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Regulation of Nuclear Receptor Transcriptional Activity by a Novel DEAD Box RNA Helicase (DP97)*

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We have identified a novel DEAD box RNA helicase (97 kDa, DP97) from a breast cancer cDNA library that interacts in a hormone-dependent manner with nuclear receptors and represses their transcriptional activity. DP97 has RNA-dependent ATPase activity, and mapping studies localize the interacting regions of DP97 and nuclear receptors to the C-terminal region of DP97 and the hormone binding/activation function-2 region of estrogen receptors (ER), as well as several other nuclear receptors. Repression by DP97 maps to a small region (amino acids 589–631) that has homology to a repression domain in the corepressor protein NCoR2/SMRTe. This region of DP97 is necessary and sufficient for its intrinsic repression activity. The N-terminal helicase region of DP97 is, however, dispensable for its transcriptional repressor activity. The knockdown of endogenous cellular DP97 by antisense DP97 or RNA interference (siRNA for DP97) results in significant enhancement of the expression of estradiol-ER-stimulated genes and attenuation of the repression of genes inhibited by the estradiol-ER. This implies that endogenous DP97 normally dampens stimulation and intensifies repression of estradiol-ER-regulated genes. Our findings add to the growing evidence that RNA helicases can associate with nuclear receptors and function as coregulators to modulate receptor transcriptional activity.

Nuclear receptors comprise a superfamily of transcription factors that activate or repress gene transcription in a manner that is dependent on the nature of the hormonal ligand and coregulator proteins (coactivators or corepressors) that are recruited to the ligand-receptor complex (1). Among the steroid hormone receptors, estrogen receptors (ERs)[1] mediate the diverse stimulatory and repressive biological actions of estrogens and antiestrogens. These ligands, which are naturally occurring as well as synthetic, display a spectrum of activities ranging from full agonist to full antagonist that are reflective of changes in receptor conformation engendered by the ligand and the distinct coactivator/corepressor proteins recruited. These observations have led to the designation of some of these ligands as selective ER modulators (SERMs) (2, 3).

Many coactivator proteins have been identified, and these assemble into several dynamic multiprotein complexes (1, 4–7). These coactivator complexes include the SRC/p160 family of proteins, CREB-binding protein (CBP) and/or p300, and other factors that are recruited in a temporally ordered fashion (4) and up-regulate nuclear receptor activity, at least in part, through enhanced histone acetyltransferase activity (5–7). ATP-dependent chromatin remodeling complexes, such as BRG1/hBrm, and the TRAP-DRIP-ARC complex, which act sequentially or combinatorially, also enhance gene transcription by facilitating RNA polymerase II recruitment to promoters (4).

In contrast to the coactivators, far fewer corepressors are known. Most fully characterized are NCoR (nuclear receptor corepressor) and SMRT (silencing mediator of retinoic acid and thyroid hormone receptor), which function as major negative regulators of several members of the nuclear receptor family, including thyroid and retinoic acid receptors. These corepressors exert much of their repressive activities through recruitment of histone deacetylases to promote a repressive chromatin state (6–10). The key determinant role of these coregulators in mediating repression by steroid hormone nuclear receptors, including the estrogen, progesterone, androgen, glucocorticoid, and mineralocorticoid receptors, is less clear (11–13). In the case of the ER, a few additional negative coregulator proteins have been identified; these are the repressor of estrogen receptor activity (REA) (14, 15), a repressor of tamoxifen transcriptional activity denoted RTA (16), and a metastasis-associated protein corepressor, MTA1 (17), which play important roles in determining the pharmacology and inhibitory effectiveness of ER ligands.

To search for other factors that are involved in regulating the activity of the ER with estrogen agonist and antagonist ligands, we used 2-hybrid interaction screening with antiestrogen liganded-ER as bait. Through this screen, we have isolated a novel DEAD box RNA helicase, DP97, a 97-kDa protein, that acts as a corepressor of the liganded ER, and also of other nuclear hormone receptor superfamily members. This protein is a member of DNA/RNA helicase superfamily 2, which includes the DEAD box and DEAH box proteins (18, 19) that have roles in ribosome biogenesis, mRNA splicing, and transcriptional regulation (20, 21).

In this study, we have characterized this novel DEAD box protein and shown that it has several intriguing properties.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY148094.

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[1] The abbreviations used are: ERs, estrogen receptors; CAT, chloramphenicol acetyltransferase; DP97, DEAD box protein 97; E_2, estradiol; Luc, luciferase; NCoR, nuclear receptor corepressor; siRNA, short interfering RNA; SMRT, silencing mediator of retinoic acid and thyroid hormone receptor; SMRTe, N-terminally extended SMRT; GST, glutathione *S*-transferase; PBS, phosphate-buffered saline; ERE, estrogen response element; TOT, *trans*-hydroxytamoxifen; REA, repressor of estrogen receptor activity; RTA, repressor of tamoxifen transcriptional activity; MTA1, metastasis-associated protein corepressor; NLS, nuclear localization signal; CHO, Chinese hamster ovary; NR, nuclear receptor; RT-PCR, real time PCR.

DP97 has RNA-dependent ATPase activity, consistent with its being an RNA helicase. DP97 interacts in a ligand-dependent manner with ERs and with other nuclear receptors, and represses their transcriptional activity. The transcriptional repression by DP97 maps to a small region that shows significant homology to a repression domain in the well-characterized nuclear receptor corepressor, NCoR2/SMRTe.

EXPERIMENTAL PROCEDURES

Isolation and Cloning of DP97—A yeast two-hybrid screen employing a cDNA library from MCF-7 human breast cancer cells (14) was used to identify a 3′ partial length clone of DP97, which interacted in a ligand-dependent manner with the ligand binding/activation function 2 (E and F) regions of wild-type ERα. The entire open reading frame was isolated from a human muscle cDNA library (Origene Inc., Rockville, MD). Three clones of DP97 were obtained. One contained the entire open reading frame; another was a shorter 5′ sequence corresponding to amino acids 33–865. A third clone, missing amino acids 453–533, may be a product of alternative splicing.

Plasmids and Constructs—For creating GST fusion proteins, the desired cDNA inserts were subcloned into the pGEX-4T-1 expression vector (Amersham Biosciences, Piscataway, NJ) in-frame with the N-terminal GST protein. For the *in vitro* transcription and translation reactions, the plasmids contained a T7 promoter upstream of the translated sequence. pBluSK-ERα (14), pBluSK-ERα(ABC) (15), and pCR2.1-ERβ (22), have been described earlier. pBluSK-ERα(DEF) and pBluSK-ERα(EF), pBluSK-ERβ(DEF), pBluSK-PRb(DEF), and pBluSK-GR(DEF) were generated by PCR using the sequence from the pCMV construct as template (15), and all constructs were confirmed by sequencing.

For transient transfection reporter gene transactivation experiments, the expression vectors for the human estrogen receptor (ER), pCMV5-ERα and pCMV5-ERβ, human progesterone receptor (PR), pCMV5-PRb, human glucocorticoid receptor (GR), pRSV-GR, and pCMV-β, the β-galactosidase internal control vector, have been described earlier (14). pRSV-RARα was a kind gift from Christopher Glass (University of California at San Diego, CA). The reporter plasmid 2ERE-pS2-Luc was created by inserting a PCR-amplified 2ERE-pS2 product, engineered with flanking restriction sites (MluI): f, 5′-GCTG-TTTAACGCGTTATTCGGCCG-3′ and (*Bgl*II): r, 5′-GGGAATTGGAGA-TCTGAGCTT-3′, into the pGL2-Basic luciferase vector (Promega, Madison, WI). The other estrogen responsive constructs TGFβ3-CAT and complement 3-Luc have been described (15). 2PRE-TK-Luc was a gift from Kathryn Horwitz, University of Colorado Health Sciences Center, Denver, CO. SV-DR5-CAT, a retinoic acid receptor responsive promoter, was a gift from Ron Evans, Salk Institute, San Diego, CA. 2Gal4-pS2-CAT has previously been described (23). The reporter pGal4-SV40-Luc was kindly provided by Mitchell Lazar (University of Pennsylvania, Philadelphia).

For the preparation of FLAG-tagged DP97, the entire DP97 coding sequence (1–865) as well as sequences encoding the truncated DP97 proteins, DP97-(1–412), DP97-(413–865), DP97-(413–656), and DP97-(657–865), were amplified by PCR using pCMV5-DP97 as a template, and with 5′ *Bam*HI and 3′ *Xho*I sites engineered into the primers. Fragments were ligated into *Bam*HI-*Xho*I-digested pCMV-Tag 2 vector (Stratagene, La Jolla, CA) so that they are expressed in-frame with the FLAG tag. The DP97-(1–412) also had an SV40 nuclear localization sequence 5′-PKKKRK-3′ (24) incorporated at its C terminus. The pCMV5-Antisense DP97 was generated from the pCMV-Tag 2-DP97 using the *Eco*RI and *Bam*HI restriction enzyme sites.

For the mammalian two-hybrid assays, full-length DP97 as well as DP97-(1–412), DP97-(413–656), DP97-(657–865), DP97-(589–631), and DP97-(Δ589–631) were cloned into the *Eco*RI and *Bam*HI pM vector of the Mammalian Matchmaker two-hybrid assay system (Clontech, Palo Alto, CA), creating fusions to the Gal4 DNA binding domain. The DP97-(1–412) insert was generated by PCR so as to incorporate a nuclear localization signal (NLS) at its C terminus and used forward primer (*Bam*HI): f, 5′-GGAATTCATGGCCCAGTGGAGG-3′ and reverse primer (*Xho*I): r, 5′-AATCTCGAGTCACTTCCGCTTCTTCTT-GGGACGGCCCACG-3′ with the SV40 NLS underlined. All constructs were sequenced to check accuracy.

The mammalian two-hybrid assays also used pCMV5-VP16-ERα that was generated earlier (25), and pG5-CAT reporter, pVP16, pM3-VP16 (three Gal4 DNA binding domains fused to a VP16 transcriptional activation domain), and pM-p53 from Clontech (Palo Alto, CA).

Generation and Purification of DP97 Rabbit Polyclonal Antibody—DP97 antibody was generated against the peptide YRPKDFDSERGL-SISG (amino acids 668–683), which was chosen based on its hydrophobicity and likely antigenicity. The peptide was conjugated to MUC-1 keyhole limpet hemocyanin. Rabbits were immunized according to standard protocol at the Pocono Rabbit Farm and Laboratory Inc. (Canadensis, PA). ELISA assays were done to measure the titer of serum response to the conjugated peptide. Serum was purified and tested by Western blot analysis using MCF-7 whole cell extracts. Pooled serum from days 139, 146, 153, and 160 was purified on an affinity column against the immunizing peptide.

Overexpression and Purification of Recombinant GST Fusion Proteins—All pGEX4T1 (GST-DP97) constructs were transformed into *E. coli* BL21(DE3) (Novagen, Madison, WI). Bacterial culture (500 ml) expressing the recombinant GST fusion protein was grown at 37 °C to an OD_{600} of 0.6, then the temperature was brought to 25 °C. The cells were treated with 0.1 mM isopropyl-b-D-thiogalactopyranoside (IPTG) at an OD_{600} of 1.0. After 3.5 h, bacteria were resuspended in PBST (PBS, 1% Triton, 2 mM EDTA, 0.1% β-mercaptoethanol, and protease inhibitors (4.0 μg/ml of aprotinin, 2.0 μg/ml of leupeptin, 1.0 μg/ml of pepstatin A, and 0.2 mM phenylmethylsulfonyl fluoride), sonicated, and centrifuged. Soluble extracts were incubated with a glutathione-agarose matrix (Sigma) for 1 h at 4 °C, washed three times with PBST and then washed twice with 50 mM Tris-HCl, pH 8.0. The fusion protein was eluted from the beads with 5 mM glutathione and dialyzed to remove glutathione using dialysis buffer (25 mM Tris-HCl, pH 8.0, 125 mM NaCl, 1.25 mM EDTA, 20% (v/v) glycerol, and 20 mg/ml dithiothreitol).

ATP Hydrolysis Assays—The methods used were based on those of Askjaer *et al.* (26). Reactions containing 250 ng of GST-DP97 fusion protein in 5 μl of GST dialysis buffer (see above) were mixed on ice with $MgCl_2$, RNA, and unlabeled ATP and 0.1 μl of [α-^{32}P]dATP (PerkinElmer Life Sciences, 3 Ci/μmol) in a final volume of 15 μl. The optimal concentrations for the hydrolysis of ATP were 600 μM $MgCl_2$, 100 μM dATP, and 100 ng of MCF-7 total RNA. For the competition assays, a 10-fold excess of various unlabeled nucleoside triphosphates were added separately to each tube. The samples were incubated at 37 °C for 60 min and reactions stopped by placing on ice and adding 1 μl of 0.5 M EDTA. A portion (3.5 μl) of the reactions was spotted on PEI cellulose F thin layer chromatography plates (E. Merck). The plates were air-dried briefly, prewetted with ethanol, and developed in 0.5 M KH_2P0_4. The amount of [α-^{32}P]dATP hydrolysis was determined by PhosphorImager analysis. Images were quantitated using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

In Vitro Protein Interaction Assays—^{35}S-radiolabeled nuclear receptors were generated by *in vitro* transcription and translation using the TNT Quick kit from Promega. GST, GST-DP97-(1–865), or GST-DP97-(664–865) was bound to glutathione-agarose and equilibrated with 1× GST binding buffer (20 mM HEPES, pH 7.9, 100 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, 10% v/v glycerol, and protease inhibitors (4.0 μg/ml of aprotinin, 2.0 μg/ml of leupeptin, 1.0 μg/ml of pepstatin A, and 0.2 mM phenylmethylsulfonyl fluoride) for 2 h. Then, 5 μl of [^{35}S]methionine-labeled receptor was added to the immobilized GST fusion proteins in 100 μl of 1× GST binding buffer plus the proper ligands. The incubations proceeded for 2 h at 4 °C with rotary shaking. The beads were washed three times with 1× GST binding buffer (0.5 ml) and twice with 50 mM Tris (pH 8.0) (0.5 ml) buffer. Bound proteins were eluted with 10 mM reduced glutathione in 50 mM Tris buffer. Eluted proteins were resolved by SDS-PAGE, dried onto Whatman 3MM paper, and visualized by autoradiography.

Cell Transfections and Gene Transactivation Assays—Chinese hamster ovary (CHO) cells were maintained and transfected as previously described (27). Cells were plated at a density of 3×10^4 cells/well of a 24-well plate and incubated for 24 h at 37 °C with 5% CO_2. Some transfections also included increasing amounts of pCMV-FLAG-DP97 along with 300 ng of an internal β-galactosidase reporter gene to correct for transfection efficiency and empty pCMV-FLAG to 1000 ng. Cells were incubated with lipofectin (Invitrogen)-transferrin (Sigma)-complexed DNA for 7–8 h in serum-free medium and then washed with growth medium and given subsequent ligand treatment in growth medium. Cells for chloramphenicol acetyltransferase (CAT) assays were harvested 24 h after ligand treatment and lysed by 3 cycles of freezing on dry ice and thawing at 37 °C. β-galactosidase activity was measured to normalize for transfection efficiency and CAT assays were performed as described (28). Cells to be processed for luciferase assays were rinsed once in PBS and frozen in the presence of 100 μl of lysis buffer (Promega) at −80 °C. Luciferase activity was measured using a Dynex (Chantilly, VA) MLX luminometer and the Promega luciferase assay system 1.

Cell Studies Employing Short Interfering RNAs (siRNA)and Quantitative RT-PCR—siRNA oligonucleotides were designed using the Oli-

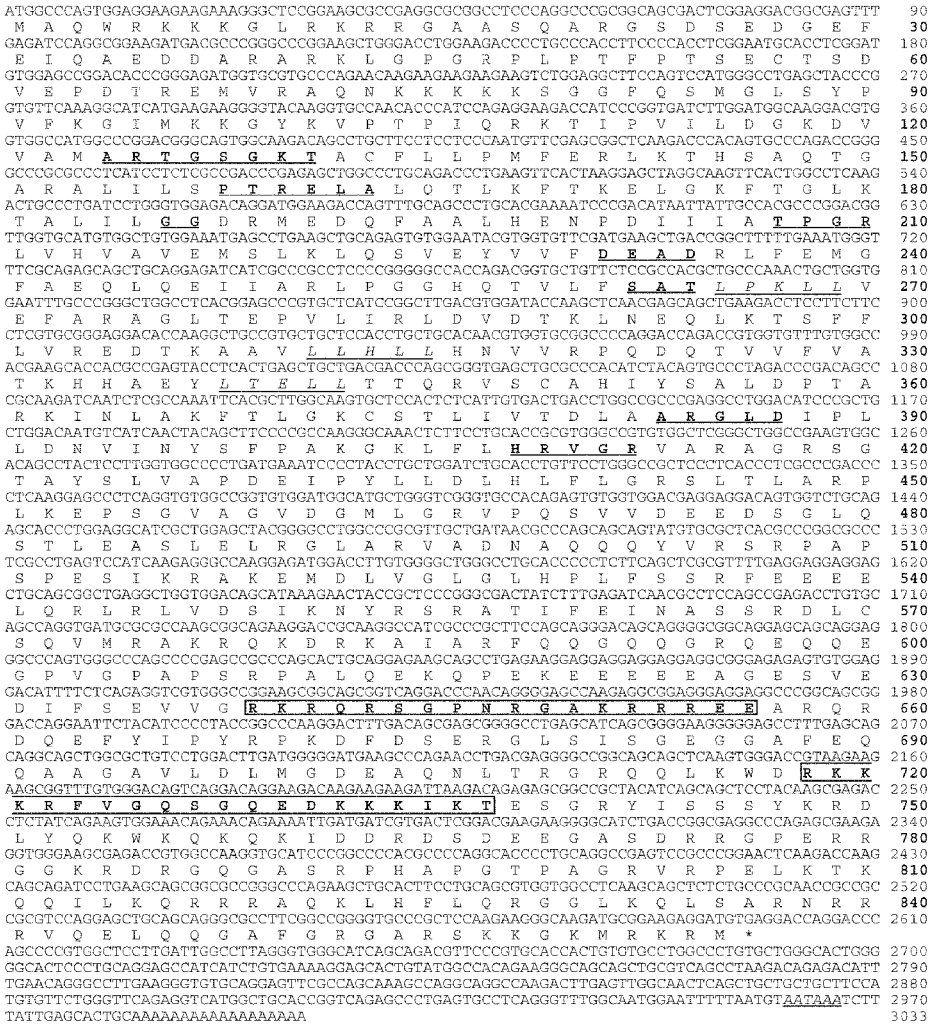

FIG. 1. **Amino acid and nucleotide sequence of human DP97.** Some notable features are the 8 DEAD box RNA helicase motifs (*bold* and *underlined*), 2 bipartite nuclear localization signals (*bold* and *boxed*), and 3 NR box LXXLL motifs (*underlined italics*). The polyadenylation signal in the 3′-untranslated region is also shown (*underlined italics*).

goengine program (oligoengine.com) and were obtained from Dharmacon Research (Lafayette, CO) as duplexed 2′ unprotected, desalted, and purified siRNA. The sequence used for DP97 siRNA was GAAGAAGUCUGGAGGCUUCdTdT. A scramble oligonucleotide was used as a negative control.

MCF-7 cells were grown to near confluency in 150-cm^2 flasks in Minimal Essential Medium (MEM) containing phenol red supplemented with 5% calf serum and antibiotics. Cells were then split into 24-well plates at a dilution of 1:500 per well in MEM minus phenol red supplemented with 5% charcoal-dextran-stripped calf serum, but without antibiotics. Cells were allowed to adhere overnight and then transfected with 0.12 nmol/well of either the DP97 siRNA duplex or scramble siRNA duplex using Oligofectamine (Invitrogen) At 48 h after transfection, cells were treated with 10 nM E_2, or the 0.1% ethanol vehicle. The cells were harvested at either 8 or 24 h after E_2 treatment. Total RNA was isolated using Trizol Reagent (Invitrogen) according to the manufacturer's instruction. 1 μg of total RNA was reverse-transcribed in a total volume of 20 μl using 200 units of reverse transcriptase, 50 pmol of random hexamer, and 1 mM dNTP (PerkinElmer Life Sciences). The resulting cDNA was then diluted to a total volume of 100 μl with sterile H_2O. The expression of DP97 and of the estrogen-regulated genes *pS2*, *WISP2*, and c-*erbB2*, were measured by real time PCR using the SYBR Green PCR System (Applied Biosystems, Foster City, CA). Each real-time PCR reaction consisted of 1 μl of diluted RT product, 1× SYBR Green PCR Master Mix, and 50 nM of forward and reverse primer. The primers used in the real time PCR were for DP97: DP97 2763f, 5′-AGGAGTTCGCCAGCAAAGC-3′ and DP97 2840r, 5′-ACCCAGAACACATGGAAGCAG-3′; for pS2: pS2 255f, 5′-ATACCATCGACGTCCCTCCA-3′ and pS2 401r, 5′-AAGCGTGTCTGAGGTGTCCG-3′; for WISP2: WISP2 8f, 5′-GCACACCGAAGACCCACCT-3′ and WISP2 94r, 5′-AGGTACATGGTGTCGGGCA-3′; for c-erbB2: c-erbB2 925f, 5′-GGATCCTGCACCCTCGTCT-3′ and c-erbB2 1009r, 5′-GCTTGCTGCACTTCTCACACC-3′; and for 36B4: 36B4 574f, 5′-GTGTTCGACAATGGCAGCAT-3′ and 36B4 657r,5′-GACACCCTCCAGGAAGCGA-3′. Reactions were carried out in an ABI Prism 7700 Sequence Detection System (Applied Biosystems) for 40 cycles (95 °C for 15 s, 60 °C for 1 min) following an initial 10-min incubation at 95 °C. The fold change in gene expression was calculated using the ΔΔCt method with the ribosomal protein 36B4 mRNA (29) as an internal control.

Immunocytochemical Detection of DP97 and ERα in CHO and MCF-7 Cells—CHO cells were transfected using the FuGENE 6 transfection reagent (Roche Pharmaceuticals). MCF-7 cells were not transfected. At 48 h after transfection, cells were rinsed with calcium- and magnesium-free phosphate buffered saline (CMF-PBS) and fixed in 1.6% formaldehyde (Polysciences Inc., Warrington, PA) at room temperature. Cells were then washed three times for 5 min each in CMF-PBS and permeabilized in 0.1% Triton X-100 detergent (Pierce Chemical) for 5 min. Cells were treated with 2.8 ng/μl of affinity-purified α-DP97 rabbit polyclonal antibody as well as 2 ng/μl α-ERα H222 rat monoclonal antibody. After washes, cells were treated with fluorescein isothiocyanate-conjugated goat anti-rabbit secondary antibody and Texas Red goat anti-rat Affinipure IgG (Jackson ImmunoResearch, West Grove, PA). Cells were again washed and then mounted in anti-fade solution (Molecular Probes, Eugene, OR). Images of the MCF-7 and CHO cells were collected on an inverted light microscope (IMT2, Olympus, Success, NY) with a cooled, slow-scan CCD camera (Photometrics, Tucson, AZ) as described previously (30). Optical sections of nuclei were collected and deconvoluted as described (31).

RESULTS

Identification and Characterization of an ER-interacting Protein, DP97—In order to identify proteins that might repress the activity of the ER in a ligand-dependent manner, we used domains E and F of ERα complexed with the antiestrogen *trans*-hydroxytamoxifen (TOT) as bait in a yeast two-hybrid

screen with a cDNA library prepared from MCF-7 human breast cancer cells. In this manner, we identified a partial 3′ clone that interacted with the hormone-occupied ER. It showed preference for interaction with TOT-ER over estradiol (E_2)-ER, and it did not interact with the unliganded ER. The entire open reading frame, which was subsequently isolated, encodes a protein of 97 kDa, consisting of 865 amino acids (DP97, Fig. 1). A Kozak consensus sequence is found in the 5′-region that would allow expression of the DP97 protein (32, 33). A shorter variant of this sequence, also isolated in library screening, has the corresponding coding sequence with amino acids 453–533 removed, probably due to alternative splicing. Bioinformatic analysis of the DP97 sequence using the BLAST search of the human genome demonstrates that the DP97 sequence is located at chromosome 12q22–12q23. Interestingly, the corepressor NCoR2/SMRTe has been localized to the region 12q24 in the human genome (34).

DP97 contains two bipartite nuclear localization signals and three nuclear receptor boxes (L*XX*LL motifs) thought to be important for coactivator interactions with nuclear receptors (35). There is also a possible CoRNR box (amino acids 245–249), important for corepressor interactions with nuclear receptors (36, 37). Stretches of glutamate and lysine residues, of unknown significance, also exist throughout the sequence and the C-terminal region of DP97 is very basic. A nonredundant standard protein-protein homology BLAST search reveals DP97 to have a high degree of homology to the DEAD box family of ATP-dependent RNA helicases. In fact, DP97 has all of the eight consensus motifs (*bold* and *underlined* in Fig. 1) that are the hallmark of proteins in this family (19). Therefore, DP97 appears to be a novel member of the DEAD box family of ATP-dependent RNA helicases.

Northern analysis of DP97 expression showed a predominant mRNA species of 3.1 kb (85%) and another less abundant mRNA of 4.3 kb (15%) in several human cell lines including MDA-MB-231 breast cancer cells, HepG2 hepatoma cells, and MCF-7 breast cancer cells (data not presented). A human tissue mRNA Master Blot (Clontech, Palo Alto, CA) revealed that DP97 mRNA is expressed in all tissues examined, with the highest level of expression in the pancreas and lung (data not presented).

Biochemical Characterization of DP97 as a Putative ATP-dependent RNA Helicase—We performed RNA-dependent ATPase assays, measuring ATP hydrolysis to AMP by purified GST-DP97 fusion protein in the presence of increasing amounts of MCF-7 total RNA. As seen in Fig. 2*A*, we observed an RNA concentration-dependent increase in ATP hydrolysis. In contrast, poly(U) RNA (Fig. 2*A*) and RNase-treated MCF-7 total RNA (not shown) did not stimulate the hydrolysis of ATP by DP97.

We also assessed the ability of other nucleoside triphosphates (at 10-fold excess concentration) to function as competitors in the hydrolysis of [α-^{32}P]dATP by DP97 in the presence of MCF-7 RNA (Fig. 2*B*). GST-DP97 caused hydrolysis of [α-^{32}P]dATP, whereas the GST protein alone, as expected, did not. Competition by unlabeled nucleoside triphosphates was only seen with ATP and dATP. Therefore, we conclude that DP97 hydrolyzes ATP in preference to other nucleoside triphosphates. These findings provide support for DP97 as an ATP-dependent RNA helicase, suggested by the phylogenetic motifs in its sequence.

In Vitro Interaction of DP97 with Nuclear Receptors—To characterize the interaction of DP97 with ER, we performed interaction assays in the absence of ligand, or in the presence of the estrogen estradiol (E_2) or the antiestrogen TOT. Wild type ERα interacted with the full-length DP97 only in the presence of ligand, and more effectively with TOT-ER than with E_2-ER

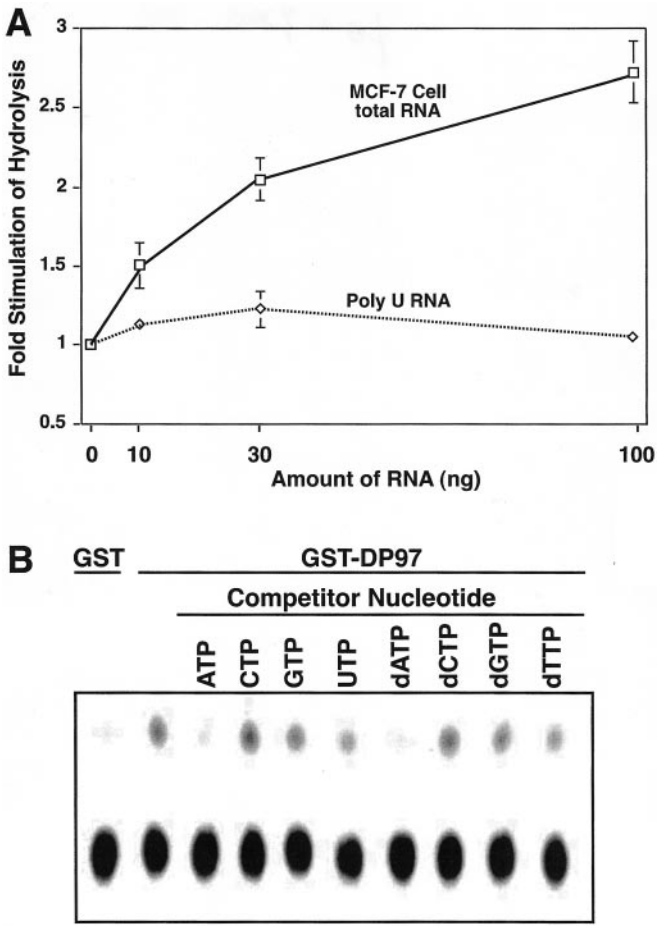

FIG. 2. **Characterization of DP97 RNA-dependent ATPase activity.** *A*, the ability of GST-DP97 to hydrolyze [α-^{32}P]dATP in the presence of increasing amounts of MCF-7 total cell RNA (or poly(U) RNA) was determined. The level of hydrolysis, monitored by thin layer chromatography and PhosphorImager quantification, is plotted relative to the level of hydrolysis without RNA present. Values are means ± S.D. from three independent determinations. *B*, hydrolysis by DP97 was specific for ATP, as shown by competition of hydrolysis of [α-^{32}P]dATP with a 10-fold excess of radioinert ATP or dATP, but not by the other nucleoside triphosphates tested. *Left-most lanes* show GST or GST-DP97 with [α-^{32}P]dATP and no competitor nucleoside triphosphate.

(Fig. 3*A*). To determine the region of interaction between the ER and DP97, we created GST fusion proteins with full-length DP97-(1–865) and with the truncated DP97 proteins DP97-(1–656), DP97-(413–656), and DP97-(664–865). These proteins were purified over a glutathione-agarose resin and were analyzed by SDS-PAGE to verify their sizes. GST pull-down experiments revealed that only the full-length DP97 and the DP97-(664–865) interacted with ERα (Fig. 3*A*).

We then examined which region of ERα interacted with DP97 using *in vitro* translated products: ERα-(domains ABC) containing the receptor N-terminal A/B activation function-1 and DNA-binding domains, ERα-(DEF) containing the hinge and hormone-binding/activation function-2 regions, and ERα-(EF) containing the hormone binding/activation function 2 domain and C-terminal F domain (Fig. 3*A*). DP97 did not interact with ERα-(ABC), but it did interact with ERα-(DEF) and ERα-(EF) (Fig. 3*A*). Interaction did not require domain F nor E domain helix 12, as the ER ligand binding domain E truncated at amino acid 530 interacted as well as did ERα-(EF) (data not shown). We also see a preference for DP97 interaction with full-length ERα and ERα-(EF) in the presence of TOT, and no interaction when ER is unliganded (Fig. 3*A*). This result is consistent with the original yeast two-hybrid screen where the

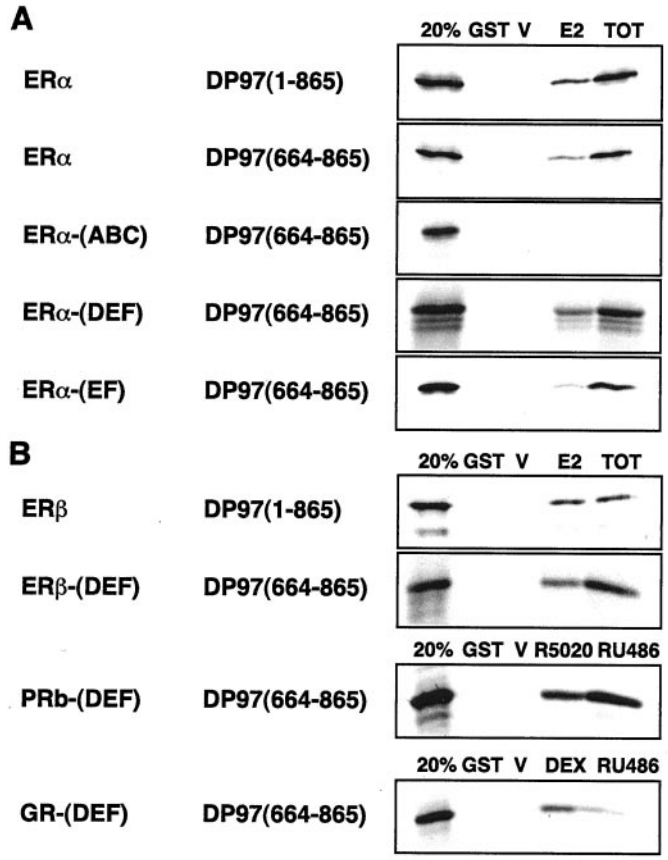

FIG. 3. **Examination of the interaction of DP97 with nuclear receptors.** Interaction of DP97 with ERα (*panel A*) as well as ERβ, progesterone receptor (PRb), and glucocorticoid receptor (GR) (*panel B*) was assessed in the presence of agonist ligand, antagonist ligand, or in the absence of ligand. *Panel A* examines interaction of full-length ERα and ERα domains ABC, DEF, and EF with DP97. *Panel B* examines interaction of DP97 with ERβ as well as DP97-(664–865) with the DEF regions of ERβ, PRb, and GR. *In vitro* translated [^{35}S]methionine-labeled receptor proteins were incubated with purified GST or GST-DP97 fusion proteins and with glutathione-agarose beads in the presence of 0.1% control ethanol vehicle, 10^{-6} M ligand (E_2 and TOT for ER, R5020 and RU486 for PR, and dexamethasone (*Dex*) and RU486 for GR). Lanes (*left* to *right*) show 20% input; incubation with GST alone; incubation with GST-DP97 fusion protein and control vehicle (*V*), or with agonist ligand, or with antagonist ligand.

ERα-(EF) bait interacted with the C terminus of DP97 with increased affinity in the presence of TOT.

The other ER subtype, ERβ, and its DEF region also interacted with GST-DP97 in the presence of E_2 or TOT (Fig. 3*B*). The DEF regions of the progesterone receptor (PRb) and the glucocorticoid receptor (GR) also interacted with DP97 in the presence of agonist and antagonist ligand, but not in the absence of ligand (Fig. 3*B*).

Mammalian Two-hybrid Interaction of DP97 with ERα—The interaction between DP97 and ERα was also confirmed in cells using a mammalian two-hybrid system. DP97 was expressed as a fusion protein with the Gal4 DNA binding domain and ERα was expressed as a fusion protein with the VP16 activation domain (AD) in CHO cells. A robust activation of the Gal4-regulated reporter gene indicated that the two proteins interact in the presence of E_2 and TOT, but not in the absence of ligand (Fig. 4), which is consistent with the GST pull-down interaction data.

DP97 Represses the Transcriptional Activity of Nuclear Receptors—Using transfection and reporter gene transactivation assays in mammalian cells, we observed that DP97 repressed the transcriptional activity of ERα on a variety of promoters having different estrogen responsive regions. This included the pS2 promoter with two consensus estrogen response elements (EREs) (Fig. 5*A*); the complement component 3 (C3) promoter, which contains a mix of consensus and nonconsensus EREs (Fig. 5*B*), and the TGFβ3 promoter, which has an estrogen-responsive region very different from an ERE and where ER works by tethering to other DNA-bound protein factors (Fig. 5*C*). These data demonstrate that DP97 can repress ERα stimulation at diverse estrogen-regulated gene sites. In order to determine if DP97 inhibits ER activity by interfering with the DNA binding function of ER, we performed a promoter interference assay in MDA-MB-231 cells (38). This revealed that DP97 did not inhibit the ability of ERα to bind to estrogen response elements (data not shown).

To determine whether the interaction between DP97 and various nuclear receptors has a functional consequence, we examined the effect of DP97 on the transcriptional activity of several nuclear receptors. DP97 repressed by 60–90% the transcriptional activity of ERβ (Fig. 6*A*), PRb (Fig. 6*B*), GR (Fig. 6*C*), and retinoic acid receptor α (RARα) (Fig. 6*D*). Therefore, DP97 repressed both type I (steroid) as well as type II (retinoic acid) nuclear hormone receptors. DP97; however, did not repress all transcription factors, as it failed to repress the activity of p53 (Fig. 5*E*) and the viral protein VP16 (Fig. 5*F*). Differences in the extent of transcriptional repression by DP97 among different nuclear receptors might reflect differences in affinities for the nuclear receptors, or differences in the potency of DP97 in repression of different promoter-response element gene constructs by the different nuclear receptor-ligand complexes. There is substantial evidence for different efficacies of coregulators being dependent on promoter and cell context (39–42).

Characterization of the Region of DP97 Required for the Repression of ERα—To identify the region of DP97 responsible for its repression, we analyzed the ability of truncated DP97 proteins to repress the activity of ERα (Fig. 7). First, we confirmed that FLAG-DP97-(1–865) repressed ERα to a similar extent as did DP97 without the FLAG tag (not shown). We found that FLAG-DP97-(1–412), which contains all of the DEAD box motifs, did not affect the transcriptional activity of the ER. However, the FLAG-DP97-(413–865) construct repressed ERα even slightly better than the full-length DP97. Since DP97-(657–865) interacted with ERα, we divided DP97-(413–865) into two portions. Neither portion, FLAG-DP97-(413–656) nor FLAG-DP97-(657–865), could repress ERα on its own. Thus, both the region of DP97 that interacts

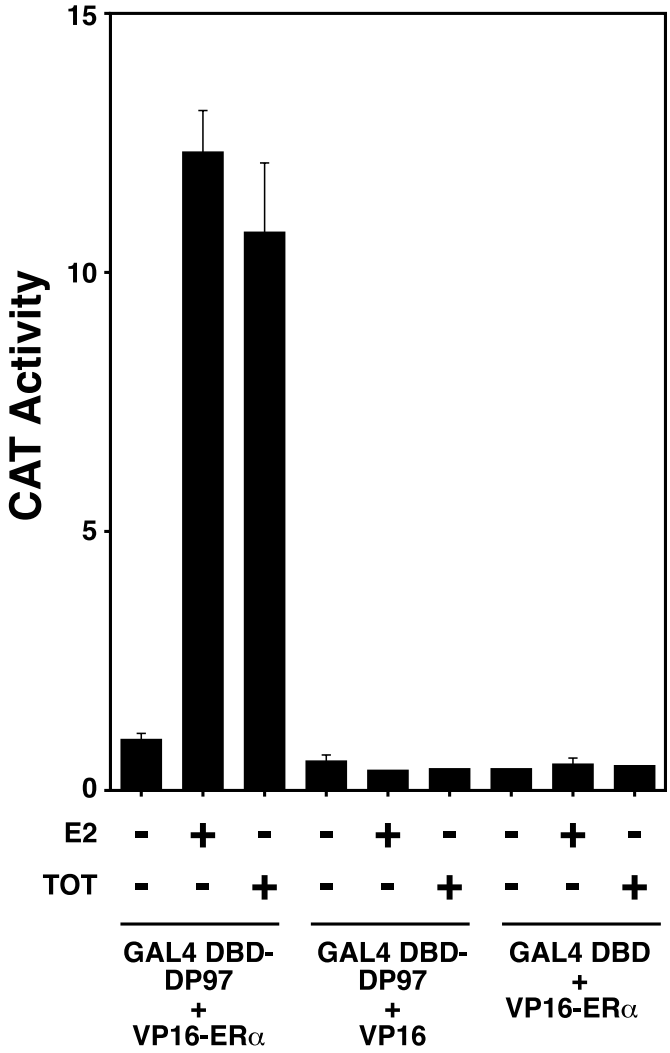

FIG. 4. **Mammalian two-hybrid interaction of ERα and DP97.** Interaction between DP97 expressed as a fusion protein with the Gal4 DNA binding domain and ERα expressed as a fusion with the VP16 activation domain was assessed in CHO cells in the absence of hormone and in the presence of 10^{-8} M E_2 or 10^{-8} M TOT using a reporter construct containing 5 Gal4 response elements upstream of the *CAT* gene. All transfections contained a β-galactosidase internal control reporter to normalize for transfection efficiency. Values are means with S.D. from four independent determinations.

with ERα (amino acids 657–865) and the region of DP97 from 413–656 were required for transcriptional repression of ERα.

DP97 Has Intrinsic Transcription Repression Activity and a Separable Repression Domain with Homology to NCoR2/SMRTe—Corepressors can inhibit the activity of nuclear receptors by several mechanisms. The nuclear receptor corepressors NCoR and SMRT have been shown to repress the basal activity of promoters that they are recruited to as Gal4 DNA binding domain fusion proteins (34, 43). We tested the intrinsic repression activity of DP97 in this manner and found that the DP97-Gal4 DNA binding domain fusion protein repressed a constitutively active SV40 promoter with 5 upstream Gal4 binding sites (Fig. 8*A*). Thus, DP97 has the ability to repress promoters by recruitment alone.

Gal4 DNA binding domain fusions were also made with DP97-(1–412), DP97-(413–656), and DP97-(657–865) to determine which region(s) retained the intrinsic repression activity of the entire protein. Gal4-DP97-(413–656) was able to repress the Gal4-SV40-Luc reporter, but neither the Gal4-DP97-(1–412) nor the Gal4-DP97-(657–865) affected the reporter gene activity (Fig. 8*A*). Using a protein BLAST homology search for short nearly exact matches, we found a glutamine-proline-glutamate rich sequence (amino acids 589–631) within DP97-(413–656) that bears significant sequence similarity with two regions in NCoR2/SMRTe (Fig. 8*B*). The first is a glutamine-proline-glutamate rich region in the SANT domain of NCoR2, and the second is a proline-glutamate rich region in repression domain 2 (RD2) of NCoR2.

We, therefore, examined the role of this 43 amino acid region (amino acids 589–631) in the transcriptional repression activity of DP97 (Fig. 8*C*). As seen before in Fig. 8*A*, the Gal4 DNA binding domain fused to DP97 repressed the SV40 promoter. Of note, the region with homology to NCoR2/SMRTe within DP97-(589–631) also had intrinsic repression activity (Fig. 8*C*). Furthermore, removal of this small region from DP97-(Δ589–631) abolished the ability of DP97 to repress the constitutively active SV40 promoter (Fig. 8*C*) and also to repress the transcriptional activity of the ER (data not shown). Therefore, the region encompassing amino acids 589–631 functions as the transcriptional repression domain of DP97.

Reversal of the Effect of DP97 on ER Transcriptional Activity by Antisense DP97or siRNA for DP97—Introduction of antisense DP97 into cells resulted in an enhanced stimulation of ER transcriptional activity in the presence of estradiol, consistent with the hypothesis that endogenous DP97 recruited to the hormone-occupied ER normally suppresses the response to estradiol (Fig. 9*A*).

To further examine the effect of DP97 on the expression of estrogen-regulated genes, we used MCF-7 breast cancer cells and employed siRNA molecules (44). Transfection of cells with siRNA reduced DP97 mRNA to 25% of control levels, while the scramble siRNA had no effect on DP97 RNA level, as monitored by quantitative real-time RT-PCR (Fig. 9*B*). Reduction of DP97 levels in MCF-7 cells enhanced estradiol-stimulated expression of pS2 and WISP2, two genes that are known to be up-regulated by estrogen in these cells (45–48). This was seen at 8 h of E_2 treatment for the primary response gene *pS2*, and at both 8 and 24 h of hormone treatment for the *WISP2* gene. Likewise, silencing of DP97 substantially reduced the magnitude of estradiol repression of *erbB2* gene expression (Fig. 9*B*), *erbB2* being a gene that is down-regulated by the E_2-ER complex in these cells (49). Hence, the findings with DP97 antisense and siRNA provide strong evidence that endogenous DP97 plays a role in modulating ER transcriptional activity in cells, normally dampening estrogen-stimulated gene expression and increasing the effectiveness of the E_2-ER in suppressing down-regulated genes.

DP97 Localizes to the Nucleolus and Nucleoplasm of Cells—We generated a polyclonal antibody against a peptide present in the DP97 sequence. In Western blot analysis, this antibody recognizes predominantly a 97-kDa protein in MCF-7 cell extracts (Fig. 10*A*). We used this antibody and the anti-ERα monoclonal antibody H222 to determine the localization of endogenous DP97 and ERα in MCF-7 cells, and of DP97 and ERα in CHO cells that had been transfected with plasmids expressing DP97 and ERα (Fig. 10*B*). Untransfected CHO cells do not contain ER and if they contain endogenous DP97, it is not detected with our antibody to human DP97, perhaps because of the species difference of the cells. In both types of cells, DP97 was localized in the nucleus; it was present throughout the nucleoplasm and was concentrated at the nucleoli. As expected, ERα was nuclear and was present throughout the nucleoplasm but little was nucleolar. There was overlap of DP97 and ER in speckled structures in the nucleoplasm, structures that may be associated with RNA processing events. Hence, the interaction between ER and DP97 may

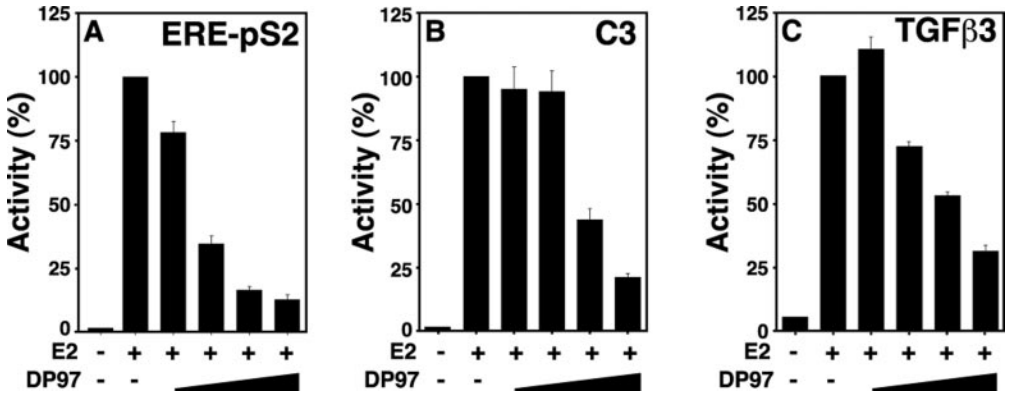

FIG. 5. **DP97 suppresses the transcriptional activity of ERα when ER is acting as a direct DNA-binding transcription factor as well as when it is tethered via other transcription factors at non-estrogen response element DNA sites.** CHO cells were transfected with an expression vector for ERα and the estrogen-responsive reporter gene construct indicated in the absence of DP97 or with increasing amounts of DP97 expression plasmid (10, 100, 500, or 1000 ng). Cells were (+) or were not (−) exposed to 10^{-8} M E_2. In *A*, the reporter plasmid was 2ERE-pS2-Luc; in *B*, complement component 3-Luc (C3, −1030 to +58); in *C*, transforming growth factor β3-CAT (TGFβ3-CAT). All transfections contained a β-galactosidase internal control reporter to normalize for transfection efficiency. Values are means with S.D. from four independent determinations.

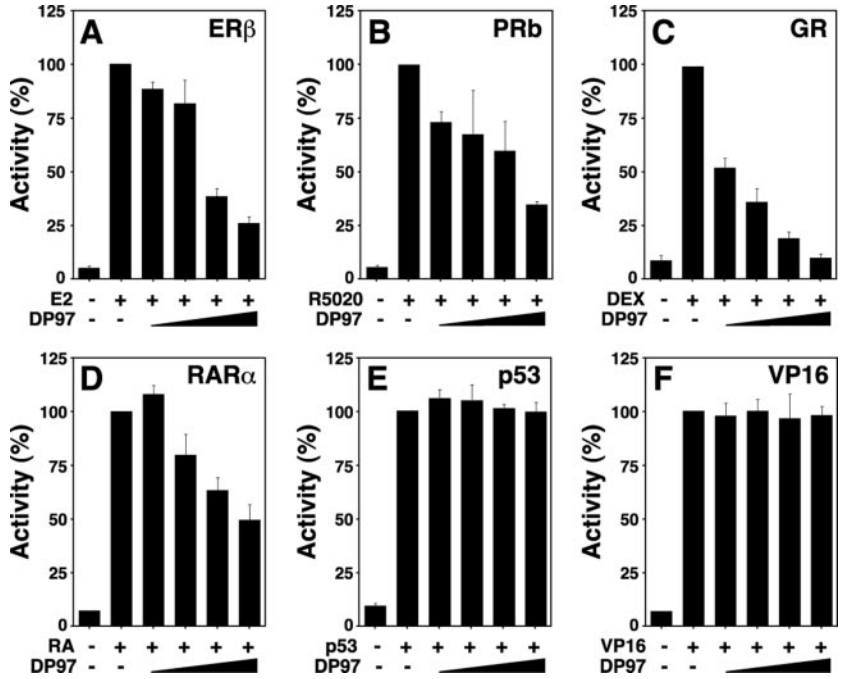

FIG. 6. **DP97 is able to repress the transcriptional activity of various nuclear receptors but not p53 or VP16.** CHO cells were transfected with plasmids expressing either ER β (*A*), progesterone receptor b (*B*), glucocorticoid receptor (*C*), retinoic acid receptor α (*D*), p53 (*E*), or the VP16 transcriptional activator (*F*); and the reporter gene construct: ERβ, 2ERE-pS2-Luc; PR-b, 2PRE-TK-Luc; GR, 2PRE-TK-Luc; RARα, DR5-RARE-CAT; p53, 2Gal4-pS2-CAT; VP16, 2Gal4-pS2-CAT. Transfections also included increasing amounts of DP97 plasmid (10, 100, 500, or 1000 ng). All transfections contained a β-galactosidase internal control reporter to normalize for transfection efficiency. Values are means with S.D. from four independent determinations.

occur at the edge of the nucleoli and/or with the portion of DP97 that exists in the nucleoplasm (see "Discussion").

DISCUSSION

In this work, we describe the identification of a novel DEAD box protein, DP97, that interacts in a hormone-dependent manner with the estrogen receptor and other nuclear receptors and has several interesting properties. DP97 has RNA-dependent ATPase activity, consistent with its being an RNA helicase; it interacts with and represses the activity of nuclear receptors; and it has a small region of sequence homology with NCoR2/SMRTe that is responsible for its transcription repression activity. Analysis of the repression activity of truncated forms of DP97 shows that the DEAD box helicase motifs are dispensable for DP97 repression activity, indicating that its RNA helicase and corepressor activities represent distinct, separable functions.

Nuclear receptor corepressors, such as NCoR1 or NCoR2, typically have separable functional domains for nuclear receptor interaction and for transcriptional repression (50, 51). This is the case, as well, with DP97. Its repression activity maps to a small region (589–631), while a more C-terminal part of DP97-(664–865) is the region that interacts with the C-terminal portion of nuclear receptors encompassing the ligand binding/AF-2 regions. Although DP97 contains 3 NR boxes (L*XX*LL motifs) and a possible CoRNR box through which many coregulators interact with nuclear receptors, these receptor interaction motifs are in the N-terminal half of DP97, not in the C-terminal region that interacts with nuclear receptors, indicating that other sequences in DP97 are responsible for its receptor interaction. This is consistent with findings from peptide phage display and studies with other coregulators (14–16), showing that peptide sequences in addition to L*XX*LL motifs and CoRNR boxes can interact with nuclear receptors with high affinity (52, 53).

Comparisons of the Transcription Repression and the Ligand-dependent Interactions of DP97 and Other Corepressors—We have shown that DP97 has a compact intrinsic transcription repression region (amino acids 589–631). Like DP97, several other transcriptional repressor proteins have small regions with active transcriptional repression activity. These include

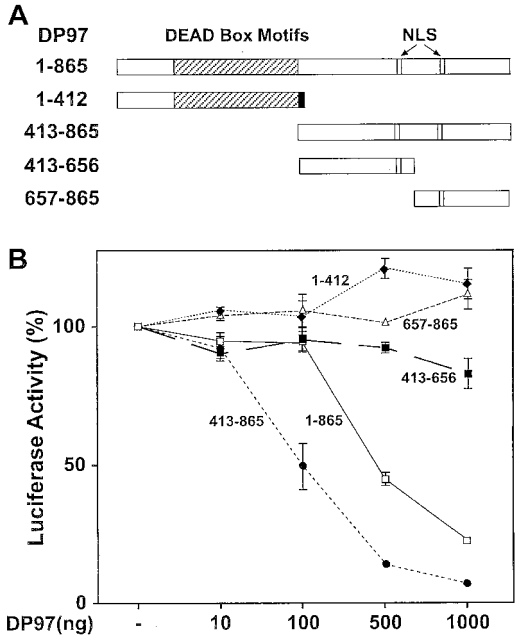

FIG. 7. **Effect of truncated DP97 proteins on transcriptional activity of ERα.** *A*, schematic of DP97 constructs tested. NLS, nuclear localization signals. Since DP97-(1–412) lacked the NLS, we cloned an NLS (indicated in *black*) at the C terminus of this construct. *B*, full-length DP97 and DP97-(413–865) repressed ERα transcriptional activity, while DP97-(1–412), DP97-(413–656), or DP97-(657–865) did not. Transfections were performed in CHO cells with a 2ERE-pS2-Luc reporter gene construct and ERα expression plasmid and increasing amounts of each DP97 construct. Cells were treated with 10^{-8} M E_2. All transfections included a β-galactosidase internal control reporter to normalize for transfection efficiency. Values are means with S.D. from four independent determinations.

Nab-1, a direct acting corepressor of the NGFI-A family of zinc finger transcription factors (54), Sim-2, a protein involved in midline development in mice (55), and AREB6, a zinc-finger homeodomain transcription factor (56).

It is of note that the repression region of DP97-(589–631) shows considerable homology with two regions in the well known corepressor, NCoR2/SMRTe, amino acids 498–534 and 813–839 (34, 43, 51, 57). The latter (813–839) is within the second repression domain (RD2) of NCoR2 (34), whereas the former (498–534) encompasses a polyglutamine and acidic/basic region (43) that is between SANT(SWI3, ADA2, NCoR, TFIIIB) domains A and B. Since this former region (498–534) has only been examined as a Gal4 fusion along with other repressive regions of NCoR2, such as the SANT domains and the region of high similarity between NCoR and NCoR2 (51), it is not known whether NCoR2-(498–534) has intrinsic repression activity on its own. Nevertheless, our observations with DP97 expand the utilization of these motifs for nuclear receptor regulation beyond SMRT/NCoR to a new corepressor, DP97.

The ligand requirements for interaction of SMRT and NCoR with nuclear receptors is quite different from that observed with DP97. SMRT and NCoR interact preferentially with type II nuclear receptors, including retinoic acid and thyroid hormone receptors, and interaction with these receptors is observed primarily in the absence of ligand, with ligand occupancy resulting in dissociation of SMRT or NCoR. These corepressor proteins have also been reported to interact with estrogen receptors and progesterone receptors, but only in the presence of antagonist ligands (4, 13). In contrast, DP97 interacts with nuclear receptors in the presence of agonist or antagonist ligand, and shows no interaction in the absence of ligand. Hence, SMRT/NCoR and DP97 may serve distinct transcriptional regulatory activities that differ in their hormonal requirements, providing potentially multiple combinatorial regulatory mechanisms (11). Indeed, there is already evidence that NCoR and SMRT preferentially regulate different receptors (36) and that they exhibit distinct promoter and cell-type specificity.

With the exception of the small repression region (amino acids 589–631) of DP97, DP97 shows no structural resemblance to NCoR or SMRT or to three other proteins known to be negative coregulators for nuclear receptors (REA, MTA1, and RTA). Like DP97, REA preferentially interacts with antiestrogen-liganded ER but it also interacts with estrogen-occupied ER. However, in sharp contrast to DP97, REA is an ER-selective coregulator (14, 15, 27). RTA (16) and MTA1 (17) bind to ER as well as other nuclear hormone receptors, as does DP97, but RTA interacts through the N-terminal region of the receptors in a ligand-independent manner, whereas DP97 interaction with receptor, like that of RTA and MTA1, is via the hormone binding/activation function-2 domain and is ligand-regulated. MTA1 is identical to NuRD-70, a component of nucleosome remodeling complexes (17). Of interest, RTA contains RNA recognition motifs that are required for its repressor function, indicating a role for RNA binding in regulation of nuclear receptor activity (16). For DP97, its repressor activity is physically and functionally separable from its DEAD box motif-containing region.

DP97 Variants, Subcellular Localization, and Comparisons with other DEAD Box RNA Helicases and Coregulators Known to Modulate Nuclear Receptor Activity and RNA Processing— DP97 may exist in several splice variant forms in cells. We found that DP97 mRNA was present as 3.1-kb and 4.3-kb species. The 3.1-kb message corresponds to the clone shown in Fig. 1, and the 4.3-kb message appears in the GenBank™ (accession number NM024072.2) as a similar clone, isolated from human placenta, that contains in addition a 1.2-kb 3′-untranslated region accounting for the 4.3-kb size. No publication associated with this GenBank™ entry has appeared. The 3.1-kb mRNA is the predominant form, accounting for 85% of the total cellular DP97 mRNA in the several human cell lines we examined (MCF-7, MDA-MB-231, and HepG2). Also, in our isolation of a full-length DP97 clone from a cDNA library, we found an alternatively spliced form that would encode a protein missing amino acids 453–533. It is of note that RNA-binding proteins that modulate ER activity, such as RTA, also exist in multiple alternatively spliced forms (16).

Two other DEAD box RNA helicase proteins have been shown to be specifically involved in modulating nuclear receptor transcriptional activity. RNA helicase p68 interacts with the N-terminal A/B region of ERα and selectively potentiates the activity of ERα; this coactivator does not interact with or serve as a coactivator of either ERβ or other nuclear receptors (58, 59). Another DEAD box RNA helicase, DP103, interacts with the orphan nuclear receptor, steroidogenic factor-1 (SF-1), and potentiates its transcriptional repression (23). In contrast, DP97 interacts with the C-terminal hormone binding region of ERα, ERβ and other nuclear receptors, and functions more broadly as a corepressor of these nuclear hormone receptors. Our findings with DP97 antisense and siRNA provide evidence that endogenous DP97 normally dampens the stimulation and intensifies the repression of estradiol-ER-regulated genes, such that the knockdown of DP97 enables greater estrogen stimulation of up-regulated genes and attenuates the repression of genes that are normally inhibited by the E_2-ER complex.

Our identification of DP97 localization in nucleoli, as well as throughout the nucleus, is consistent with the reported intracellular localization of most RNA helicases. Since ERα has been

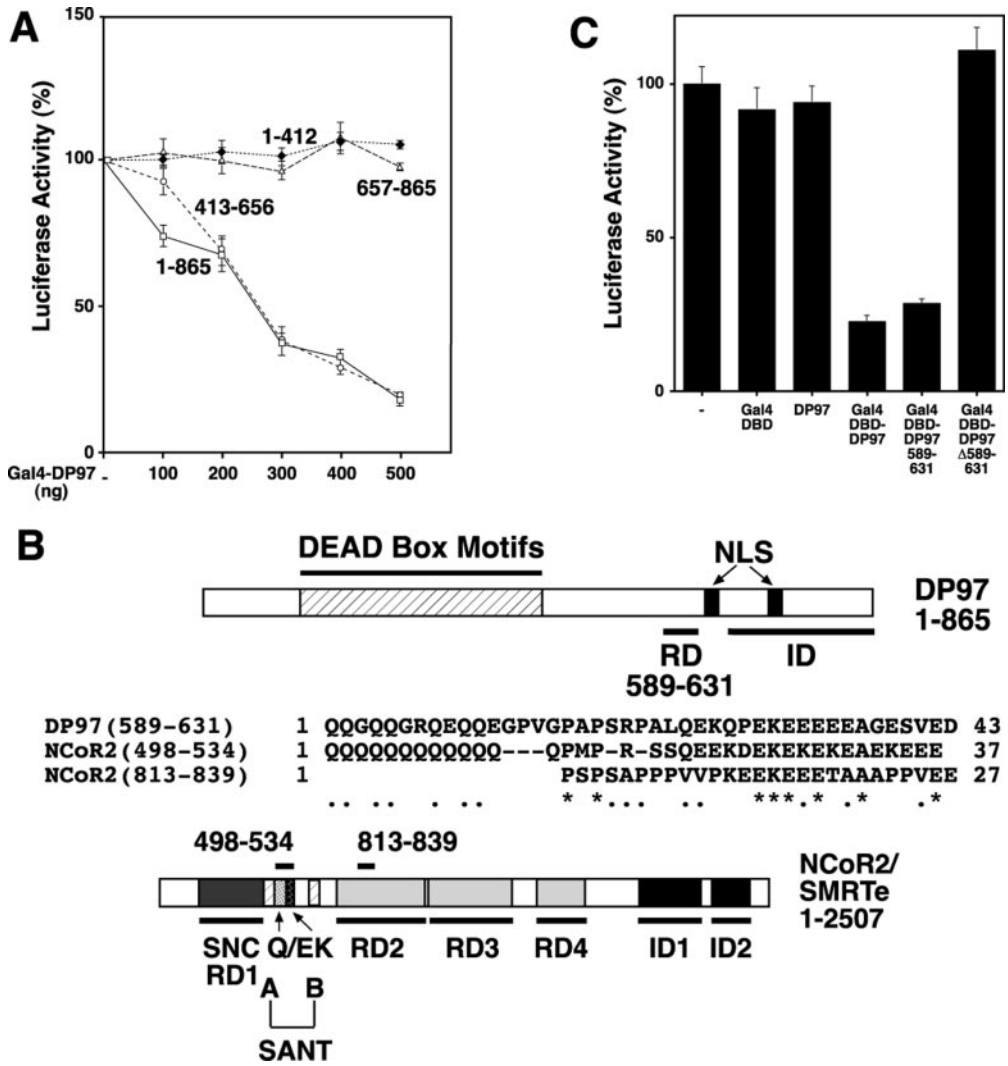

FIG. 8. **Identification of intrinsic transcription repression activity of DP97 and a region of DP97 with repression activity that shows homology to the corepressor NCoR2/SMRTe.** *A*, Gal4 DNA binding domain-DP97 fusion proteins were tested for their ability to repress the 5-Gal4 DNA binding sites-SV40-luciferase reporter gene. DP97-(413–656) repressed the SV40 promoter as well as the full-length protein. *B*, protein BLAST analysis of DP97-(413–656) reveals significant sequence similarity of DP97 amino acid region 589–631 with two regions in NCoR2. *, same amino acid in DP97 and both regions of NCoR2/SMRTe; •, same amino acid in DP97 and one of the regions in NCoR2/SMRTe. *RD*, repression domain; *ID*, receptor interaction domain. *C*, Gal4-DP97-(589–631) maintains the intrinsic repression activity of the Gal4-DP97 protein, and Gal4-DP97 deleted of these 42 amino acids (Δ589–631) loses intrinsic repression activity. All transfections contained a β-galactosidase internal control reporter to normalize for transfection efficiency. Values are means with S.D. from four independent determinations.

previously shown to be nuclear and present in the nucleoplasm, but largely excluded from nucleoli, the interaction between ERα and DP97 may occur either at the edge of the nucleoli and/or with the portion of DP97 that exists within the nucleoplasm. Many nucleolar proteins are dynamic, and constantly cycle between the nucleolus and nucleoplasm (60), and this may be the case for DP97. The presence of DP97 in the nucleolus suggests that it may have regulatory roles, possibly in RNA and ribosome biosynthesis and processing, in addition to its role in regulation of nuclear hormone receptor activity. In this regard, it is of note that the coregulator PGC1, originally identified as a coregulator of PPARγ-mediated transcriptional activity, but shown more recently to also serve as a coregulator of the ER (61), exerts dual regulation in that, in addition to its transcriptional regulatory role, it also mediates mRNA splicing (62). Such dual regulatory functions may prove to be a common theme among nuclear receptor coregulators.

DEAD-box RNA helicases, as well as certain RNA species themselves, may be involved more broadly than previously envisioned in the actions of nuclear receptor superfamily members and in the regulation of transcription and RNA splicing (63, 64). In addition to the DEAD box RNA helicase DP103 that represses SF-1 activity (65), and the RNA helicase p68 that functions as a specific hormone-independent coactivator of ERα (66), steroid receptor activator (SRA), itself a novel RNA species, has been shown to function as a coactivator for steroid receptors (59, 67); and SHARP, an RNA-binding corepressor protein can bind to SRA and regulate nuclear receptor activity (68). Three proteins that associate with thyroid hormone receptors (TLS, PSF, and NonO/p54^{nrb}) each contain RNA recognition motifs and may play possible roles in RNA processing (69, 70). One of these, PSF, functions as a transcriptional repressor. These findings, plus observations that CBP, which is often a component of nuclear receptor transcriptional complexes, binds RNA helicase A and that the tumor suppressor protein BRCA1 is linked to the RNA polymerase II holoenzyme complex via RNA helicase A (71), imply that RNA helicases and additional proteins that bind RNA may play crucial roles in the actions of nuclear receptor superfamily members and in the regulation of transcription in normal and cancer cells (1).

Our findings add to the growing evidence for RNA helicases associating with distinct activation function regions of nuclear

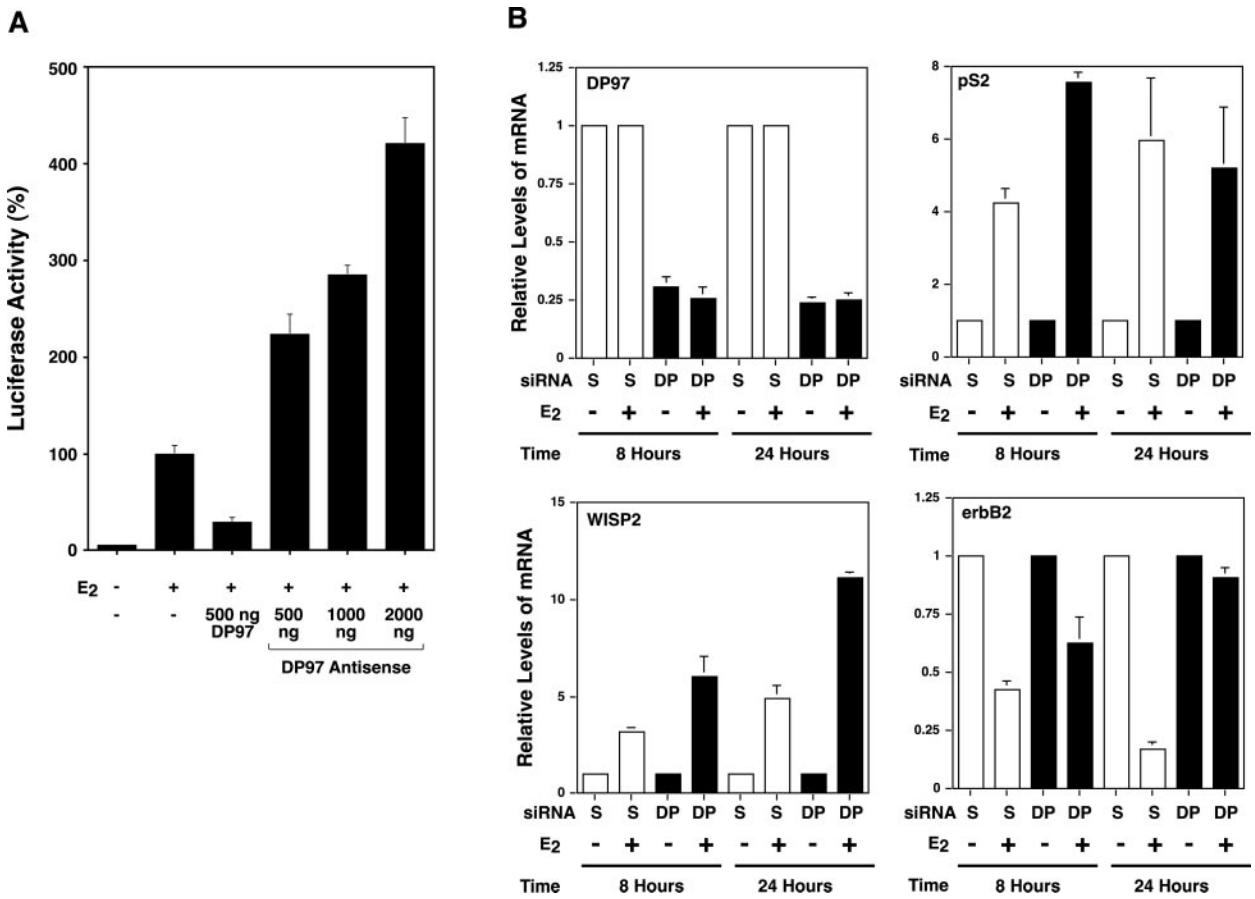

FIG. 9. **The effect of antisense DP97 or siRNA for DP97 on estrogen-regulated gene expression.** *A*, an antisense DP97 plasmid was transfected along with ERα expression plasmid into MDA-MB-231 breast cancer cells and after 48 h, cells received 10^{-8} M E_2 or 0.1% ethanol vehicle for 8 h. The activity of ERα on a 2ERE-pS2-Luc reporter gene construct was then monitored. All transfections contained a β-galactosidase internal control plasmid to normalize for transfection efficiency. Values are means with S.D. from four independent determinations. *B*, MCF-7 cells were exposed for 48 h to either a scramble (*control*, *open bars*) siRNA or to DP97 siRNA (*filled bars*) to reduce the levels of endogenous DP97. The cells were then treated with either 0.1% ethanol vehicle ($-E_2$) or 10^{-8} M estradiol ($+E_2$) for 8 or 24 h and the levels of mRNA for DP97, pS2, WISP2, or c-erbB2 were monitored by quantitative real-time RT-PCR. *S* and *DP* denote scramble or DP97 siRNA exposure, respectively.

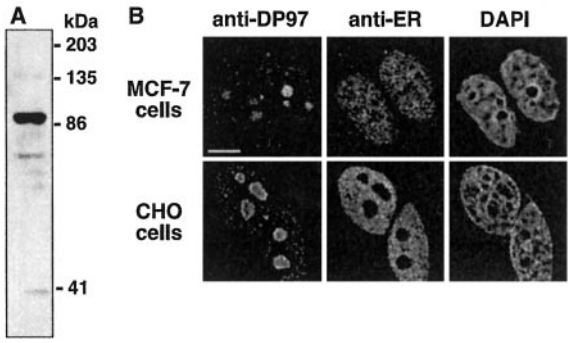

FIG. 10. **Cellular localization of DP97.** *A*, antibody to DP97 detects primarily a single protein of the correct size on SDS-PAGE gels of MCF-7 cell extracts. The gel marker kDa sizes, run in a lane adjacent to the cell extract, are indicated. *B*, endogenous DP97 is localized to the nucleoli and nucleoplasm in MCF-7 cells, and transfected DP97 is localized similarly in CHO cells. CHO cells were transfected with DP97 and ERα expression plasmids. MCF-7 cells were not transfected. Cells were incubated with an antibody to DP97 (anti-DP97 rabbit polyclonal), an antibody to ERα (H222 monoclonal rat antibody) followed by secondary antibody conjugated to flourescein or Texas Red, respectively, and DAPI (to stain nuclear DNA). Cell images shown are from cells without hormone treatment. However, the distributions of DP97 and ER were not changed upon exposure to estrogen or antiestrogen.

receptors and serving as coregulators that can either up- or down-modulate the activity of these transcription factors. Since RNA helicases are known to be involved in many aspects of RNA metabolism, including RNA transcription, processing and transport, and ribosome biogenesis, it is tempting to speculate that there is a linkage (through coregulator proteins such as DP97) between RNA processing and transcriptional activity of the nuclear hormone receptors. Further investigations are needed to explore this relationship.

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