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13. ABSTRACT ***(Maximum 200 Words)***

The androgen receptor (AR) is a ligand-regulated transcription factor that stimulates both normal and malignant prostate cell growth. To understand the mechanism of AR function, we sought to identify novel factors that interact with AR N-terminus by use of a yeast two-hybrid system. A 157-amino acid protein termed ART-27 was cloned and shown to interact with an AR N-terminal subdomain (residues 153-336), localize predominantly to the nucleus and increase the transcriptional activity of AR when overexpressed in cultured mammalian cells. ART-27 overexpression also enhanced the transcriptional activation by $AR_{153-336}$ fused to LexA DNA binding domain, but not other AR N-terminal subdomains, lending support to the notion that ART-27 mediates its effect via an interaction with a defined region of the AR N-terminus. ART-27 overexpression also lowers the concentration of androgen required for AR-dependent transcriptional activation. ART-27 gene maps to a region of the X-chromosome near the AR locus, which is amplified in a subset of prostate cancers, suggesting that overexpression of ART-27 may facilitate malignant cell growth by increasing the sensitivity and activity of AR to androgen in target cells.

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INTRODUCTION

Our overall objective is to elucidate the molecular mechanisms of transcriptional regulation by the androgen receptor (AR) in prostate cancer. AR is a hormone-dependent transcription factor involved in the regulation of both normal and malignant prostate cell growth by controlling target genes and signaling pathways involved in cellular proliferation. Although androgens, such dihydro testosterone, act as the primary signal in activating AR's transcriptional regulatory functions, AR-mediated transactivation is also controlled by associations with as yet unidentified cofactors involved in transcription regulation, termed coactivators and corepressors. We will identify and characterize proteins that interact with the AR N-terminal transcriptional and define their effects on AR transcriptional activity in prostate cancer cells. In addition, we will determine if the concentration of specific AR AF-1-interacting proteins varies in AR-expressing prostate tumors using antibody and nucleic acid probes. Understanding how AR AF-1-interacting factors govern AR transcriptional activation in prostate cancer will likely uncover novel markers and points of intervention to be exploited in the development of new therapies for AR-dependent prostate cancer.

BODY

Statement of Work (Year 1).
Task1. Identification and Characterization of AR AF-1 Interacting Proteins

a. Completing yeast two-hybrid screen for AR Interaction Trap (ART)
b. Specificity tests on ARTs
c. Expression pattern of ART mRNA
d. Cloning of full-length ARTs

<u>A yeast two-hybrid screen for proteins that interact with the AR N-terminus (Task 1a)</u>

To identify proteins that interact with the AR N-terminus, we used a modified yeast two-hybrid system that allows for the identification of factors expressed in the prostate that associate with transcriptional activators. An androgen-stimulated LNCaP prostate cancer cell cDNA library fused to the LexA DNA binding domain was screened for proteins that interact with the AR N-terminal transcriptional activation domain encompassing receptor residues 18 through 500 from rat AR. We chose to search

for AR interacting proteins using this library for several reasons. First, it is prostate-specific, being derived from a well-characterized AR-expressing androgen-dependent prostate cancer cell line. Second, AR in LNCaP cells activates transcription of a *bona fide* AR-responsive gene (e.g., prostate specific antigen (PSA) promoter), which implies that the AR cofactors required for its regulation are present. Third, choosing androgen-stimulated LNCaP cells as the source of mRNA from which the library was produced also allows for the enrichment and detection of androgen-inducible AR-associated factors. In principle, androgen-regulated AR-interacting cofactors may represent a means through which AR-dependent transcriptional activity is modulated. Finally, since LNCaP cells are androgen-dependent for growth, the use of this library increases the likelihood of identifying cofactors that regulate the AR mitogenic response.

Out of ~1 x 10^6 library transformants, eight clones have been isolated that interact with the AR N-terminus. We have termed these factors ARTs, for Androgen Receptor Trapped. We sequenced the eight ART clones and subjected them to a database search using the BLAST program. A quantitative liquid β-galactosidase assay was used to measure the relative strength of interaction between the AR N-terminus and the ARTs using the yeast two-hybrid system. The levels of expression of the ARTs in yeast were similar as determined by immunoblotting using an antibody to the LexA DNA-binding domain that is common to all the ARTs (not shown). Figure 1A summarizes the relative strength of interaction and the results of the database search. The strongest AR N-terminal interacting proteins, in decreasing order of affinity, are ART-37, ART-5 and ART-27. A BLAST search of the Genebank database revealed that ART-37 and ART-5 are proteins of unknown function represented in the Expressed Sequence Tag (EST) database, whereas ART-27 is located on the X chromosome (Xp11.23-11.22) and is identical to a recently identified ORF, termed ubiquitously expressed transcript (UXT), prevalent in tumor tissues (2). Intermediate strength interacting proteins include ART-6, an EST, and ART-15, which is identical to ATBF1a, a transcription factor containing multiple zinc finger and homeodomain motifs that was isolated in a screen for proteins that bind to the alpha-fetoprotein enhancer (3). Weak interacting proteins include ART-9, which corresponds to ZNF160 (1), a zinc finger containing protein of unknown function and ART-2 and ART-3, which are present in the EST database. We chose to continue the characterization of the three strongest AR N-terminal interacting factors.

ART interaction specificity (Task 1b)

To analyze the specificity of ART interaction, we examined the capacity of the strongest AR N-terminus-interacting factors to associate with a panel of transcriptional regulatory proteins in the modified yeast two-hybrid assay. ART-5, -27 and -37 were tested for interaction with: Sp1 (A and B domains), the cyclic AMP response element binding protein (CREB), TBP-associated factor 130 ($TAF_{II}130$), the AF-1 of the glucocorticoid receptor (GR) and the steroid receptor coactivator-1 (SRC-1). As shown in Figure 1B, ART-5 interacts exclusively with the AR N-terminus, whereas ART-27 interacts with the AR and GR N-termini, as well as with Sp1 and with $TAF_{II}130$, but not with SRC-1 or CREB. No interaction between the AR ligand binding domain (LBD) and ART-5 and ART-27 was observed in either the absence or presence of hormone, whereas ART-37 interacted with the AR LBD in the presence of hormone. In contrast, ART-37 is relatively promiscuous, interacting with virtually all of the transcriptional regulators examined. These results indicate that ART-5 interacts rather specifically with the AR N-terminus, ART-27 displays less selectivity, interacting with the AR N-terminus and with certain other transcriptional regulatory factors including $TAF_{II}130$, whereas ART-37 is unable to discriminate among the factors examined.

Expression pattern of ART mRNA (Task 1 c)

Using ART-5, ART-27 and ART-37 cDNAs as nucleic acid probes, we performed Northern blot analysis on mRNA isolated from androgen-independent (PC-3) and androgen-dependent (LNCaP) prostate cancer cells, either untreated or stimulated for 72 hours with the synthetic androgen R1881 at the indicated concentrations (Figure 2; right). ART-37 mRNA (~1.2-kb) is highly expressed in PC-3 cells relative to LNCaP cells. Likewise, the mRNA level of ART-27 (~0.9-kb) is slightly elevated in PC-3 relative to LNCaP cells, while ART-5 (~1.4-kb) steady state mRNA concentration is similar in both cell types. We also examined whether androgens regulate ART expression in LNCaP cells. While ART-27 and ART-5 showed a small increase in steady state mRNA expression in LNCaP cells in response to increasing concentrations of androgen, ART-37 RNA levels were not affected. We have also probed multiple human tissue blots for ART expression (Figure 2; left). ART-5, ART-27 and ART-37 appear to be widely expressed in human tissues, including normal human prostate. ART-27 mRNA

appears uniformly expressed in the tissues examined. In contrast, ART-37 and -5 mRNA expression varies among tissues, with the highest level of ART-37 mRNA in the testis and the lowest in the thymus, while ART-5 expression is greatest in the small intestine and least in the colon. These results indicate that ART-5, -27 and -37 are expressed in a variety of normal human tissues and display differential patterns of expression in prostate cancer cell lines.

Cloning and characterization of full length ART-27 (Task 1 d)

The full-length cDNA of ART-27 was cloned and contains an insert of 984 bp, in which the largest open reading frame (ORF) encoding a protein of 154 amino acids with an estimated MW of ~18kDa. The putative genomic structure of ART-27 shows that the gene is composed of six exons interrupted by five introns (Fig. 3A). ART-27 contains potential phosphorylation sites for protein kinase A, protein kinase C and casein kinase II, but no other obvious motifs as determined by PROSITE (Fig. 3B). Secondary structure prediction algorithms suggest that ART-27 is composed of multiple, successive alpha helices. ART-27 also appears to be conserved throughout evolution, with model organisms *M. musculus, D. melanogaster and C. elegans*, displaying 92%, 49% and 26% percent similarity, respectively, with human ART-27.

An antibody against AR-27 was raised by injecting rabbits with a peptide that corresponds to the C-terminal region from amino acids 142-157. Western blot analysis of COS-1 cell lysates transfected with ART-27 shows that the antibody recognizes full-length ART-27 and that the cloned ART-27 migrates at the same apparent molecular weight as endogenous ART-27 from HeLa and PC-3 cell nuclear extracts, confirming that the predicted ORF from sequence analysis was correct (Fig. 3C). Specificity of the antibody was also demonstrated in immuno precipitation assays with this antibody and pre-immune sera from the same rabbit (not shown). We also investigated the pattern of subcellular localization of ART-27. Indirect immunofluorescence demonstrates that full length ART-27 localizes predominantly to the nucleus, although some diffuse staining is apparent in the cytoplasm of cells expressing high levels of the protein (Fig. 3D). Such predominant nuclear distribution of ART-27 is consistent with its role as a potential transcriptional regulatory protein.

ART-27 is involved in androgen receptor transcriptional activation (Task 2)

Since ART-27 interacts with the AR N-terminus, we anticipate that ART-27 would play a role in AR-dependent transcriptional regulation. To establish whether overexpression of ART-27 affects AR transcriptional activation, AR-deficient HeLa cells were transfected with a constant amount of full length AR and increasing concentrations of an expression vector encoding a full length HA-tagged ART-27 along with an AR-responsive luciferase reporter gene. As shown in Figure 6A, hormone-dependent AR transcriptional activation was increased in a dose-dependent manner when ART-27 is overexpressed. This effect was AR-dependent, since in the absence of AR, ART-27 did not influence reporter gene activity (Figure 4). To ensure that this enhanced transcriptional activity was not the result of increased AR protein production, we monitored protein expression and found that AR levels are not affected by ART-27 coexpression (not shown). The effect of ART-27 on AR was not restricted to a single cell type, since overexpression of ART-27 in PC-3 and COS-1 cells also resulted in a dose-dependent increase in AR transcriptional activity (Figure 6B and not shown). Thus, ART-27 overexpression enhances the AR-dependent transcriptional response, which suggests that ART-27 can act as a regulator of AR transcriptional activity in mammalian cells.

KEY RESEARCH ACCOMPLISHMENTS:

- Completed yeast two-hybrid screen for AR Interacting factors (ARTs).
- Determined specificity of ART interactions.
- Determined mRNA expression patterns for a subset of ARTs.
- Cloned the full AR N-terminal interacting protein, ART27.
- Shown that ART27 protein is located in the nucleus.
- Shown that ART27 overexpression increases AR-dependent transcriptional activation, suggesting it may function as a new AR AF-1 coactivator.

REPORTABLE OUTCOMES:

None, however a manuscript describing these results is in preparation.

CONCLUSIONS:

We had identified ART27 as a novel AR N-terminal interacting protein, which may serve as a receptor coactivator. Validating ART27 as an AR regulatory protein in vivo is ongoing.

REFERENCES

1. Halford, S., M. G. Mattei, S. Daw, and P. J. Scambler. 1995. A novel C2H2 zinc-finger protein gene (ZNF160) maps to human chromosome 19q13.3-q13.4. Genomics 25:322-3.

2. Schroer, A., S. Schneider, H. Ropers, and H. Nothwang. 1999. Cloning and characterization of UXT, a novel gene in human Xp11, which is widely and abundantly expressed in tumor tissue. Genomics 56:340-3.

3. Yasuda, H., A. Mizuno, T. Tamaoki, and T. Morinaga. 1994. ATBF1, a multiple-homeodomain zinc finger protein, selectively down- regulates AT-rich elements of the human alpha-fetoprotein gene. Mol Cell Biol 14:1395-401.

Figure 1

A.

ART#	β-galactosidase units	Sequence Information
37	2533	EST
5	1019	EST
27	432	UXT_{1-157}
15	82	$ATBF\text{-}1a_{560-960}$
6	n/a	EST
9	5	ZNF160
2	4	EST
3	1	EST

B.

Regulatory Factors	ART-5	ART-27	ART-37
AR_{18-500}	+	+	+
$AR_{579-901}$	-	-	+
$GR_{107-237}$	-	+	+
$SRC\text{-}1_{374-800}$	-	-	+
$TAF130_{270-700}$	-	+	+
$Sp1_{83-262}$	-	+	+
$Sp1_{263-542}$	-	+	+
$CREB_{3-296}$	-	-	+

Figure 1: **Results of the modified yeast two-hybrid screen for AR N-terminus-interacting factors.**

(A) Quantitative analysis of ART interactions with AR N-terminus. ARTs expressed as fusion proteins with the LexA DNA binding domain were analyzed for their ability to interact with AR_{18-500}. The relative strength of interaction is determined by a quantitative liquid _-galactosidase assay after a 12 hr incubation in galactose-containing media at 30°C. The LexA vector alone gives 1 unit of activity. Eight ART clones were partially sequenced and these sequences were used to search the NCBI and Swissprot databases using the BLAST search program for homologies to known proteins. ART-27 corresponds to UXT, an ORF of unknown function on the X-chromosome, ART-15 corresponds to alpha-fetoprotein enhancer binding protein (ATBF1a), and ART-9 is identical to ZNF160, a zinc-finger containing protein of unknown function, whereas ART-37, ART-5, ART-6, ART-3 and ART-2 represent proteins of unknown identity found in the expressed sequence tag (EST) database. (B) Specificity of AR-ART interactions. Interaction of ART-5, ART-27 and ART-37 with AR N-terminus (N-T) (AR_{18-500}), AR ligand-binding domain (LBD) ($AR_{579-901}$) and other transcriptional regulatory factors was analyzed using the modified yeast two-hybrid assay. Regulatory factors include the glucocorticoid receptor _1 ($GR_{107-237}$), steroid receptor coactivator-1 ($SRC\text{-}1_{374-800}$), TBP-Associated Factor 130 ($TAF_{II}130_{270-700}$), Sp1 A ($Sp1_{83-262}$), Sp1 B ($Sp1_{263-524}$) and cAMP response element binding protein ($CREB_{3-296}$). The strength of interaction is determined by a qualitative plate _-galactosidase assay after a 24 hr incubation on galactose X-gal plates at 30°C. Interactions (+) represent blue colonies and (-) represents no interactions above background "vector only" control (white colony).

Figure 2

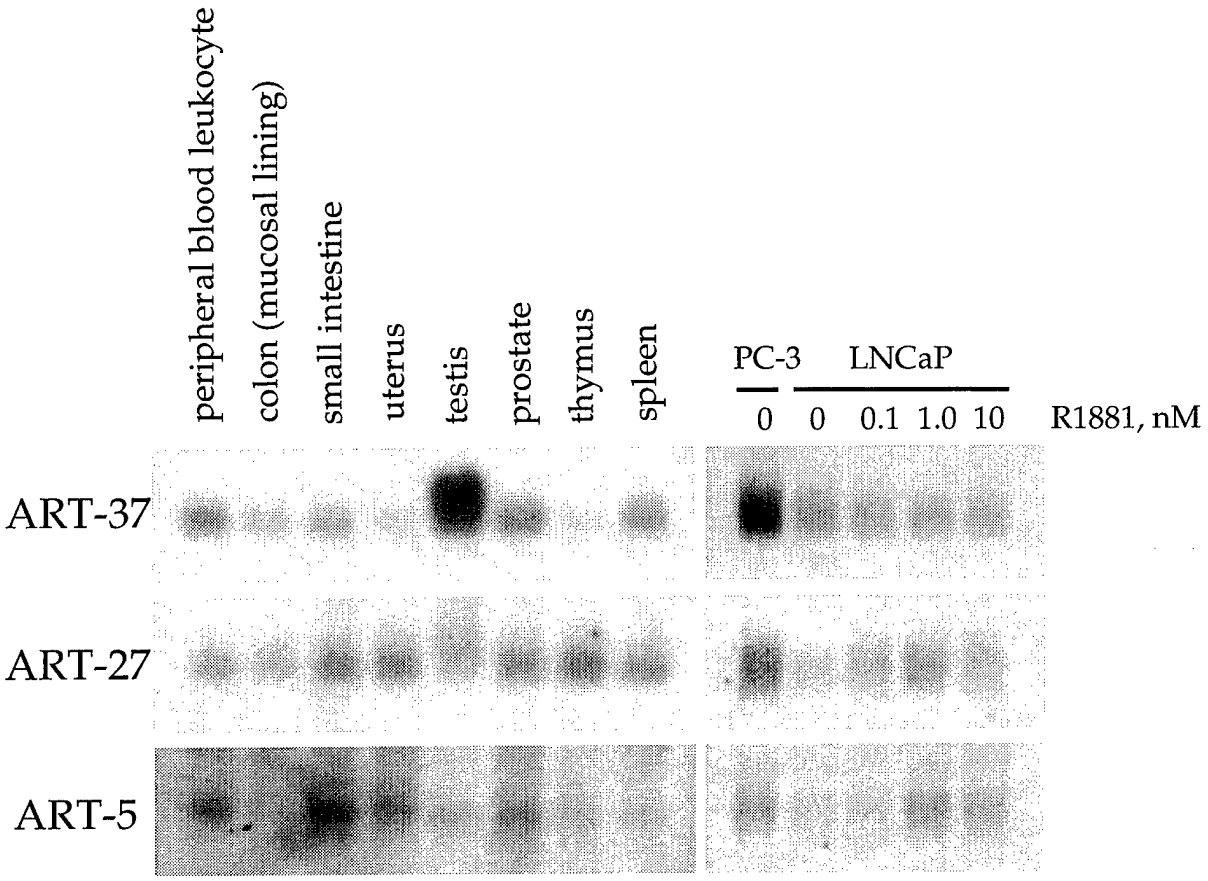
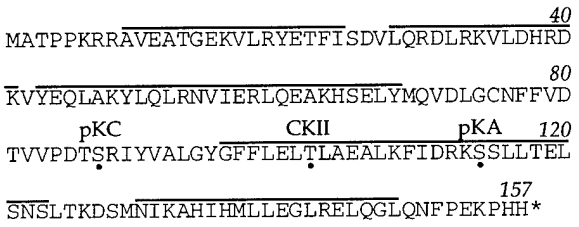


Figure 2: **ART mRNA expression in prostate cancer cells and in human tissues.**
Total RNA was extracted from PC-3 and LNCaP cells cultured in the absence or presence of indicated doses of androgen (R1881) for 72 hr. Equal amounts of RNA were separated on denaturing formaldehyde-agarose gels (see Materials and Methods), transferred to Duralon nylon membrane and hybridized to ^{32}P-labeled cDNA probes corresponding to ART-37, ART-27 and ART-5 (right panel). Equal loading for each lane is determined by ethidium bromide staining of the 28S rRNA (not shown). A human multiple tissue northern blot (Clontech: MTN Blot IV) containing 2 μg of poly A+ mRNA from the tissues indicated was hybridized with ^{32}P-labeled probes corresponding to ART-37, ART-27 and ART-5 (left panel).

Figure 3

A.

B.



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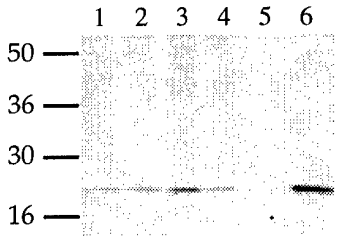

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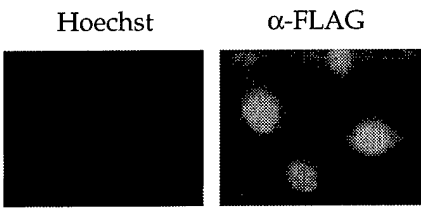

Figure 3. **Cloning and characterization of ART27.**
(A) The putative genomic organization of human ART27 is shown. Grey boxes represent exons and the exon numbers are noted inside their respective boxes. B) Human ART27 protein. The sequence of the human ART27 protein is shown. Filled dots indicate potential serine/theronine phosphorylation sites: protein kinase A (pKA), protein kinase C (pKC) and casein kinase II (CKII). Lines above the amino acid residues indicate predicted α-helices. (C) Western blot analysis of nuclear extracts from HeLa cells (lane1), HeLa cells treated with TPA (12-O-tetradecanoylphorbol-13-acetate) (lane2); HeLa cells, serum-stimulated (lane 3); PC-3 androgen-insensitive prostate cell line, (lane4); COS-1 cells transfected with either empty vector (*lane 5*), or a vector encoding for full length ART-27 (*lane 6*). (D) ART-27 subcellular localization. HeLa cells were transfected with a FLAG-ART-27 expression construct, fixed, permeabilized and incubated with an anti-FLAG primary antibody, a corresponding rhodamine-conjugated secondary antibody and the nuclear DNA was stained with Hoechst dye H334211. The rhodamine and Hoechst fluorescent signals were visualized using a Zeiss Axioplan 2 fluorescence microscope. Note that the FLAG-ART-27 fluorescence is localizes predominantly to the nucleus.

Figure 4

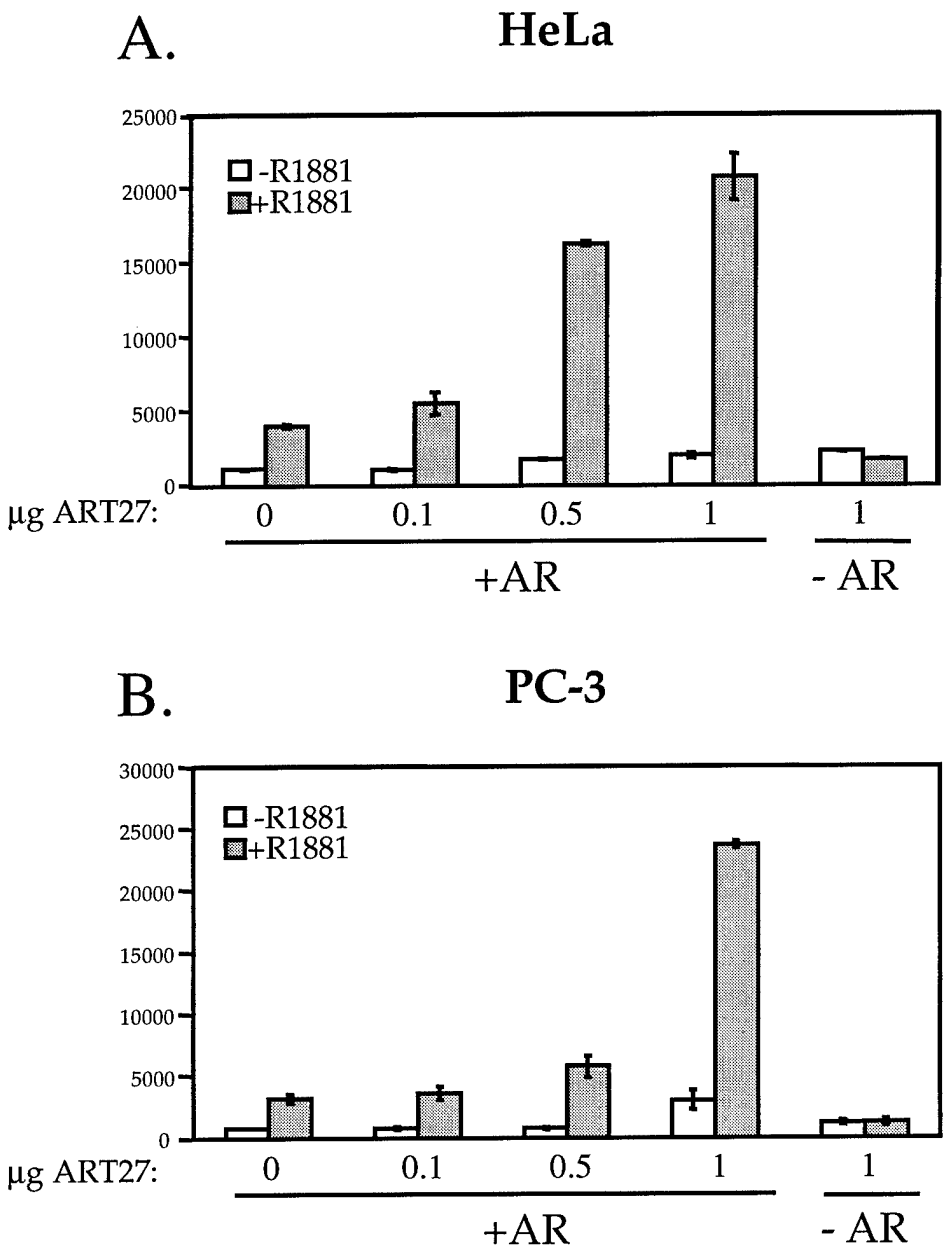

Figure 4: **ART-27 enhances AR -dependent transcriptional activation.**

HeLa cells (A), and PC-3 cells (B), both AR deficient, were transfected with expression plasmids for human AR (+AR) and ART-27 at the indicated amounts along with a MMTV-Luciferase reporter construct (2 µg/dish) and CMV-β-galactosidase (0.5 µg/dish) as an internal standard for transfection efficiency. Adding empty expression vector equalized the total amount of DNA per dish. Cells were treated with 100 nM R1881 (gray bars) or the ethanol vehicle (white bars) for 12 hr and AR transcriptional activation was assayed as described in Materials and Methods, normalized to β-galactosidase activity and expressed as relative luminescence units (RLU). The average of three independent experiments is shown with standard error.