 h. Cells were then grown in starvation medium for a further 2 days. To activate AR signaling, mibolerone (a non-metabolizable synthetic analog of dihydrotestosterone) was added to a final concentration of 10 nM for 24 h. Cells were washed twice and collected in ice-cold PBS before being pelleted by centrifugation. Total RNA was prepared using the RNeasy kit (Qiagen) following the manufacturer's instructions. One microgram of RNA was reversed transcribed using a Superscript II kit (Invitrogen), and expression of *KLK2*, *PSA*, *TMPRSS2*, *DRG1*, and *GAPDH* was quantified using FAM™-labeled oligonucleotide sets on an ABI 7500 instrument (Applied Biosystems).

GST Pulldown Interaction Assays—Protein/protein interaction assays were performed essentially as described previously (32).

Immunofluorescence and Confocal Microscopy—LNCaP:HEYL cells were grown in RPMI medium on sterile glass coverslips in 24-well dishes to 50% confluence for 72 h in the presence or absence of 1 μM doxycycline. Cells were washed and incubated in starvation medium for a subsequent 48 h in the presence or absence of doxycycline (1 μM). To assess translocation of the AR, vehicle (ethanol) or mibolerone was added to a final concentration of 10 nM for 2 h after which cells were washed in ice-cold PBS and fixed/permeabilized in ice-cold 100% acetone for 10 min at −20 °C. Cells were washed twice in PBS and blocked in PBS with the addition of 1% BSA and 0.05% Tween (PBS-T) for 60 min at room temperature. After two to three washes in PBS-T, cells were incubated with primary antibody at room temperature for 60 min. Cells were washed (three to five times) in PBS-T before incubation with Alexa Fluor secondary antibody for 1 h at room temperature. Cells were washed extensively in PBS-T, mounted on slides with VECTASHIELD containing 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories), sealed, and air-dried before microscopy. RWPE-1 and BPH-1 cells were grown in 24-well plates on glass coverslips, fixed, and processed as above. For mapping of nuclear localization and export sequences, COS-1 cells were seeded as above and transfected with either 200 ng/well pEGFPC1-HEYL, pEGFPC1-HEYL(132–152) (containing an nuclear export sequence (NES), pEGFPC1-HEYL(42–51) (containing a nuclear localization sequence (NLS)), pEGFPC1-HEYLmutNLS, or pEGFPC1-Empty as a control using FuGENE (Roche Applied Science). The following day, cells were fixed in 10% formaldehyde for 10 min at room temperature before being washed extensively in PBS. Fixed cells were subsequently stained and mounted on slides as described above. Slides were analyzed on a Zeiss Meta 512 confocal microscope.

Tissue Immunohistochemistry—Needle biopsies of prostate tissue from patients with biopsy-proven adenocarcinoma (St. Mary's Hospital, London, UK) were examined. All tissues were obtained with local ethics committee approval and informed consent. Tissue was formalin-fixed, paraffin-embedded, sectioned (3 μm), subsequently dewaxed in xylene, and then rehydrated through a series of increasingly lower percentage alcohol washes. After several washes in PBS, antigen retrieval was performed in a Tris/EDTA buffer (10 mM Tris, 1 mM EDTA, 0.05% Tween 20, pH 9) by boiling tissue for a total of 20 min in 5-min intervals. Slides were left to cool to room temperature and washed several times in PBS. All further steps were performed with the Histostain third generation immunohistochemistry detection kit (Invitrogen) according to the manufacturer's instructions. Sections were stained with mouse anti-HEYL (Abnova) at a concentration of 1:400 in 1% BSA and PBS overnight at 4 °C. Sections were counterstained with hematoxylin (Invitrogen), dehydrated, and mounted in DEPEX solution (BDH). Results shown are representative of between three and 15 scores per patient.

RESULTS

HEYL Is a Potent AR Corepressor and Interacts with Activation Function-1—HEYL is 328 amino acids in length and exhibits high sequence similarity at the protein level to HEY1 (supplemental Fig. S2). To further understand the role of HEY family members in AR signaling, we amplified full-length *HEYL* from MCF7 cDNA to create mammalian expression vectors pSG5-HEYL and pCDNA4TO-FLAG-HEYL. Transient transfection experiments in COS-1 cells with an AR-responsive luciferase reporter (TAT-GRE-E1B-LUC) indicated that HEYL was a more potent repressor of AR activity than HEY1 (Fig. 1*A*), repressing ligand-induced activity to ~20% of maximum; furthermore, this repression was dose-dependent (Fig. 1*B*). Both HEY1 and HEY2 interact with HEYL; however, in combination experiments, there were no additive or synergistic effects on AR activity, suggesting that HEY proteins might repress AR signaling via independent mechanisms (supplemental Fig. S2). Furthermore, it was clear that both HEY1 and HEY2, separately or together, appear to have weaker repressor activity than HEYL alone (Ref. 25 and supplemental Fig. S2). We assessed the effect of exogenous overexpression of HEYL on a second AR-responsive reporter construct, Probasin-LUC, which contains a non-consensus androgen response element, and also on TAT-GRE-E1B-LUC in cell lines that express either endogenous AR (MCF7) or stably transfected AR (PC3wtAR). Repression of Probasin-LUC in COS-1 cells and of TAT-GRE-E1B-LUC in MCF7 cells was evident although more modest (~50 and 40%, respectively; Fig. 1, *C* and *D*). The repressive effect was more striking in PC3wtAR, a prostate cell line, in which AR activity was repressed by up to ~90% (Fig. 1*E*). To confirm that endogenous HEYL can affect endogenous AR activity, we screened a panel of AR-positive cell lines for HEYL expression (data not shown). HEYL mRNA levels were very low in the prostate cancer lines tested (suggesting that loss of expression may occur during malignant progression) but were ~3 times higher in the AR-positive breast cancer line MCF7. We therefore depleted cellular levels in this cell line using transiently transfected

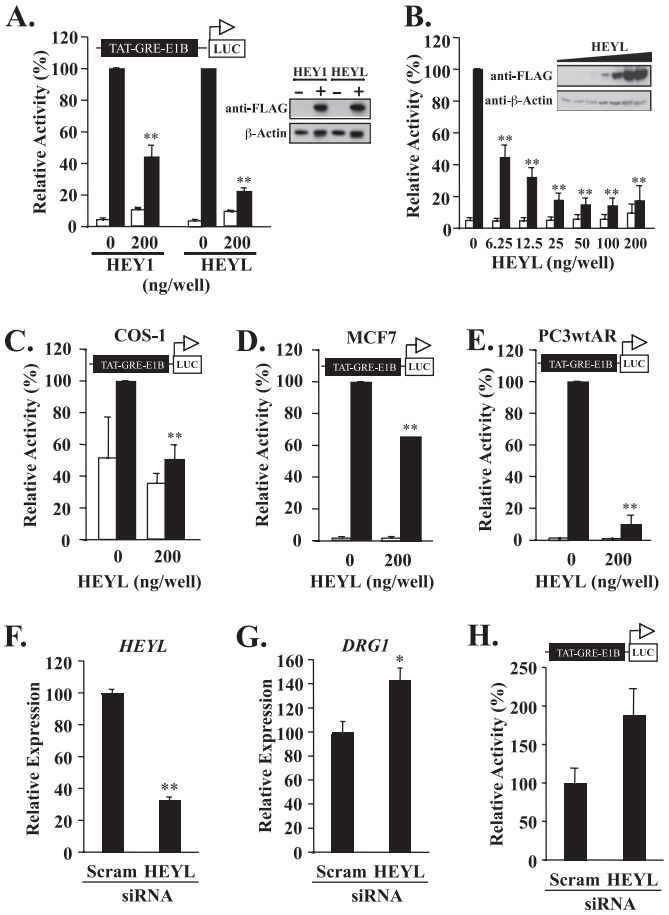

FIGURE 1. **Repression of AR signaling by HEYL.** *A*, COS-1 cells were co-transfected with expression plasmids for β-galactosidase (50 ng), AR (50 ng), AR-responsive reporter construct TAT-GRE-E1B-LUC (1 μg), and either pCDNA4TO-FLAG-HEY1, HEYL, or empty vector (200 ng). After transfection, cells were washed and incubated for 24 h with either vehicle (EtOH; *white bars*) or hormone (10 nM mibolerone; *black bars*) for 24 h after which cells were lysed, and luciferase activity was calculated and normalized to β-galactosidase expression. Values are expressed as a percentage of AR activity in the presence of 10 nM mibolerone and are the average + 1 S.E. of three independent experiments performed in duplicate. *Inset*, cell lysates were separated by SDS-PAGE, transferred to nitrocellulose, and probed with either anti-FLAG or anti-β-actin antibody. *B*, COS-1 cells were transfected as for *A* but with a range of amounts of HEY expression vector (0–200 ng/well) and empty vector added to normalize total DNA added. *Inset*, cell lysates were resolved as stated in *A* and probed with either anti-FLAG or anti-β-actin antibody. *C*, COS-1 cells were transfected and treated as stated in *A* but with 1 μg Probasin-LUC reporter construct. *D* and *E*, MCF7 (*D*) or PC3wtAR (*E*) were co-transfected with FuGENE 6 with expression plasmid for β-galactosidase (50 ng), AR-responsive reporter construct TAT-GRE-E1B-LUC (250 ng), and a plasmid encoding HEYL (200 ng) and treated as stated in *A*. *F–H*, MCF-7 cells were seeded in 6-well dishes at a cell density of 400,000 cells/well. In serum starvation medium, cells were transfected with either scrambled (*Scram*) or specific HEYL siRNA for a total of 96 h. After 48 h, cells were co-transfected with AR-reporter construct TAT-GRE-E1B (1 μg) and an expression plasmid for β-galactosidase (250 ng). After 72 h, mibolerone was added to a final concentration of 10 nM and incubated for a further 24 h. RNA was extracted, and levels of *HEYL* (*F*) and *DRG1* (*G*) were quantified by qRT-PCR. *H*, cells were treated as stated above and lysed, and luciferase activity was calculated and normalized to β-galactosidase expression. A *t* test was used to determine the significance of the difference to the corresponding result (*, $p < 0.05$; **, $p < 0.01$).

siRNA and achieved ~70% reduction in HEYL mRNA levels (Fig. 1*F*). Assessing the effect of this depletion on expression of AR target genes, we observed a significant increase in androgen-dependent expression of the endogenous androgen-regulated gene *DRG1* (Fig. 1*G*) and also increased expression of the transfected AR-dependent luciferase reporter (Fig. 1*H*).

The AR is unusual among steroid hormone receptors in that the major transactivation function is activation function-1 (AF1) situated within the N-terminal domain rather than AF2 in the ligand-binding domain (LBD), which is relatively weak in the AR (6, 31, 33). Furthermore, AF1 can be subdivided into two discrete, overlapping activation domains, the usage of which is context-dependent. Although almost the entire N terminus (residues 1–494) is required for full activity of the full-length receptor, a core that contributes 50% of the activity is located between residues 101 and 360, and this region has been termed TAU1. However, in the absence of the LBD, a different region mediates activation, and this region is termed TAU5 (residues 370–494) (31) (Fig. 2*A*). We performed pulldown interaction assays to determine the interaction of full-length HEYL with full-length and deletion constructs of AR. These showed that GST-HEYL was able to interact strongly with full-length AR expressed in COS-1 cells (Fig. 2*B*). Furthermore, a robust interaction was also observed between full-length HEYL and GST-NTD, which contains the AR N-terminal domain (residues 1–556) but lacks the LBD and most of the DNA-binding domain (DBD), indicating that HEYL likely interacts with AR via the N-terminal transactivation domain (Fig. 2*B*). In support of this, no interaction was seen between HEYL and GST fused to the LBD in the presence or absence of ligand. To determine whether HEYL also represses AR-AF1 activity, we transfected COS-1 cells with an AR deletion construct lacking amino acids 654–919 (ARΔLBD), which is constitutively active. HEYL could significantly repress the activity of ARΔLBD on TAT-GRE-E1B-LUC (Fig. 2*C*) and Probasin-LUC promoter constructs (data not shown). Together, these data indicate that HEYL interacts with and represses AR activity largely through AF1. To narrow down the functional domain affected, we next assayed the ability of HEYL to repress AR deletion constructs lacking either TAU1 (ARΔτ1) or TAU5 (ARΔτ5) (Fig. 2*D* and supplemental Fig. S3). This showed that deletion of either domain resulted in reduced relative repression of AR activity by HEYL; however, the repression of ARΔτ1 (with intact TAU5) by HEYL was proportionately less than that of ARΔτ5 (with intact TAU1), indicating that TAU1 (residues 101–360) is likely to be the functional domain that is primarily affected by HEYL in the context of the full-length AR.

HEYL Inhibits AR and SRC1 Loading on PSA Enhancer—We have previously shown that HEY1 interacts with the bHLH-PAS domain of SRC1e and that overexpression of HEY1 can competitively repress SRC1e "coactivation" of AR activity (25). To assess whether HEYL may repress AR activity via a similar mechanism, we transfected cells with expression plasmids for AR, SRC1e, and increasing amounts of pSG5-HEYL and studied the activity of an AR-responsive reporter. As shown in Fig. 3*A*, SRC1e potentiated the activity of ligand-activated full-length AR ~5-fold. Increasing amounts of pSG5-HEYL reduced the effect of SRC1e such that, at the highest level of pSG5-HEYL (200 ng/well), AR activity was at background levels (Fig. 3*A*). The ligand-binding domain/AF2 is not required for this effect because we observed a similar competitive effect on activity of ARΔLBD when we transfected increasing amounts of pSG5-HEYL; at 100 ng/well, the positive effect of SRC1e on AR activity was essentially negated (Fig. 3*B*). This suggests that

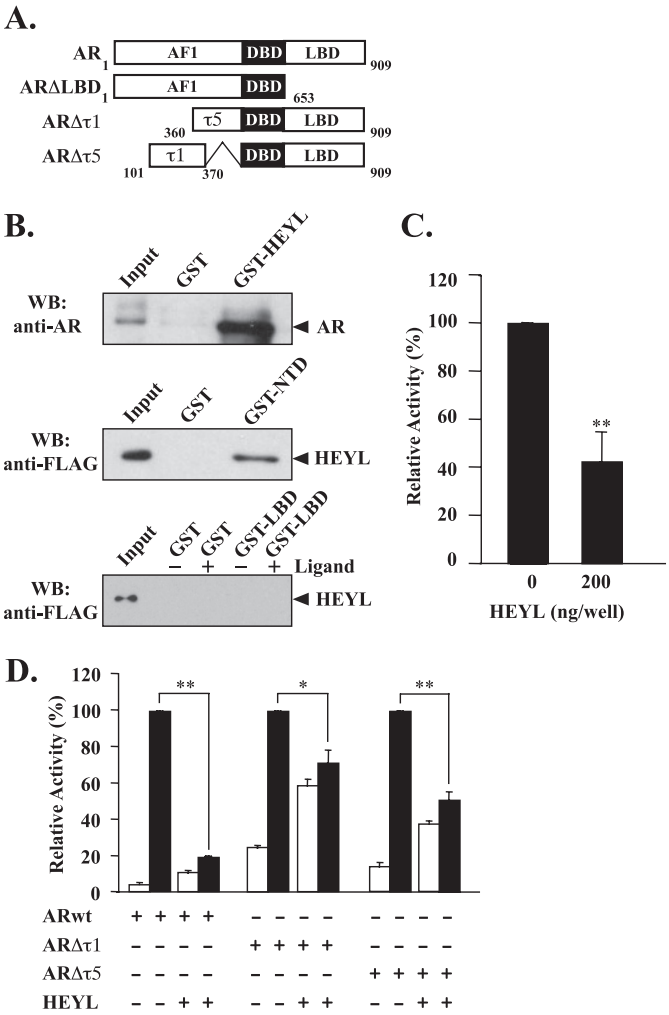

FIGURE 2. **HEYL represses hormone-independent AR signaling and interacts with AF1 domain.** *A*, a schematic representation of AR deletion constructs used in Fig. 2, *B–D*. *B*, COS-1 cells were transfected with expression plasmids for either AR or FLAG-tagged HEYL. Whole cell lysates were prepared and incubated with either GST alone, HEYL (*GST-HEYL*), or the NTD or LBD domains of AR fused to GST (*GST-NTD* or *GST-LBD*, respectively). Complexes were incubated with Sepharose beads, and specific interactions were detected by probing Western blots (*WB*) with anti-AR or anti-FLAG antibody. 5% of whole cell lysate was loaded for *Input*. *C*, COS-1 cells were co-transfected with expression plasmids for β-galactosidase (50 ng), ARΔLBD (50 ng), HEYL or empty vector (200 ng), and AR-responsive reporter construct TAT-GRE-E1B-LUC (1 μg). After transfection, cells were washed and incubated for a further 24 h after which cells were lysed, and luciferase activity was calculated and normalized to β-galactosidase expression. Values are expressed as a percentage of AR activity in the absence of HEYL and are the average + 1 S.E. of three independent experiments performed in duplicate. *D*, COS-1 cells were transfected as stated in *C* but with either ARwt, ARΔτ1, or ARΔτ5. A *t* test was used to determine the significance of the difference to the corresponding result for COS-1 cells transfected with 0 ng of HEYL (*, $p < 0.05$; **, $p < 0.01$).

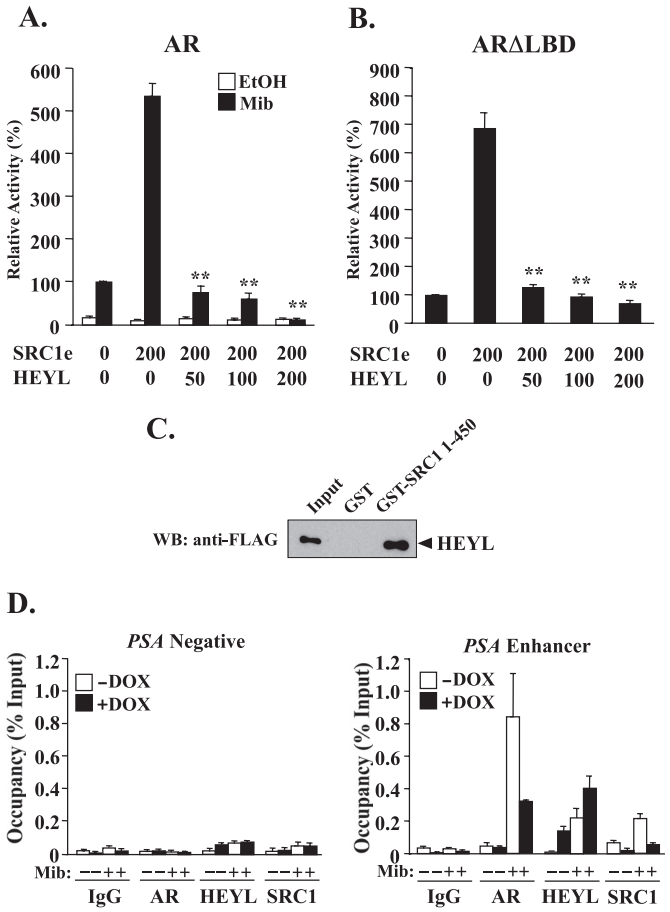

FIGURE 3. **HEYL attenuates AR and SRC1 loading on *PSA* enhancer.** *A* and *B*, COS-1 cells were co-transfected with expression plasmids for β-galactosidase (50 ng), full-length AR (50 ng) (*A*) or LBD deletion mutant ARΔLBD (50 ng) (*B*), and AR-responsive reporter construct TAT-GRE-E1B-LUC (1 μg) and plasmids encoding SRC1e (200 ng) and increasing concentrations of HEYL (50–200 ng). After transfection, cells were washed and incubated for a further 24 h with vehicle (*EtOH*; *white bars*) or hormone (10 nM mibolerone (*Mib*); *black bars*) in *A* or with fresh medium in *B* after which cells were lysed, and luciferase activity was calculated and normalized to β-galactosidase expression. Values are expressed as a percentage of AR activity in the presence of 10 nM mibolerone (*A*) or the absence of SRC1 and HEYL (*B*) and are the average + 1 S.E. of three independent experiments performed in duplicate. A *t* test was used to determine the significance of the difference to the corresponding result for cells transfected with 200 ng of SRC1e (**, $p < 0.01$). *C*, COS-1 cells were transfected with expression plasmids for FLAG-tagged HEYL. Whole cell lysates were prepared and incubated with either GST alone or GST fused to amino acids 1–450 of SRC1 (*GST-SRC1 1–450*). Complexes were incubated with Sepharose beads, and specific interactions were detected by probing Western blots with anti-FLAG antibody. 5% of whole cell lysate was loaded for *Input*. *D*, occupancy of AR, HEYL, and SRC1 on the *PSA* enhancer was assessed by chromatin immunoprecipitation as stated under "Experimental Procedures." A representative result of three independent experiments is shown.

HEYL may repress AR activity by functional competition with (AF1) coactivators such as SRC1. In support of this, pulldown assays demonstrated an interaction between full-length HEYL and the N-terminal 450 amino acids of SRC1 spanning the bHLH-PAS domain of this protein (Fig. 3*C*). Pulldowns also confirmed that full-length HEYL can interact with both HEY1 and HEY2 (supplemental Fig. 2C), supporting the consensus that HEYL may act in the form of homo- or heterodimers (34). To assess functional SRC1/AR/HEYL interactions further in the presence of DNA, we performed chromatin immunoprecipitation experiments on LNCaP cells (LNCaP:HEYL; described below) inducibly expressing HEYL (Fig. 3*D*). In the absence of exogenous HEYL (−doxycycline), we observed loading of AR and SRC1 on the *PSA* enhancer after 2 h in the presence of hormone (10 nM mibolerone). Strikingly, when HEYL levels were increased (+doxycycline) for 72 h, we observed an increase in HEYL occupancy at the *PSA* enhancer and a concomitant decrease in occupancy by both AR and SRC1 in the presence of hormone, suggesting that HEYL inhibits the loading of these factors during transcriptional regulation *in vivo*, thus attenuating AR-dependent gene regulation.

Overexpression of HEYL Represses Androgen Target Gene Expression and Inhibits Growth of Prostate Cancer Cell Lines—Because the commonly used prostate cancer cell lines, including LNCaP, express the *HEY* genes at low or negligible levels (data not shown), to examine the potential role of HEYL in prostate cells, we created a stable line exogenously expressing FLAG-tagged HEYL. In this LNCaP-derived line (LNCaP:HEYL), increased expression of HEYL (at mRNA and protein levels) was inducible by the addition of doxycycline in a dose-dependent manner (Fig. 4*A* and supplemental Fig. S1). At the highest concentrations of doxycycline used (0.01–1 μM), *HEYL* mRNA was expressed ~30–40-fold higher than in the absence of the antibiotic, and protein levels appeared maximal at 0.001 μM and above. To assess the consequences of HEYL overexpression on AR-regulated genes, we treated LNCaP:HEYL cells with or without doxycycline for 72 h, serum-starved the cells for a further 48 h, and then stimulated the activity of the AR by addition of 10 nM mibolerone for 24 h. Again, *HEYL* expression was induced ~35-fold in the presence of mibolerone but to a lesser extent (~10-fold) in the absence of hormone. Exogenous HEYL expression was confirmed at the protein level by probing total cell lysates with an anti-FLAG monoclonal antibody (Fig. 4*B*). We next quantified the expression of the AR-regulated genes *KLK2*, *PSA*, *DRG1*, and *TMPRSS2* by qRT-PCR. In vehicle-treated cells, expression of AR-regulated genes was minimal, but as expected, expression was induced by the synthetic androgen mibolerone (Fig. 4*C*). When exogenous *HEYL* expression was switched on by addition of doxycycline, androgen-dependent *KLK2*, *PSA*, *DRG1*, and *TMPRSS2* expression was decreased (in the case of *PSA* and *TMPRSS2* in a statistically significant manner; $p < 0.05$), indicating that HEYL can repress AR activity *in vivo* (see also supplemental Fig. S1).

As prostate tumors grow in response to AR signaling, we examined the effects of doxycycline on growth rates of LNCaP:HEYL in comparison with control LNCaP:Empty cells. Cells in hormone-depleted medium were cultured for 6 days with or without the addition of androgen and/or doxycycline. LNCaP:HEYL cells treated with 10 nM mibolerone in the presence or absence of doxycycline were probed with anti-FLAG and anti-β-actin antibodies to confirm HEYL protein levels (Fig. 4*E*, *inset*). As expected, cell growth was only observed in the presence of androgen (Fig. 4, *D* and *E*). Addition of doxycycline had no effect on the androgen-dependent growth of the parental cell line LNCaP:Empty (Fig. 4*D*). However, addition of doxycycline resulted in reduced androgen-dependent growth of the LNCaP:HEYL cells (Fig. 4*E* and supplemental Fig. S1), suggesting that HEYL inhibits androgen-dependent growth likely by repression of AR activity.

Characterization of HEYL Repression Domains—From the above experiments, it was apparent that HEYL is a potent repressor of AR signaling. To identify the domains of HEYL responsible for repression, we created a variety of HEYL deletion constructs expressing the N-terminal bHLH region, the central Orange domain, and C-terminal variable domain, respectively (Fig. 5*A*), fused to Gal4-DBD and tested their ability to repress in *trans* the activity of a strong transactivation domain (VP16) fused to LexA-DBD using a luciferase reporter downstream of several LexA and Gal4 binding elements (Fig. 5*B*). The addition of the Gal4-DBD alone did not effect a change in luciferase activity; however, transfection of Gal4-HEY1 dramatically reduced (by ~75%) the activity of luciferase, indicating that Gal4-HEY1 as reported previously (25) can repress VP16-LexA transactivation of the reporter construct. We then compared the repressive activity of full-length HEYL and observed that Gal4-HEYL repressed VP16-LexA activity by almost 90%, indicating that HEYL is a more potent repressor than HEY1 in this assay also. The deletion constructs encompassing the N-terminal bHLH domain and the C-terminal variable domain both repressed the reporter although to a lesser extent than full-length HEYL, indicating that the protein contains two distinct repressive domains that likely both contribute to the repressive activity of the full-length protein. However, the middle 55 amino acids, which encompass the Orange domain, increased rather than decreased activity of the reporter (to ~150%.) The differences in repressive activity of Gal4-fused proteins were not due to differences in expression levels as confirmed by Western blotting of cell extracts with an anti-Gal4-DBD antibody (data not shown). Furthermore, the differences were not due to altered localization of proteins because all of these constructs were nuclear on visualization by fluorescence microscopy (data not shown) presumably due to the presence of an NLS within the Gal4-DBD fragment.

As outlined above, active repression of transactivation can be achieved by recruitment of specific proteins and complexes that contain HDAC activity. We hence performed the *trans*-repression assay in the absence or presence of 100 nM trichostatin A (TSA), a potent HDAC Class I/II inhibitor. The inhibitory activity of TSA was confirmed by probing cell lysates with anti-acetylated Histone H3 and anti-β-actin antibodies, which showed an increase in relative acetylated Histone H3 levels in TSA-treated cells (Fig. 5*C*, *inset*). The addition of TSA had a small but significant ($p = 0.03$) effect on repression by full-length Gal4-HEYL, increasing luciferase activity from 17 to 25% (Fig. 5*C*). However, there were no differences in luciferase activity in the presence of 100 nM TSA when amino acids 1–110 (bHLH domain) and 110–165 (Orange domain) were transfected. Interestingly, the repressive effect of amino acids 165–328 was reduced although not significantly ($p = 0.07$) upon TSA treatment, suggesting that the repression by the C-terminal domain of HEYL may involve, but not depend upon, recruitment of proteins with HDAC activity. To confirm these observations, further Class I/II HDAC inhibitors were also tested, including sodium butyrate and valproic acid, and similar results were observed (data not shown). Hence, it appears that HEYL represses transcription largely by mechanisms that do not depend on these classes of HDACs.

To determine which of the intrinsic repression domains is responsible for repression of AR activity, the effect of the deletion constructs on AR activation of the TAT-GRE-E1B-LUC reporter was assayed in COS-1 cells. As shown in Fig. 6*A*, full-length HEYL repressed ligand-activated AR activity as expected. Of the deletion constructs, only the C-terminal domain (residues 165–328) retained the ability to repress AR activity. Basic helix-loop-helix proteins can repress transcription in a variety of ways, including physical "blocking" or dissociation of activation factors from DNA or by direct recruitment

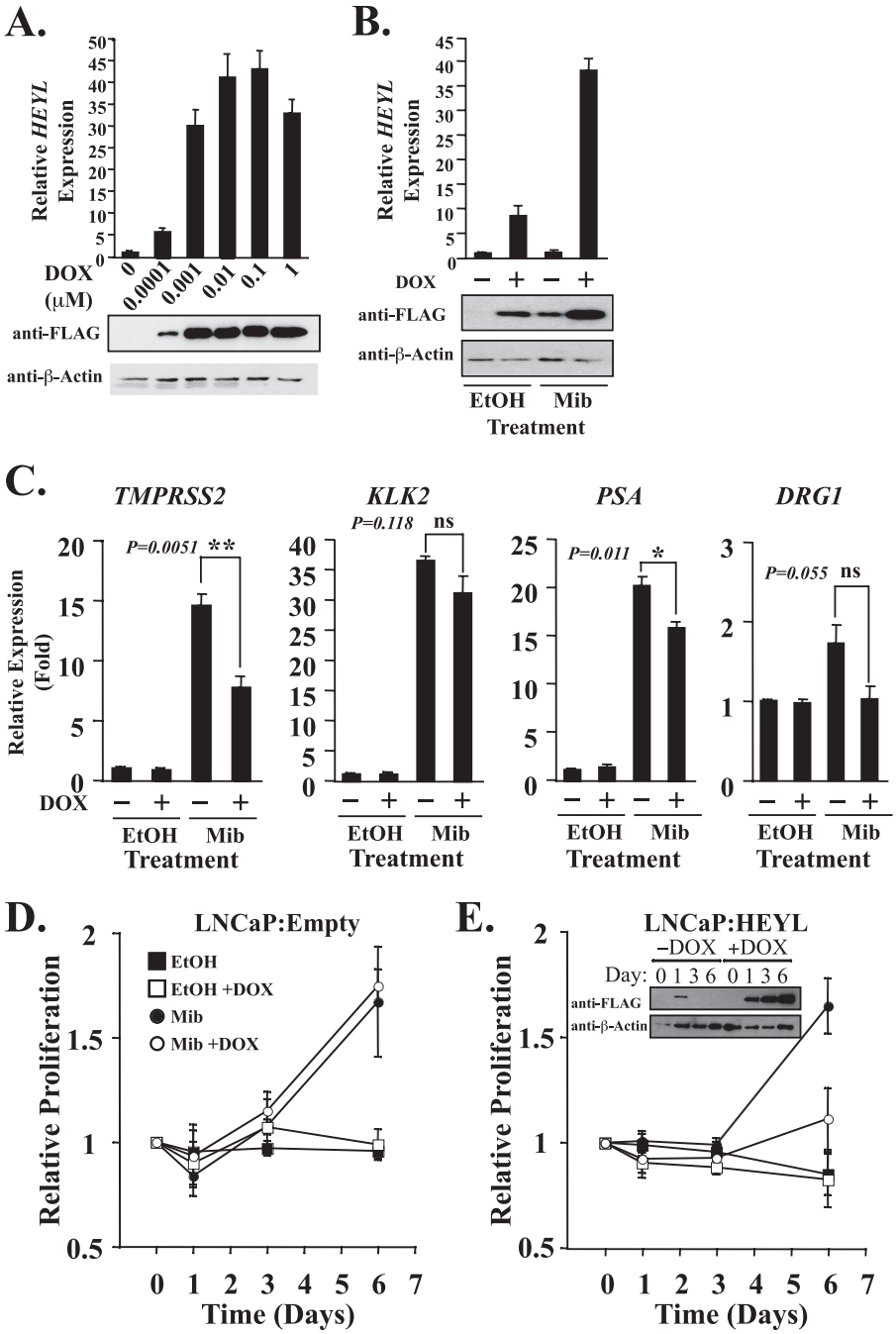

FIGURE 4. **Overexpression of HEYL represses AR-regulated genes and hormone-dependent prostate cancer cell line growth.** *A*, LNCaP:HEYL cells were plated in 6-well plates in RPMI medium and cultured to 60–70% confluence. *HEYL* expression was induced by the addition of a range of doxycycline concentrations (0, 0.0001, 0.001, 0.01, 0.1, and 1 μM) for 24 h after which total RNA was extracted, and qRT-PCR was performed. *HEYL* expression was normalized to *GAPDH* and set to 1 in the absence of doxycycline. Data shown are the average + 1 S.E. of three independent experiments performed in triplicate. *Bottom*, LNCaP:HEYL cells were treated as above but were induced for 48 h with a range of doxycycline concentrations after which whole cell lysates were prepared and Western blotted. Membranes were probed with either anti-FLAG or anti-β-actin antibody. *B*, LNCaP:HEYL cells were cultured in RPMI medium for 72 h in the presence or absence of 1 μM doxycycline to induce *HEYL* expression. Cells were then washed and serum-starved for 48 h before being treated for 24 h with either vehicle (*EtOH*) or hormone (10 nM mibolerone (*Mib*)). *HEYL* expression was quantified by qRT-PCR and normalized to *GAPDH* levels. *HEYL* expression in the absence of hormone and doxycycline was set to 1. Data shown are the average + 1 S.E. of three independent experiments performed in triplicate. *Bottom*, LNCaP:HEYL cells were treated as above but were induced for 48 h with 1 μM doxycycline after which whole cell lysates were prepared and Western blotted. Membranes were probed with either anti-FLAG or anti-β-actin antibody. *C*, LNCaP:HEYL cells were treated as in *B*, and levels of AR-regulated genes were quantified by qRT-PCR. Data shown are the average + 1 S.E. of three independent experiments performed in triplicate. A *t* test was used to determine the significance of the difference to the corresponding result for hormone-treated LNCaP:HEYL cells in the absence of doxycycline (*, $p < 0.05$; **, $p < 0.01$; *ns*, not significant). *D* and *E*, LNCaP:Empty (*D*) or LNCaP:HEYL (*E*) cells were seeded in 24-well dishes and treated with vehicle (*EtOH*; *black boxes*), vehicle plus 1 μM doxycycline (*DOX*; *white boxes*), hormone (10 nM mibolerone (*Mib*); *black circles*), or hormone plus 1 μM doxycycline (*white circles*) for 0, 1, 3, and 6 days. Proliferation was measured by sulforhodamine B assay, and growth at day 0 was set to 1. Data shown are the average + 1 S.E. of three independent experiments performed in quadruplicate. *Inset*, LNCaP:HEYL cells were treated with hormone in the absence or presence of 1 μM doxycycline for 0, 1, 3, or 6 days. Whole cell lysates were prepared and resolved by SDS-PAGE and Western blotting and probed with either anti-FLAG or anti-β-actin antibody.

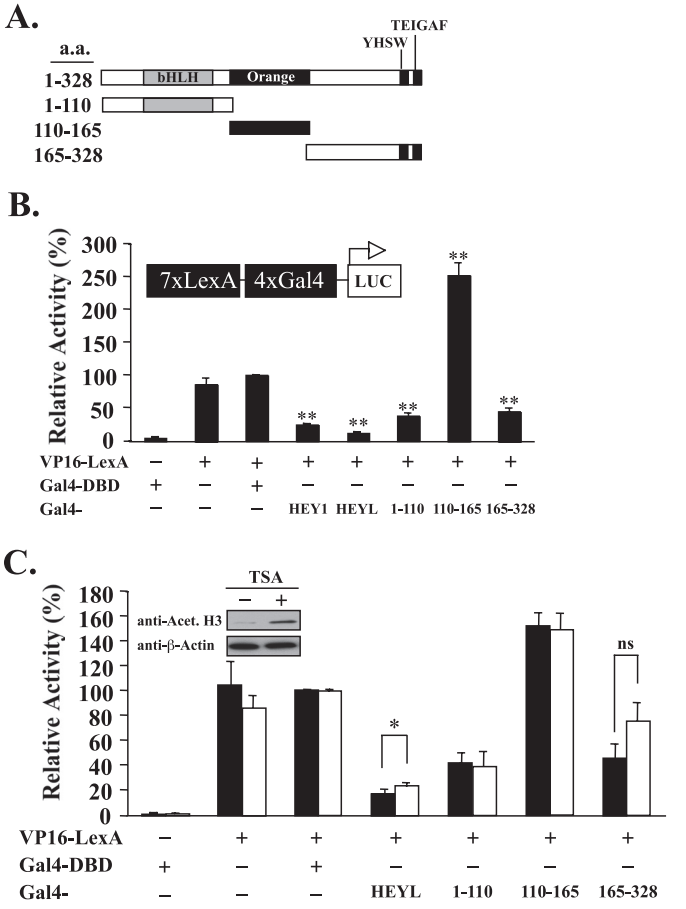

FIGURE 5. **Mapping of HEYL-repressive domains.** *A*, schematic representation of HEYL domain constructs representing the N-terminal bHLH domain (amino acids (*a.a.*) 1–110), central Orange domain (amino acids 110–165), and C-terminal variable domain (amino acids 165–328). *B*, COS-1 cells were co-transfected with a luciferase reporter construct containing 7xLexA and 4xGal4 response elements, $LexA_{(7)}$-$Gal4_{(4)}$-LUC (1 μg), and expression plasmids for β-galactosidase (50 ng), LexA-VP16 (100 ng), and either Gal4-DBD alone or Gal4-DBD fused to full-length HEY1, HEYL, or domains of HEYL (100 ng). After transfection, cells were washed and incubated for 24 h after which cells were lysed, and luciferase activity was calculated and normalized to β-galactosidase expression. Values are expressed as a percentage of VP16-LexA activity in the presence of empty Gal4-DBD and are the average + 1 S.E. of three independent experiments performed in duplicate. *C*, COS-1 cells were transfected as in *B* and in addition treated for 24 h with either vehicle (EtOH; *black bars*) or 100 nM TSA (*white bars*). Luciferase activities were quantified and expressed as in *B*. A *t* test was used to determine the significance of the difference to the corresponding result for vehicle-treated COS-1 cells in the absence of TSA (*, $p < 0.05$; **, $p < 0.01$; *ns*, not significant). *Inset*, cell lysates were separated by SDS-PAGE, transferred to nitrocellulose, and subsequently probed with either anti-acetylated (*Acet.*) H3 or anti-β-actin antibody.

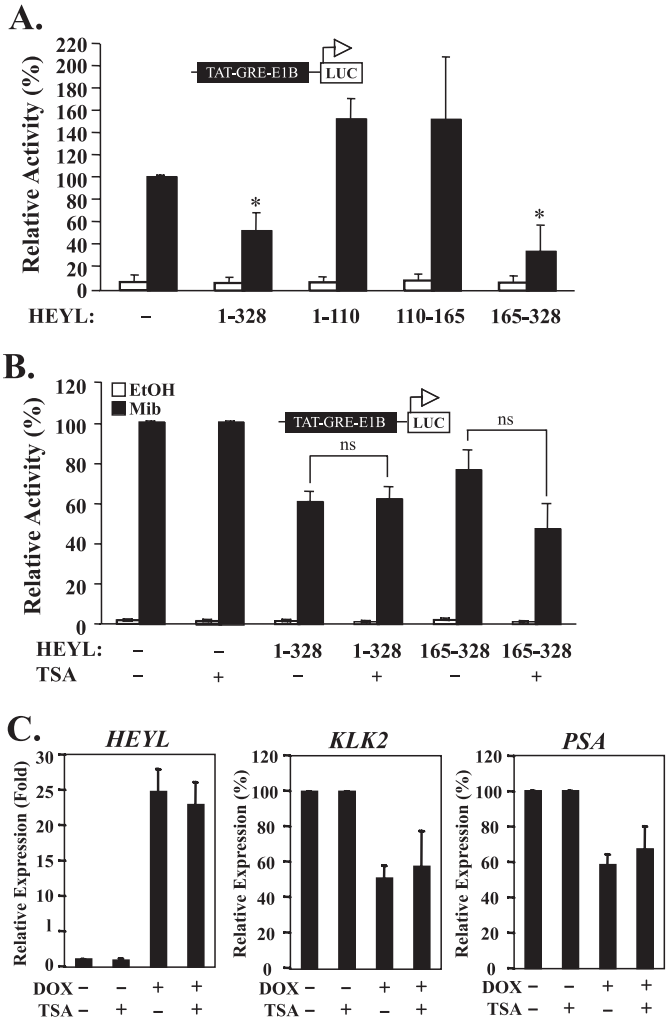

FIGURE 6. **Mechanism of AR repression by HEYL is HDAC-independent.** *A*, COS-1 cells were co-transfected with expression plasmids for β-galactosidase (50 ng), AR (50 ng), and AR-responsive reporter construct TAT-GRE-E1B-LUC (1 μg) and plasmids encoding full-length HEYL or domains (100 ng). After transfection, cells were washed and incubated for 24 h with either vehicle (*EtOH*; *white bars*) or hormone (10 nM mibolerone (*Mib*); *black bars*) for 24 h after which cells were lysed, and luciferase activity was calculated and normalized to β-galactosidase expression. Values are expressed as a percentage of AR activity in the presence of 10 nM mibolerone in the absence of HEYL and are the average + 1 S.E. of three independent experiments performed in duplicate. *, $p < 0.05$. *B*, COS-1 cells were co-transfected with expression plasmids for β-galactosidase (50 ng), AR (50 ng), and AR-responsive reporter construct TAT-GRE-E1B-LUC (1 μg) and plasmids encoding full-length HEYL or C-terminal variable domain (100 ng). After transfection, cells were washed and incubated for 24 h with either vehicle (*EtOH*; *white bars*) or hormone (10 nM mibolerone; *black bars*) in the absence or presence of 100 nM TSA for 24 h after which cells were lysed, and luciferase activity was calculated and normalized to β-galactosidase expression. Luciferase activities were quantified, and values are expressed as a percentage of AR activity in the presence of 10 nM mibolerone in the absence of HEYL and are the average + 1 S.E. of three independent experiments performed in duplicate. *C*, LNCaP:HEYL cells were cultured in RPMI medium for 48 h in the presence or absence of 1 μM doxycycline (*DOX*) to induce *HEYL* expression. Cells were then treated for 24 h with either vehicle (*EtOH*) or 100 nM TSA. *HEYL*, *KLK2*, and *PSA* expression was quantified by qRT-PCR and normalized to *GAPDH* levels. *HEYL* expression in the absence of doxycycline was set to 1. *KLK2* and *PSA* expression levels in the absence of doxycycline were set to 100%. Data shown are the average + 1 S.E. of three independent experiments performed in quadruplicate. *ns*, not significant.

of repressive complexes that include HDACs, Sin3A, and NCoR (22). Surprisingly and in contrast to Fig. 5*B*, the N-terminal bHLH domain had no repressive effect in this assay, suggesting that this domain has the ability to repress transcriptional activation but is redundant in repression of AR activity. We next tested whether TSA can relieve repression of TAT-GRE-E1B-LUC activation by repressive HEYL constructs. These experiments indicated that in this context the recruitment of Class I/II HDACs did not contribute to AR repression by HEYL as no relief of AR repression was observed in the presence of TSA (Fig. 6*B*). Furthermore, we assessed changes in expression of AR-regulated genes in the presence of TSA in the LNCaP:HEYL cell line. First, we quantified *HEYL* expression by qRT-PCR and verified that TSA treatment did not significantly alter the level of induction (Fig. 6*C*). Next, we quantified the expression of

KLK2 and *PSA* in response to doxycycline (in full serum conditions, *i.e.* with activated AR) and observed a reduction in levels as noted previously in Fig. 4*C*. After treatment with TSA for 24 h, we observed no significant relief of this repression, suggesting that HEYL repression of androgen-dependent activation of the target genes *KLK2* and *PSA* is not via HDAC I/II-dependent mechanisms (Fig. 6*C*).

Taken together, these results indicate that the N-terminal bHLH and C-terminal domains have intrinsic repressive ability and that the C-terminal domain may repress partly via HDAC-dependent mechanisms. However, the N-terminal domain was dispensable for AR-dependent repression, which is thus mediated mainly by the C-terminal domain. Furthermore, HEYL (unlike HEY1) repressed AR via TSA-insensitive mechanisms.

HEYL Localization Is Altered in Cancer Tissue—We have previously shown that in prostate cancer biopsies HEY1 expression is predominantly cytoplasmic rather than nuclear, and we hypothesized that this cellular localization provides a growth advantage in cancer cells, circumventing the repressive effects of nuclear HEY1 (25, 26). To understand better the mechanism(s) of repression of AR by HEY proteins, we examined the subcellular distribution of HEYL protein by confocal microscopy with a monoclonal antibody. In normal and benign transformed prostate epithelial lines, RWPE-1 and BPH-1, respectively, endogenous HEYL was entirely nuclear (Fig. 7*A*). In our inducible LNCaP:HEYL cell line treated with doxycycline, the exogenously expressed HEYL was also nuclear, demonstrating that the transfected HEYL behaves similarly to endogenous protein, and furthermore, we observed a co-localization of HEYL and AR in the nucleus of these cells in the presence of androgen (Fig. 7*A*, *bottom panel*). In addition, we verified the specificity of the HEYL antibody by immunofluorescence and Western blotting of LNCaP:HEYL cells treated with or without doxycycline (Fig. 7*A*). To examine expression patterns of endogenous HEYL in human prostate tissue, we used prostate needle biopsies from patients with prostate cancer and stained them for HEYL. As shown in Fig. 7*B*, endogenous HEYL was expressed in the epithelial cell compartment of the prostate and showed nuclear localization in benign cells (Fig. 7*B*, *left panels*) with little expression detected in the cytoplasm. However, in all stages of cancer examined, HEYL nuclear intensity decreased, in most cases correlating with increasing cytoplasmic expression (Fig. 7, *B* and *C*). The expression score for each patient is plotted in Fig. 7*C*. This shows that nuclear expression decreased in all stages of the disease studied but remained high in adjacent benign tissue. Furthermore, expression of HEYL in the cytoplasm remained low in adjacent tissue but at the same level or higher in prostate cancer. Collectively, these data suggest that HEYL is excluded from the nuclei of cancer cells but not benign cells, indicating that nuclear exclusion of HEYL occurs in the early stages of prostate cancer formation or progression.

Identification of Nuclear Localization and Export Sequences in HEYL—Recently, we identified an NLS within HEY1 that when mutated results in a cytoplasmic distribution of protein that is also unable to repress AR (32). Using a bioinformatics approach, we identified putative NLS and NES in HEYL. As shown in Fig. 8*A*, the region of HEY1 that has been shown to be

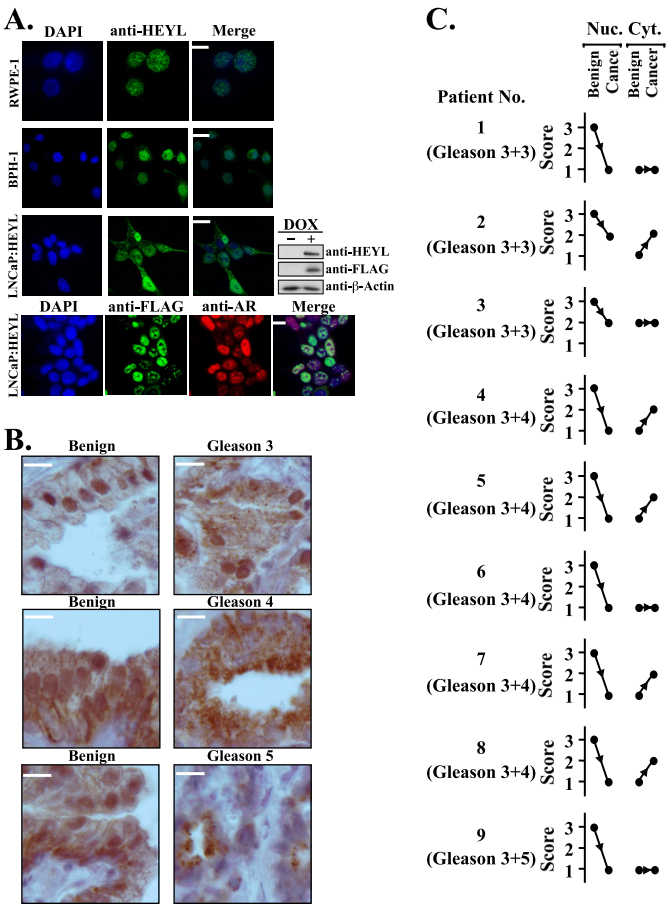

FIGURE 7. **HEYL expression in human prostate cancer tissue.** *A*, RWPE-1, BPH-1, and LNCaP:HEYL cells were cultured on sterile glass coverslips and stained with anti-HEYL as stated under "Experimental Procedures." LNCaP:HEYL cells were treated with 1 μM doxycycline (*DOX*) for 72 h to induce HEYL expression. The *first column* shows nuclei stained with DAPI (*blue*), the *second column* shows the same nuclei stained positive for HEYL (*green*), and these images were merged to create the image in the *third column*. *Bottom panels*, LNCaP:HEYL cells were cultured as above in the presence of 1 μM doxycycline for 72 h and serum-starved for a further 48 h. Cells were then treated with hormone (10 nM mibolerone) for 2 h and processed as stated under "Experimental Procedures." The *first panel* shows nuclei stained with DAPI (*blue*), the *second panel* shows the same nuclei stained for HEYL (anti-FLAG; *green*), the *third panel* shows the same cells stained for AR (*red*), and these images were merged to create the image in the *fourth panel*. The *scale bar* represents 10 μm. *Inset*, LNCaP:HEYL cells were cultured as above, lysed, resolved by SDS-PAGE, and probed with either anti-HEYL, anti-FLAG, or anti-β-actin antibody. *B*, prostate needle biopsies were immunostained with anti-HEYL as described under "Experimental Procedures." Adjacent tissue for each patient is shown in the *first column* (benign), and in the *second column*, areas that contain regions of cancer are indicated by the corresponding Gleason grade. Areas positive for HEYL are *brown*, and negative nuclei are *blue*. *C*, patient tissues were scored for nuclear (*Nuc.*) and cytoplasmic (*Cyt.*) intensity and plotted. The scoring was performed by a pathologist as follows: *1*, 5–10 positive per four high power fields in a section; *2*, 10–20 positive; *3*, 20–30 positive; *4*, 30–40 positive. The *scale bar* represents 20 μm.

important in nuclear localization (^{49}ARKRRR^{54}) exhibits similarity to the same region in HEY2 (^{48}ARKKRR^{53}) and HEYL (^{43}ARKKRR^{48}). These regions are basic in nature and highly homologous to the SV40 large T antigen NLS (^{126}PKKKRKV^{132}). Furthermore, using NetNES (a bioinformatics tool that identifies regions that exhibit NES properties; Ref. 35), we identified regions within HEY1 (amino acids 138–160), HEY2 (amino acids 137–159), and HEYL (amino acids 132–155) that have multiple spaced leucine/hydrophobic amino acids and are

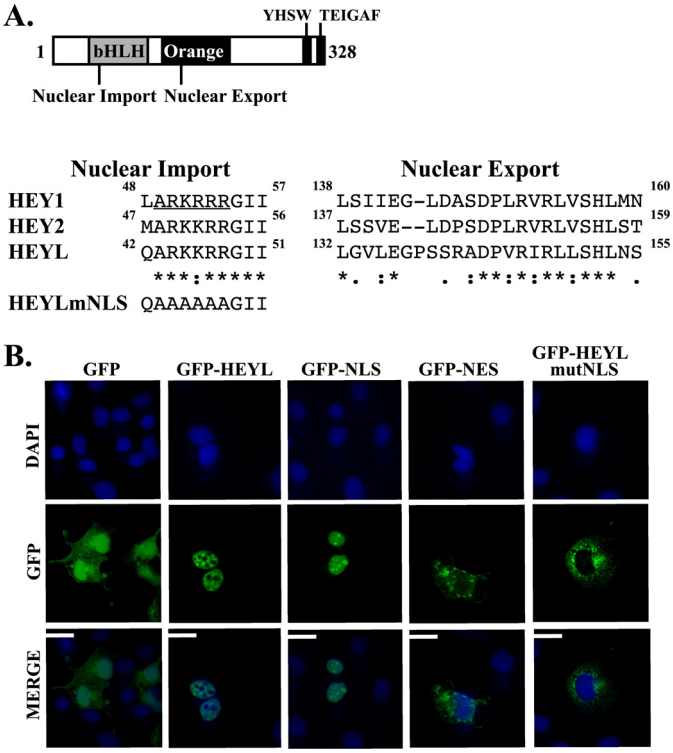

FIGURE 8. **Identification of putative nuclear localization and export sequences in HEYL.** *A*, schematic representation of putative nuclear localization and export sequences in HEY proteins identified through bioinformatics approaches. The previously identified NLS in HEY1 is *underlined* (ARKRRR) (32). *Asterisks*, conserved amino acids; *double dots*, conserved substitutions; *dots*, semiconserved substitutions. *B*, COS-1 cells were transfected with expression plasmids (200 ng) for either GFP alone (*GFP*), GFP fused to HEYL (*GFP-HEYL*), amino acids 42–51 representing the NLS of HEYL (*GFP-NLS*), amino acids 132–155 representing the NES of HEYL (*GFP-NES*), or GFP fused to full-length HEYL with mutated NLS (*GFP-HEYLmutNLS*). After 48 h, cells were fixed and processed as stated under "Experimental Procedures." The *upper row* shows nuclei stained with DAPI (*blue*), the *middle row* shows the same cells imaged directly for GFP (*green*), and these images were merged to create the image in the *bottom row*. The *scale bar* represents 10 μm.

suggestive of an NES (Fig. 8*A*). To examine the putative NLS and NES regions of HEYL, we created GFP-HEYL, GFP-NLS (containing HEYL residues 42–51), GFP-NES (containing HEYL residues 132–155), and GFP-HEYLmutNLS constructs and transfected these into COS-1 cells. By confocal microscopy, we found that addition of the short stretch of amino acids HEYL(42–51) was sufficient to alter the nuclear/cytoplasmic localization of GFP to entirely nuclear (Fig. 8*B*, compare *first* and *third columns*), an expression pattern similar to that observed for endogenous HEYL in RWPE-1 and BPH-1 cells, transfected wild-type HEYL in LNCaP:HEYL cells (Fig. 7*A*), and GFP fused to full-length HEYL in COS-1 cells (Fig. 8*B*, *second column*). Furthermore, we confirmed the role of amino acids 42–51 in nuclear localization by mutating basic amino acids to alanine residues to create GFP-HEYLmutNLS and observed that expression was cytoplasmic (Fig. 8*B*, compare *second* and *fifth columns*). In contrast, GFP-NES was seen solely in the cytoplasm, confirming that amino acids 132–155 of HEYL contain a region that can function as an NES (Fig. 8*B*).

DISCUSSION

The activity of the AR is controlled at various stages, including ligand binding, protein degradation/stabilization, post-translational modification, DNA binding, and recruitment of coregulator proteins (2). To activate transcription in response to ligand, the AR must recruit a variety of coactivator proteins to the regulatory regions of target genes. The best characterized coactivators include members of the SRC/p160 family, which potentiate transcriptional activation in part by directly acetylating chromatin (36). To fine-tune transcriptional control and gene expression, the AR can also recruit corepressor proteins and complexes that contain proteins with HDAC activities, which recondense chromatin by deacetylating histone tails (21). We have previously studied two members of the HEY family of corepressors and found that HEY1 and, to a lesser extent, HEY2 are AR-specific corepressors (25). Until now, the role of HEYL in regulating steroid signaling was unknown; therefore, we cloned full-length *HEYL* from an MCF7 cDNA library and examined the effects on AR activity by exogenous expression in a variety of mammalian cell lines. We found that, like HEY1, HEYL represses AR activity and is indeed a more potent corepressor in a variety of cell lines, including prostate cancer cells. From previous studies, it appears that HEY proteins tend not to be co-expressed in the same cells with the exception of some cell types during cardiac/vascular development and that there is some redundancy between the family members as evidenced by the non-lethal phenotype of single knock-outs (34, 47). We found that addition of HEY1 or HEY2 did not increase the repressive effect of HEY2 on AR signaling, a preliminary indication that functional redundancy of HEY proteins also extends to their effect on androgen signaling.

Furthermore, we found that HEYL was able to repress a strong transactivation domain in *trans* and contains two distinct repression domains, an N-terminal HDACI/II-independent domain that is dispensable for AR-specific repression, and a C-terminal domain. Repression of androgen target genes was not significantly altered by TSA, suggesting that HEYL represses AR activity via HDAC-independent or "passive" mechanisms, although we cannot rule out recruitment of Class III HDACs. This suggests differences in modes of action of the HEY proteins because intrinsic HEY1 repression was largely TSA-sensitive and therefore HDACI/II-dependent, although the effect of TSA on AR target genes was not specifically investigated (25). The C-terminal region of HEYL, which appears to be instrumental in AR repression, contains two short amino acid motifs showing conservation between HEY family members. YHSW is positioned similarly to a WRPW motif in the related Hairy/Enhancer of split repressors where it mediates interaction with the Groucho family of repressors (for a review, see Ref. 23). However, Groucho and related repressors showed no functional interaction with HEY proteins (for a review, see Ref. 37), and deletion of the homologous YRPW and TEIGAF motifs from HEY1 did not affect its repressive activity (25). Interestingly, the Orange domain also did not appear from our assays to be involved in repression (either intrinsic or AR-specific) despite Orange domains, which are unique to this bHLH-Orange family of proteins, being linked to repression of the ARNT and MASH transcription factors (38, 39). However, it has been suggested that the Orange domain is required for specificity of the bHLH-PAS proteins (40); hence, it may have a more subtle role in transcriptional repression. Castella *et al.*

(38) postulate that the Orange domain in Hairy/Enhancer of split factors interacts with the WRPW motif to promote repression via this domain, and such an intramolecular interaction could explain why in our assays the C-terminal domain of HEYL, although the only region capable of repressing AR activity in isolation, does not repress AR to the same extent as full-length HEYL.

Unlike other steroid hormone receptors, AF1 in the N-terminal domain of the AR is the major transactivation domain, and deletion of AF2 results in a constitutively active receptor (31, 33). We found that HEYL can repress the activity of AF1 in isolation, and furthermore, GST-HEYL was able to interact with AR through the AF1 domain (although it is not yet clear whether this interaction is direct). A characteristic of AR-AF1 is the ability of this domain to interact with the coactivator SRC1, and this interaction may be more functionally relevant than well characterized interactions of coactivators with AF2 (via L*XX*LL interaction motifs) as described for other nuclear receptors (41). We previously showed that HEY1 interacts with the bHLH-PAS domain of SRC1 and hypothesized that HEY1 can repress AR activity by blocking SRC1e/AR interactions and/or sequestering SRC1e from functional transcriptional complexes (25). In support of this, we found that HEYL was also able to effectively compete with SRC1e activation of both full-length receptor and AF1-dependent transactivation, suggesting a common mechanism of repression. Furthermore, by chromatin immunoprecipitation, we observed reduced loading of both AR and SRC1 on the *PSA* enhancer when HEYL was overexpressed. In the context of transcriptional activation, competition between a coactivator and corepressor may be an important regulatory process (21). Indeed, AR corepressors such as HEY1 (25), Cyclin D1 (42), Prohibitin (20), Filamin-a (43), and short heterodimer partner (44) have been shown to compete with p160 proteins, resulting in a down-regulation of AR activity. This may be an important step in regulating the access of coactivators to AR transcriptional complexes.

In summary, we have reported that HEYL, a member of the bHLH family of transcription factors, is an AR corepressor that down-regulates AR activity by functionally competing with SRC1 (and possibly other coactivators), resulting in attenuated SRC1 loading at the regulatory regions of AR target genes. *In vivo*, HEYL repressed several endogenous AR-regulated genes and, importantly, also inhibited the growth of the LNCaP prostate cell line, suggesting that HEYL may be an important factor in the development of prostate cancer. In human prostate tissue, HEYL was excluded from the nucleus in prostate cancer but not benign tissue in a manner similar to that previously reported for HEY1, suggesting a mechanism common to both corepressors. We hypothesize that nuclear exclusion of HEY family proteins may be a direct result of or may be directly involved in disease progression. We previously found that a cytoplasmic mutant form of HEY1 can act as a coactivator rather than as a corepressor of AR, indicating that mislocalization of such cofactors may radically alter their function and could thus contribute to disease progression (32). In support of this, p44, an AR coregulator, is mislocalized to the cytoplasm in prostate cancer but not matched benign sections (45). Intriguingly, nuclear p44 appears to have an antiproliferative role, but cytoplasmic p44 has an opposing, proliferative role in prostate cell line growth (45, 46). Further analysis of primary tumor biopsies, sequencing of *HEY* genes for mutations in regions important for nuclear localization, and analysis of potential sites of post-translational modification may increase our understanding of the mechanisms and significance of HEY protein exclusion in prostate cancer.

Acknowledgments—We are grateful to Prof. Albert Brinkmann, Prof. Jan Trapman, Dr. Robert Kypta, Dr. Guido Jenster, Prof. Frank Claessens, and Prof. Andrew Cato for kind gifts of plasmids and cell lines. Also, we thank members of the Androgen Signalling Laboratory and Prostate Cancer Research Group for technical help, critical comments, and suggestions.

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