Androgen receptor interacts with a novel MYST protein, HBO1

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Running title: HBO1 modulates androgen receptor activity

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SUMMARY

The androgen receptor (AR), a member of the nuclear receptor superfamily, plays a central role in male sexual differentiation and prostate cell proliferation. Results of treating prostate cancer by androgen ablation indicate that signals mediated through AR are critical for the growth of these tumors. Like other nuclear receptors, AR exerts its transcriptional function by binding to cis-elements upstream of promoters and interacting with other transcriptional factors (e.g., activators, repressors and modulators). To determine the mechanism of AR-regulated transcription, we used the yeast two-hybrid system to identify AR-associated proteins. One of the proteins we identified is identical to the human origin recognition complex (ORC1)-interacting protein termed HBO1. A ligand-enhanced interaction between AR and HBO1 was further confirmed in vivo and in vitro. Immunofluorescence experiments showed that HBO1 is a nuclear protein, and Northern-blot analysis revealed that it is ubiquitously expressed, with the highest levels present in human testis. HBO1 belongs to the MYST family, which is characterized by a highly conserved C2HC zinc finger and a putative acetyltransferase (HAT) domain. Surprisingly, two yeast members of the MYST family SAS2 and SAS3, have been shown to function as transcription silencers, despite the presence of the HAT domain. Using a GAL4 DNA binding domain (DBD) assay, we mapped a transcriptional repression domain within the N-terminal region of HBO1. Transient transfection experiments revealed that HBO1 specifically repressed AR-mediated transcription in both CV-1 and PC-3 cells. These results indicate that HBO1 is a new AR-interacting protein capable of modulating AR activity. It could play a significant role in regulating AR-dependent genes in normal and prostate cancer cells.
INTRODUCTION

The androgen receptor (AR), as a member of the superfamily of ligand-dependent nuclear receptors, plays a central role in male sexual development and in normal and malignant prostate cell proliferation (1-4). Ligand-dependent nuclear receptors typically contain three domains: a carboxy-terminal ligand-binding domain (LBD), an amino-terminal transactivation domain (TAD), and a DNA binding domain (DBD) located between the LBD and TAD. Another transactivation domain, termed AF-2, is present in the LBD of most steroid hormone receptors (5,6), but has not been clearly defined for the AR (7,8). The AR, and other receptors in this family, forms a complex with heat-shock proteins (HSPs) before exposure to the ligand, which appears to sequester the unbound AR (9-11). Upon binding to ligand, the AR dissociates from the HSPs and translocates to the nucleus, where it binds to the androgen response element (ARE) and recruits cofactors to regulate transcription (12,13). Recent evidence suggests that upon ligand binding there may also be an interaction between the LBD and TAD which contributes to transactivation (14).

Ligand-dependent nuclear receptors function by directly or indirectly interacting with other regulatory proteins in cells (see review by Xu et al. (15)). Several co-activators have been identified that bind to the nuclear hormone receptors to modulate their transcription (16-19). A group of AR associated proteins, termed ARA24 (20), ARA54 (21), ARA55 (22), ARA70 (23) and ARA160 (24), have been identified by yeast two hybrid screening and shown to modulate AR transcriptional activation. Interactions with CBP/p300 (25,26), AP-1 (27,28), cylin D1 (29), Stat 5 (30) and a fos containing complex (31) appear to directly couple AR and other related steroid hormone receptors to other pathways. A novel RING finger protein and a testis-specific nuclear protein were also found to interact with AR and/or other nuclear receptors (32,33). Recently, Muller et al. reported that the AR interacting protein, named FHL2, binds specifically to AR and enhances AR-mediated transcription (34).
HBO1, recently identified as a protein that interacts with the human ORC (origin recognition complex) (35), shares sequence similarities with the MYST subfamily. MYST subfamily members include the yeast proteins SAS2 and SAS3 (something about silencing) (36,37), *Drosophila* MOF (males absent on the first) (38), the human MOZ (monocytic leukemia zinc finger protein) (39), the human Tip60 (HIV Tat-interacting protein of 60kDa) (40), and a new human member, MORF (monocytic leukemia zinc finger protein-related factor) (41). The MYST proteins are characterized by a highly conserved C2HC zinc finger and a putative histone acetyltransferase (HAT) domain (42-44). To date there is no evidence to show that MYST proteins can actually acetylate the nucleosomal histones that are directly involved in transcriptional activation. Of interest, full-length HBO1 protein failed to show intrinsic HAT activity (35). Finally, the yeast SAS2 and SAS3 proteins have been shown to function as transcription silencers (37,44).

Here we demonstrate that HBO1, a MYST subfamily protein (35), can specifically interact with AR in the yeast two-hybrid system. A ligand-enhanced interaction between AR and HBO1 was further confirmed *in vivo* and *in vitro*. Several lines of experimentation demonstrated that HBO1 repressed AR-mediated transcription, and the domain responsible was localized to the N-terminal region. These results indicate that HBO1 is a new AR-interacting protein capable of modulating AR activity.

**EXPERIMENTAL PROCEDURES:**

**Yeast two-hybrid screen:** The C-terminal region of human AR (amino acids 505-919), spanning both the DBD and LBD, was fused to the GAL4 DBD in the pGBT9 vector (Clontech, Palo Alto, CA). The construct was transformed into a modified yeast strain PJ69-4A (45). The cDNA library from human prostate tissues with the GAL4 transactivation domain was used in this screening (Clontech, Palo Alto, CA). Transformants were selected on Sabouraud Dextrose medium lacking adenine, leucine, and tryptophan in the presence of 100nM di-hydrotestosterone (DHT). Positive clones were subjected to the β-galactosidase assay using the colony-lift filter method.
according to the manufacturer's instructions. Specificity of interaction was further confirmed by liquid β-galactosidase (β-gal) assay.

**Plasmid construction:** The N-terminal HBO1 was generated by PCR with two specific oligonucleotide primers. The forward primer is identical to published sequence right before the ATG translation start site (5’-GCCGCTAGCCCCGAATCGGAACCGTCGGG- 3’), and the reverse primer spans between amino acid residues 245 and 252 (5’ -GTGCCTGTTGCATGCTGTTGTTG -3’). The full length HBO1 cDNA was created by ligating the N-terminal PCR fragment and the C-terminal fragment originally isolated in the yeast two-hybrid screening into the pcDNA3 vector (Invitrogen, Carlsbad, CA) in-frame with an N-terminal hemagglutinin (HA) or flag epitope tag. The constructs were sequenced to confirm open reading frames and to verify that no mutations were introduced during PCR.

The human AR cDNA, cloned into an SV40 promoter-driven expression vector, pSV-ARo, was kindly provided by Dr. Albert Brinkmann (Erasmus University, Rotterdam, The Netherlands). The reporter plasmid MMTVpA3-Luc containing the luciferase gene under the control of androgen-responsive elements (AREs) in the MMTV-LTR, was provided by Dr. Richard Pestell (Albert Einstein Medical College, NY, NY) (46). An SV40 driven β-galactosidase reporter plasmid (pSV-β-GAL) (Promega, Madison, WI) was used in this study as an internal control. A human ER expression construct (pcDNA3-ER) and a luciferase reporter plasmid with three estrogen responsive elements (ERE) were kindly provided by Dr. Myles Brown (Dana-Farber Cancer Institute, Boston, MA). A human thyroid hormone receptor β (TRβ) expression vector driven by the SV40 promoter and a luciferase reporter controlled by two TREs were kindly provided by Dr. Anthony Hollenberg (Beth Israel Deaconess Medical Center, Boston, MA) (47,48).

Human HBO1 N-terminal (encoding 1-360 amino acids) and C-terminal (encoding 230-611 amino acids) fragments were cloned downstream of the GAL4 DNA binding domain in the pSP271 expression vector (kindly provided by Dr. Donald Ayer, University of Utah, Salt Lake City, UT).
and a modified pCDNA3 vector with a nuclear targeting sequence. Luciferase reporter constructs containing the chicken myelomonocytic growth factor gene minimal promoter (-41 to +61), with or without four GAL4 binding sites, were also provided by Dr. Donald Ayer. Both deletion mutants of HBO1 and AR were cloned into pGEX-2TK (Amersham, Arlington Heights, IL) for making GST fusion proteins as described previously (31).

**Northern Blot analysis:** Blots with RNA from multiple human tissues were obtained from Clontech Inc. The PolyA RNA was prepared from prostate cancer cell lines, including LNCaP, MDA PCa2a and 2b (49), PC-3, and TSU-pr1 (50), by using an Oligotex kit (Qiagen, Valencia, CA). For Northern blotting, 2 µg of polyA RNA were electrophoresed on a 1% agarose-formaldehyde gel, transferred to Hybond-N nylon membranes (Amersham, Arlington Heights, IL) by capillary blotting in 20X SSC, and hybridized with a DNA fragment (1-234 amino acids) derived from human HBO1. β-actin or GAPDH were used to normalize loadings.

**Immunofluorescence:** CV-1 or PC-3 cells were plated onto gelatin-coated (2%) coverslips the day before transfection. A HA-tagged HBO1 cDNA in pcDNA3 vector was transiently transfected into cells. After 24 hr, cells were fixed for 10 minutes with 3% paraformaldehyde in PBS and washed with 0.1% NP-40 in PBS. The cells were then incubated with either anti-HA-monomclonal antibody (Santa Cruz Biotech, Santa Cruz, CA, Cat. # sc-7392) or anti-HA polyclonal antibody (Cat.# sc-805) for 1hr at room temperature. Cells were washed three times followed by incubation with FITC-conjugated anti-mouse (Cat. # sc2010) or Rhodamine-conjugated anti-rabbit secondary antibody (Cat. # sc816). For staining of actin-cytoskeleton, cells were incubated with Rhodamine-conjugated Phalloidin (Sigma, St. Louis, MO).

**GST–pull down assay:** Expression and purification of GST fusion proteins were performed as described previously (31). The full length human HBO1 and AR proteins were generated and labeled in vitro by the TnT-coupled reticulocyte lysate system (Promega, Madison, WI). Equal amounts of GST-AR or -HBO1 fusion proteins coupled to glutathione sepharose beads were
incubated with radiolabeled HBO1 or AR proteins at 4°C for 2 hr in the modified NETN buffer (0.2% NP-40, 1mM EDTA, 20mM Tris-Cl, 100mM NaCl, 5% glycerol, 20µM ZnCl2, 4mM MgCl2, 0.5mM DTT, 1mM PMSF, and 10µg/ml Aprotinin). Beads were carefully washed 4 times in NETN buffer and then analyzed by SDS-PAGE followed by autoradiography.

**Immunoprecipitation and Western Blotting:** The human AR expression vector pARo, alone or with a Flag-tagged pCDNA3/HBO1 expression plasmid, was transfected into CV-1 cells. Transfected cells were cultured in the presence of 10 nM R1881 for 48 hr and then harvested in a buffer containing 20mM HEPES (pH8.0), 0.5% NP-40, 100 mM NaCl, 1mM EDTA, 5mM MgCl2, 1mM CaCl2, 10µM ZnCl2, 1mM DTT, 1mM PMSF, 5µg/ml Leupeptin, and 5% glycerol). Whole cell lysates were incubated with mouse normal IgG or Flag monoclonal antibody (Sigma, St Louis, MO) at 4°C for 2 hr. Pre-equilibrated Protein-A Sepharose beads were then added and, after an hour of incubation, collected by centrifugation and gently washed 3 times with the same buffer as described above. Specific protein complexes were eluted by 100ng/ml of Flag peptide in a buffer containing 10mM HEPES, 100mM NaCl, 1mM EDTA, and 0.1% NP-40. The eluted samples were boiled in SDS-sample buffer and resolved on a 10% SDS-PAGE. The proteins were transferred onto a nitrocellulose membrane and probed with 1: 200 dilution of a polyclonal antibody against the N-terminus of AR (Santa Cruz Biotech, Santa Cruz, CA, Cat# sc-816). Proteins were detected using ECL kit (Amersham, Arlington Heights, IL).

**Cell Cultures and Transient Transfections:** Both a monkey kidney cell line, CV-1, and a human prostate cell line, PC-3, were maintained in RPMI media supplemented with 10% fetal calf serum (HyClone, Denver, CO). Transient transfections were carried out by using a LipofectAMINE transfection kit (GIBCO/BRL, Gaithersburg, MD). Approximately 3x10^4 cells were plated in a 24-well plate 16 hr before transfection. About 400 ng of total plasmid DNA per well were used in the transfection. The total amount of plasmid per dish was kept constant by adding pcDNA3 empty vector. Approximately 16 hr after transfection the cells were washed and fed medium containing 10% charcoal-stripped (steroid hormone free) fetal calf serum (HyClone, Denver, CO) in the
presence or absence of steroid hormones. Cells were incubated for another 24 hr and luciferase activity was measured as relative light units (RLU) as previously described (51). The RLU from individual transfections were normalized by β-galactosidase activity in the same samples. Individual transfection experiments were done in triplicate and the results are reported as mean RLU/β-galactosidase (+SD) from representative experiments.

RESULTS

HBO1 interacts with the AR in yeast: To identify AR interacting proteins, we screened a human prostate library with a GAL4 DBD fusion encoding the DBD and LBD of AR. Of $2 \times 10^6$ transformants, 75 positive clones were identified by both adenine and β-galactosidase production in the presence of DHT. Sequence analysis revealed that one of these clones (PG159) is identical to the C-terminus of a recently identified novel protein (GeneBank Accession Number AF074606), named HBO1 (Histone acetyltransferase Binding to the Origin recognition complex 1) (35). To confirm this interaction, we transformed the PG159 clone with various constructs containing either GAL4DBD alone, or fusion proteins with different fragments derived from AR (Fig 1A). On the adenine minus plate, yeast colonies were only observed in the samples transformed with PG159 and GAL4DBD-AR fusion construct containing both the DBD and LBD in the presence of androgen (data not shown). There were no colonies observed in the transformants with other AR fusions. Liquid β-gal assay was performed to quantify the interactions. Constructs containing the GAL4 activation domain (AD) and either the C-terminal HBO1 or ARA70 (23), an AR coactivator, showed a strong induction with the AR-DBD-LBD in the presence of DHT. An AR-LBD construct also showed an increase in β-gal in the presence of DHT (Fig.1B). These results imply that HBO1 protein interacts with AR LBD in the presence of DHT, and that the interaction is more pronounced in the presence of the DBD.

HBO1 is a new member of the MYST superfamily. It contains a unique N-terminal serine-rich region and the conserved C-terminal region with a C2HC zinc finger and putative
acetyltransferase domain (Fig 2). To generate the full-length HBO1 cDNA, primers were designed to amplify the N-terminal fragment of HBO1. A 0.7Kb fragment was generated from the Marathon Ready cDNA libraries derived from human prostate tissues that perfectly matched the published sequence of HBO1 (35). Comparison of the C-termini of Tip 60 and HBO1 revealed significant sequence identity (Fig. 2).

**HBO1 interacts with AR in vitro & in vivo:** To verify the interaction observed in the yeast two-hybrid system, GST pull-down experiments were carried out with a series of GST-AR fusion proteins (31). 35S-methionine labeled full-length HBO1 protein bound to GST-AR fusion proteins was analyzed by SDS-PAGE and detected by autoradiography. As seen in Figure 3A, a strong retention of HBO1 protein was specifically observed for the samples with GST-AR containing both the DBD and LBD (505-919 amino acids) in the presence of DHT. A weak interaction was also observed for the full LBD (676-919 amino acids), but not with AR-AD (1-563 amino acids), DBD (505-676 amino acids), and GST or beads alone. Using two additional GST-AR fusion proteins consisting of the partial LBD (676-844 amino acids) and AF2 truncated mutants (875-919 amino acids), we further determined that the full length LBD is required for the interaction (Fig. 3A, right panel). In these experiments, the interactions were not significantly affected by adding DHT (data not shown). These results are consistent with those of the yeast-two hybrid experiments and suggest that both the DBD and LBD of AR are required for the interaction with HBO1.

To map the interaction region of HBO1 protein, GST pull-down experiments were repeated with GST-HBO1 fusion proteins containing the N-terminal (1-360 amino acids) or C-terminal domains (230-611 amino acids). As shown in figure 3B, the C-terminus of HBO1 strongly precipitated in vitro-translated AR protein. A recent report has shown that the C-terminal region of Tip60, another member of the MYST subfamily, interacts with AR (52). This observation further suggests the possibility that both the C2HC zinc finger and acetyltransferase domain may contribute to the binding with AR (Fig.2).
To determine whether an interaction between HBO1 and AR occurs \textit{in vivo}, we tagged HBO1 at its amino terminus with a Flag epitope and expressed this tagged HBO1 protein together with AR protein in CV1 cells. Both AR and Flag-HBO1 proteins were detected in the transfected cells (Fig. 3C, top and middle panels). Whole cell lysates were immunoprecipitated with normal mouse IgG or an anti-Flag monoclonal antibody. To increase the specificity of protein complexes precipitated by Flag antibody, the immunoprecipitates were eluted with antigenic Flag peptide. Eluted protein complexes were then analyzed by Western-blot using AR N-terminal antibody. As shown in the bottom panel of Figure 3C, AR protein was detected in anti-Flag immunoprecipitates from cells co-transfected with AR and Flag-HBO1 (lane 4), but not in those from cells transfected with AR alone (Lane 2) or when normal mouse IgG was used (Lanes 1 and 3). These results demonstrated that interaction between the full length AR and HBO1 proteins also occurs \textit{in vivo}.

**HBO1 is a nuclear protein and highly expressed in human testis:** Northern blot analysis was carried out to detect the expression of HBO1 in human tissues with a specific probe covering the first 234 amino acid residues of HBO1 protein. A 3.6 Kb transcript of HBO1 was detected ubiquitously in various tissues but most abundantly in testis (Fig. 4A). In contrast, Iizuka & Stillman recently reported that HBO1 was highly expressed in ovarian tissue (35). To address this issue, the Northern-blot analysis was repeated following the conditions described in the previous report. Again, we found that HBO1 is highly expressed in human testis (data not shown).

We also performed Northern blot analysis on RNA samples isolated from several prostate cancer cell lines, including AR-positive cells (LNCaP, MDA PCa 2a and MDA PCa 2b) and AR-negative cells (PC-3 and TSU) (Fig. 4B). Using the same DNA probe as above, a 3.6 kb transcript of HBO1 was consistently detected in all cell lines tested (Fig. 4B).

To determine whether the AR-HBO1 interaction takes place upon AR translocation into the nucleus, immunofluorescence assays were performed to examine the subcellular localization of HBO1 protein. As shown in Figure 4C, the HBO1 protein is exclusively localized in the nuclei of
transfected CV-1 and PC-3 cells. To determine whether the nuclear location of HBO1 is regulated by androgen, the experiments were repeated in the presence or absence of DHT. There was no difference in HBO1 localization. In contrast, as previously reported by others, nuclear translocation of AR was observed in cells transfected with an AR expression vector in the presence of DHT (data not shown).

**HBO1 represses transcription upon fusion to a heterologous DNA binding protein:**
Potential functional domains of HBO1 include a unique N-terminal serine-rich region and a common C-terminal MYST domain (Fig.2). To investigate possible effects of HBO1 on transcription, fragments containing the N-terminal (1-360 amino acids) or C-terminal domains (230-611 amino acids) of HBO1 were targeted to DNA by fusion with the GAL4 DBD (53). These constructs were then tested for their ability to modulate transcription from a minimal promoter, derived from the chicken myelomonocytic growth factor gene (-41 to +61), driving transcription from a luciferase reporter gene (Luc reporter) (54).

Limited induction of the GAL4/Luc reporter versus the Luc reporter was observed upon co-transfection of a plasmid carrying the GAL4 DBD alone (Fig.5), consistent with earlier evidence of a previously described cryptic transcriptional activation domain between amino acids 97 and 147 of the GAL4 DBD (55). Fusion of the GAL4 DBD to full length HBO1 showed slight repression of this induction. Moreover, activation by the GAL4 DBD was inhibited by more than 70% upon fusion with the N-terminal region of HBO1, suggesting that there is an intrinsic repression domain within this region of HBO1. In contrast, there was no effect when the C-terminal domain of HBO1 was used, which is consistent with the previous results that showed no intrinsic transcriptional activity from the MYST domain of Tip60 (52).

**HBO1 represses AR mediated transactivation:** Given the identification of an intrinsic repression domain within the HBO1 protein, we further examined the possible effect of HBO1 on the function of AR. AR and HBO1 expression plasmids as well as a luciferase reporter plasmid
regulated by the androgen responsive elements (AREs) in the MMTV-LTR (MMTVpA3-Luc) were transfected into CV-1 cells. In the absence of transfected HBO1, an approximately 20-fold induction of AR-mediated transcriptional activity was observed in the presence of 100nM DHT (Fig. 6A). Ligand-dependent AR activation was repressed by more than 60% by co-transfection of the HBO1 expression construct at AR-HBO1 plasmid ratios of 1:4 (Fig. 6A). In contrast, co-transfection with ARA70, an AR co-activator, showed more than a 3-fold induction (Fig. 6A). The repression by HBO1 was not associated with a change in the intracellular steady state level of AR protein (data not shown), suggesting that repression resulted from direct effects on AR transactivation of its target promoter and not on down-regulation of AR expression.

To confirm the repression of AR activity by HBO1, transient transfections were repeated in CV1 cells with a luciferase reporter driven by the human prostate specific antigen (PSA) promoter which is solely regulated by AR and has been widely used to analyze AR-mediated transcription activity (13). A similar inhibitory effect of HBO1 was observed in these experiments (Fig. 6B). To further evaluate the functional interaction of AR and HBO1 in a physiologically relevant cellular context, transient transfections were repeated in PC-3 cells, a human prostate cancer cell line. As shown in Figure 6C, the ligand dependent induction by AR was repressed by co-transfection of HBO1 in PC-3 cells.

To map the repression domains of HBO1 in the AR mediated transcription, both the N- and C-terminal regions of HBO1 were transfected into CV-1 cells. Like the full length HBO1 protein, the N-terminal domain of HBO1 repressed AR-induced activation (Fig. 6D), which was consistent with the transcriptional inhibition by this domain when fused to the GAL4 DBD (Fig. 5). In contrast, the C-terminal region was shown no significant effect.

In view of the previous study showing that a truncated Tip60 protein functions as a co-activator for AR, ER, and PR (52), our finding that HBO1 represses AR-mediated transcription was quite surprising. To assess Tip60 transcriptional activity in our system, an expression vector
encoding a full length Tip60 protein was constructed and used in transient transfection experiments. As shown in Fig. 6D, Tip60 repressed AR-mediated induction, similar to that of HBO1. The differences between our results and those previously published are probably due to the use of full length versus truncated Tip60 proteins. To ensure that HBO1-mediated inhibition did not reflect toxic or other nonspecific effects of the co-transfected plasmids, luciferase expression from the above experiments was normalized using β-galactosidase production from a co-transfected plasmid in all the experiments (Fig. 6).

Finally, we asked whether HBO1 could repress transcription mediated by other nuclear receptors. A human estrogen receptor (ER) or thyroid hormone receptor (TR) expression plasmid was co-transfected with the HBO1 expression vector and the respective luciferase reporters in CV1 cells. In contrast to the observed suppression of AR-mediated transcription, HBO1 had no inhibitory effect on either ER- or TR-mediated transcription (Fig. 6E & F).

**DISCUSSION**

The results described here demonstrate that the AR interacts with HBO1, a new member of the MYST subfamily of proteins (35). The LBD and DBD of AR are required for this interaction. When bound to AR, HBO1 represses transcription from AR element containing promoters. The transcriptional repression domain was determined to be in the N-terminal region of HBO1, which contains a serine-rich domain. HBO1 protein is exclusively localized in the nucleus, consistent with a role in regulating transcription. Like most nuclear receptor co-factors, HBO1 is ubiquitously expressed, but it is expressed at particularly high levels in testis tissues. Since HBO1 was originally identified through its interaction with the human ORC, it appears that HBO1 has important roles in both DNA replication and transcription.

Others have shown that another member of the MYST subfamily, Tip60, also interacts with AR (52). Tip60 was originally identified as an interacting protein of Tat, a transcription activator of
human immunodeficiency virus (40). Like HBO1, it is ubiquitously expressed and its normal cellular functions are not yet defined. Overexpression of Tip60 results in a modest increase of Tat transactivation of the HIV promoter in transient expression assays (40). Both HBO1 and Tip60 were identified as AR-interacting proteins using the yeast two-hybrid system. We have further confirmed that the interaction between HBO1 and AR occurs in vivo by co-immunoprecipitation of these two proteins from mammalian cell lysates. Both HBO1 and Tip60 proteins showed a stronger interaction with the DBD-LBD domains of AR than with the LBD domain alone. Androgen enhances the interaction, suggesting that conformational changes in the LBD upon binding to ligand are necessary for this interaction. Using GST-pulldown assays, we further showed the C-terminal region of HBO1 mediates binding to the AR. Sequence analysis showed no LXXLL motif in this region, suggesting other unidentified motifs may contribute the interaction. In this regard, it will be interesting to determine whether other MYST subfamily members interact with AR.

In this study, we determined that the binding of both HBO1 and Tip60 to AR leads to repression of AR-mediated transcriptional activation. The repressor effect was unexpected, especially given a previous report indicating that Tip60 was an AR co-activator (52). The Tip60 clone used in those previous studies lacked the N-terminal 70 amino acids, which may be potentially important for the transcriptional repression function. We know from our studies that the repression function is most active when a truncated HBO1 containing only the first 360 amino acids is used, and that deletion of the first 230 amino acids totally abrogates the repression activity of HBO1. Thus, our finding that Tip60 acts as a transcriptional repressor may be due to the presence of the N-terminal 70 amino acids in the full-length clone used here. Many transcription factors and co-activators contain cryptic functional domains that are uncovered when deletion mutants are studied (51,54,56-58). We believe that this is the case for Tip60; the transcription co-activation function was a result of the deletion of part of the repression domain. Further work with deletion mutants will be needed to confirm this possibility.
We found that HBO1 probably does not act broadly as a nuclear hormone receptor co-factor, as it repressed AR-dependent promoter activity but not activities dependent on ER or TR. We have not tested all possibilities, so it is possible that HBO1 repressor activity will not be limited to the AR. Studies using the truncated Tip60 protein (52) indicated that it functioned as a co-activator for all nuclear hormone receptors tested (AR, ER and PR). Whether this will be true when the full-length protein is used is currently unclear. While it is clear that members of the MYST subfamily of proteins are involved as co-factors to regulate transcription from genes dependent on a number of nuclear hormone receptors, much more work needs to be done to determine the whole spectrum of effects.

The N-terminal serine-rich domain of HBO1 appears to be unique as it has not been reported in other members of MYST subfamily. This domain of HBO1 mediates repression both in trans and in cis suggesting it either contains an inhibitory domain or that it leads to recruitment of other transcription repressors to the AR transcription complex (53,59). The repression effect was observed with two different AR regulated promoters, MMTV and PSA. This result leads us to speculate that if HBO1 recruits other factors, these factors probably bind directly to the N-terminal HBO1. Understanding the mechanism for how the N-terminal domain of HBO1 represses transcription will require further studies.

Despite the presence of the HAT domain and the knowledge that acetylation is important in transcriptional regulation, there is currently no evidence showing that MYST proteins can acetylate the nucleosomal histones that are directly involved with transcriptional activation. Tip60 and HBO1 have been shown to have HAT activity, with potentially interesting substrate specificities. However, these activities are weak, and it is not entirely clear that they are intrinsic activities of the proteins (35,40,52). It is possible that Tip60, HBO1 and other members of the MYST family are part of multi-protein complexes that contain HATs, but are co-factors rather than HATs themselves.
In conclusion, HBO1, a protein initially identified as being associated with the ORC, has been found to bind to AR and to negatively regulate transcription from AR-dependent promoters. The biological importance of HBO1 in DNA replication and transcription awaits further studies.
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FOOTNOTES:

1. Abbreviations used in this paper are: AR, Androgen receptor; CBP, CREB-binding protein; AD, activation domain; DBD, DNA binding domain; LBD, ligand binding domain; DHT, dihydrotestosterone; ER, estrogen receptor; TR, thyroid receptor; GST, glutathione S-transferase; HAT, histone acetyltransferase; ORC, origin recognition complex; HBO, histone acetyltransferase bound to ORC; SAS, something about silencing; Tip60, Tat interacting protein.

2. This work was supported by National Institutes of Health Grants CA70297 (ZJS), DK47636 (BL), and American Cancer Society Grant RPG98213 (ZJS).
Acknowledgments:

We are especially grateful for the various reagents received from Drs. A Brinkmann, Richard Pestell, Myles Brown, Anthony Hollenberg, Channadurai G., Donald Ayer, and Nora Navone. We thank Dr. Donna Peehl for critical reading of the manuscript, Drs. Steven Hayes, Fajun Yang, and other members of the Laboratory for useful discussions and comments, and Homer Abaya for administrative assistance and help in preparing this manuscript.
Figure Legend:

Figure 1. Mapping the AR domain that interacts with HBO1 in the yeast two-hybrid system: (A) Schematic representation of the full length AR, and its various domains fused to the GAL4 DNA binding domain including the activation domain (AD), DNA binding domain (DBD), ligand binding domain (LBD), and DBD-LBD. Numbers correspond to amino acid residues. (B) Yeast strain PJ-69 4A was co-transformed with different domains of AR fused to the GAL4-DBD and clone PG159 (HBO1 amino acids 230-611). The transformants were plated on selective media with or without 100nM DHT. For negative control, AR-DBD-LBD was also co-transformed with the SMAD7 gene fused to the GAL4 activation domain (negative control), and plated in the presence of 100 nM DHT. Liquid β-galactosidase assay was performed for yeast transformed with different domains of AR and HBO1 clone. β-galactosidase activities were measured using the Galacto-light Plus kit (Tropix Inc, Bedford, MA) and normalized by total protein. Results shown in the figure are the mean from three separate experiments.

Figure 2. Sequence analysis of HBO1 protein: The important features of the HBO1 protein sequence are illustrated. The N-terminal serine rich region is boxed and the serine residues are underlined. The start site of yeast clone PG159 is indicated by the arrow. The C-terminal HBO1 (a.a 315-611) is 50% homologous to the C-terminal Tip60 (a.a 211-513). This region includes the unique zinc finger (boxed) and the putative HAT domain. The identical amino acids in this region are in bold type and marked by stars. The numbers on the right correspond to the amino acid of the respective proteins.

Figure 3. HBO1 interacts with AR in vitro & in vivo: (A) In-vitro translated HBO1 protein (35S-labeled) was incubated at 4°C for 2 hr with an equal amount of various GST-AR fusion proteins coupled to sepharose beads (see Materials & Methods), which include the full ligand binding domain (LBD, 676-919 amino acids), DNA binding domain (DBD) and LBD (DBD-LBD, 505-919 amino acids), activation domain (AD, 1-563 amino acids), DBD alone (505-676 amino acids), partial LBD
(pLBD 676-844 amino acids), and AF2 truncated mutants (875-919 amino acids). The beads were washed and proteins resolved on 10% SDS-PAGE gel were analyzed by autoradiography. \(^{35}\)S-labeled HBO1 is marked in the figure by an arrow. (B) GST pulldown experiments were carried out with the GST-HBO1 fusion proteins containing the N-terminal (1-360 amino acids) and C-terminal domains (230-611 amino acids) and \textit{in-vitro} translated HBO1 protein. (C) Co-immunoprecipitation of AR and HBO1 proteins were performed in the transfected CV-1 cells with AR expression construct (pARo) alone or together with a Flag-tagged HBO1 expression plasmid (pCDNA3-Flag-HBO1). 48 hr after transfection, total cell lysates were analyzed by Western-blot to check expression of AR (the top panel) and HBO1 (the middle panel) proteins. Equal amounts of cell lysates were immunoprecipitated with normal mouse IgG or anti-Flag Monoclonal antibody at 4\(^{\circ}\)C. The immune complex bound to Protein-A sepharose beads was then eluted from Protein-A sepharose beads with 100ng/ml of Flag peptide in elution buffer. The eluted fractions were then resolved by SDS-PAGE and analyzed by Western blot using anti-AR polyclonal antibody (the bottom panel).

Figure 4. Expression of HBO1 in human tissues and prostate cells: (A) A mRNA blot for multiple human tissues was hybridized with a \(^{32}\)P-labeled 700 bp DNA fragment encoding the N-terminal region of the HBO1 gene (top). The same blot was stripped and rehybridized with a \(\beta\)-actin probe to confirm equal RNA loading (bottom). (B) PolyA RNA samples isolated from AR positive prostate cancer cell lines (LNCaP, MDA PCa2a and 2b) or AR negative cell lines (PC-3 and TSU), were hybridized with the same HBO1 probe as described above. In this experiment, a human glyceraldehyde-3 phosphate dehydrogenase (GAPDH) probe was used as a control for equivalent RNA loading. The HBO1 transcript is marked by an arrow in the figure. (C) pCDNA3-HA-HBO1 construct was transfected into CV-1 (left) or PC3 cells (right). CV-1 cells were stained with anti-HA monoclonal antibody and revealed by FITC-conjugated goat anti-mouse antibody (green). To show actin-cytoskeleton, CV-1 cells were doubled stained with Rhodamine-conjugated phalloidin (red). PC3 cells were stained with anti-HA polyclonal antibody followed by Rhodamine-conjugated anti-
rabbit antibody (red). In both CV-1 and PC3 cells, HBO1-positive staining cells are marked with arrows.

Figure 5. Detection of transcription repression of HBO1 by GAL4DBD-HBO1 fusion proteins. (A) Schematic representation of HBO1 and deletion mutants fused to GAL4-DBD. The full length, N-terminal (amino acids 1 to 360), and C-terminal (amino acid 230 to 611) HBO1 were fused to GAL4 DBD (amino acids 1-147) in the pSP271 expression vector. (B) The GAL4-DBD-HBO1 expression plasmids (40ng/well) and the pSP-GAL4-VP16 activation domain plasmid were transfected into CV-1 cells with 200ng/well luciferase reporter constructs containing the chicken myelomonocytic growth factor gene minimal promoter (-41 to +61) with or without four GAL4 binding sites, which were labeled as GAL4/Luc reporter and Luc-reporter, respectively, were transfected into CV-1 cells with 0.2µg/well of GAL4-Luc or Luc-reporter constructs. Transcription activity was determined by measuring luciferase (LUC) activity 40 hr post transfection and relative light units (RLU) were corrected based on the amount of β-galactosidase production.

Figure 6. HBO1 represses AR-mediated transactivation in CV-1 and PC-3 cells. (A) CV1 cells were transiently transfected in 24 well plates with 200ng of pMMTV-Luc, 50ng of pSV40-β-gal, 10ng of pARo, and pcDNA3-HBO1 expression plasmid using LipofectAMINE transfection method as described under "Experimental Procedures". The total amount of plasmid transfected per well were kept constant by adding empty pcDNA3 vector as needed. Transfected cells were incubated in complete medium for 16-18 hr, then washed and incubated in DMEM with 5% charcoal-stripped fetal calf serum with or without 100 nM DHT for another 24 hr. Luciferase activity was reported as relative light units and represented as mean±SD. (B) The transient transfections were repeated in CV1 cells with a luciferase reporter driven by the 7Kb promoter fragment of human prostate specific antigen (PSA). 200ng of PSA-luc reporter, 100ng of β-gal, and 20ng of pARo plasmids per well were used in the experiments. (C) The effect of HBO1 on AR mediated transcription of pMMTV-Luc reporter was assayed in a human prostate cancer cell line, PC-3. The same amount of plasmids were used in the experiment as described above. (D) The same amount of pMMTV, pSV40-β-gal,
and pARo plasmids plus the full length or truncated constructs of HBO1, a full length Tip 60, and ARA70 were transfected into CV1 cells as described above. (E) A human ER expression vector, HBO1 expression vector, a luciferase reporter controlled by 3 EREs and a $\beta$-galactosidase control plasmid were transfected into CV1 cells. Cells were washed and incubated in DMEM with 5% charcoal-stripped fetal calf serum. 10 nM 17$\beta$-estradiol (E2) was used as a ligand for the ER. (F) A human thyroid hormone receptor $\beta$ expression vector and a luciferase reporter controlled by two TREs were cotransfected into CV-1 cell with different concentrations of HBO1 plasmid as described above. 100 nM T3 was used as ligand in the experiments.
A. Androgen Receptor

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B. Relative β-gal Activity/total protein

- **AR-DBD-LBD/ARA70**
  - DHT (-) [ ]
  - DHT (+) [ ]

- **AR-DBD-LBD/HBO1**
  - DHT (-) [ ]
  - DHT (+) [ ]

- **AR-LBD/HBO1**
  - DHT (-) [ ]
  - DHT (+) [ ]

- **AR-DBD/HBO1**
  - DHT (-) [ ]
  - DHT (+) [ ]

- **AR-AD/HBO1**
  - DHT (-) [ ]

- **pGBT9/HBO1**
  - DHT (-) [ ]
Figure 2

**Serine Rich Region**

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**PG159**

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Figure 3

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35S-HBO1

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132 kDa →

35S-AR

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AR-Ab

HBO1-Ab

AR

WB AR-Ab
Figure 4

A.

- HBO1
- β-actin

B.

- LNCap, MDA, PCA2a, PCa2b, PC3, TSU
- HBO1
- GAPDH
Figure 4

C.
Figure 5

RELATIVE LUCIFERASE ACTIVITY

0 2 4 6 8 10 12

GAL4DBD 1 147

VP16AD

HBO1 611

360

230 611

LUC-REPORTER

GAL4/ LUC-REPORTER
Androgen receptor interacts with a novel MYST protein, HBO1
Manju Sharma, Mark Zarnegar, Xiaoyu Li, Bing Lim and Zijie Sun

J. Biol. Chem. published online August 4, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M004838200

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