

The Linkage of Kennedy's Neuron Disease to ARA24, the First Identified Androgen Receptor Polyglutamine Region-associated Coactivator*

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Although the linkage of polyglutamine (poly-Q) repeat expansion in the androgen receptor (AR) to Kennedy's disease (X-linked spinal and bulbar muscular atrophy) was a major step forward, the detailed molecular mechanism of how the change in poly-Q length contributes to the disease remains unclear. Here we report the identification of a nuclear G-protein, Ras-related nuclear protein/ARA24, as the first AR coactivator that can bind differentially with different lengths of poly-Q within AR. In the yeast and mammalian reciprocal interacting assays, our data suggested the interaction of AR N-terminal domain with ARA24 diminishes as the poly-Q length increases. The coactivation of ARA24 also diminishes with the poly-Q expansion within AR. Deletion of the acidic hexapeptide (DEDDDL) at the C terminus of ARA24 further enhances its AR coactivation. Together, our data suggest that poor interaction and weaker coactivation of ARA24 to the longer poly-Q AR in the X-linked spinal and bulbar muscular atrophied AR could contribute to the weaker transactivation of AR. The consequence of poor interaction and weak coactivation may eventually lead to the partial androgen insensitivity during the development of Kennedy's disease.

tial AR-associated proteins that may contribute to this disease. Using the yeast two-hybrid system we were able to find an AR coactivator, ARA24, that can differentially activate the AR with different poly-Q lengths within AR. Nucleotide sequencing revealed that this AR coactivator has an identical sequence with Ran (7).

Genetic results have identified that Ran/ARA24 is involved in nuclear transport of proteins and RNA, cell cycle progression, and nuclear structure in mitotic regulation, as well as RNA and DNA synthesis (8). Ran/ARA24 was purified as a complex with a chromatin-associated DNA-binding protein, RCC1 (regulator of chromosome condensation 1). RCC1 was first identified as the product of a gene mutated in the tsBN2 (temperature-sensitive baby hamster kidney) cell line (9). RCC1 has been shown to function as a guanine nucleotide exchange factor on Ran/ARA24 by increasing its rate $\sim 5 \times 10^5$ times. On the other hand, Ran GTPase-activating protein can also increase the GTPase activity $\sim 1 \times 10^5$ times (10). During biochemical identification of Ran-associated protein, previous reports mainly revealed its functions in nuclear transport (8). Here we demonstrate that Ran/ARA24 can also interact with the AR N-terminal poly-Q region and enhance the AR transactivation.

MATERIALS AND METHODS

Yeast Two-hybrid Screening—The cDNA fragment encoding the AR poly-Q stretch and its flanking region (amino acids 11–208) (11) were inserted into a pAS2 yeast expression plasmid as bait. Expression of this AR bait in the yeast strain Y190 for the screening of associated proteins from human brain cDNA library was performed following the standard protocols described previously (12–14).

Purification of Recombinant AR and ARA24 Proteins—The His-tagged full-length human AR (ARQ17) was used exclusively unless otherwise specified) was expressed by the baculovirus system described previously (15). The His-ARA24 was expressed in *Escherichia coli* strain BL21-(DE3)-LysS transformed with pET28c-ARA24 (Novagen). Both the His-tagged AR and His-tagged ARA24 proteins were partially purified by immobilized metal affinity chromatography using TALON metal affinity resin (CLONTECH) and quantified by Western blot analysis (16). The loading of nucleotide to ARA24 was done as described previously (17).

Antibodies and Immunoprecipitation—Protein A-agarose beads conjugated with polyclonal anti-AR antibody (NH-27) were coincubated with 100 ng of AR and 500 ng of ARA24 proteins in 0.5 ml of binding buffer (20 mM HEPES/KOH, pH 7.9, 50 mM KCl, 6 mM MgCl₂, 10% glycerol, 0.002% Nonidet P-40, 0.1 mg/ml bovine serum albumin) at 4 °C overnight with mild agitation. After five extensive washes with washing buffer (20 mM HEPES/KOH, pH 7.9, 200 mM KCl, 6 mM MgCl₂, 10% glycerol, 0.002% Nonidet P-40), the immunocomplex was examined as described in Fig. 1B.

Yeast Two-hybrid Liquid Culture β -Galactosidase Assay—Yeast clones transformed with pAS2-ARN (amino acids 11–208) and pGAD10-ARA24 were streaked on –2SD selective medium (devoid of Trp and Leu), freshly inoculated in the –2SD medium, and grown at 30 °C overnight. The yeast cells were diluted and regrown in –2SD medium to an A₆₀₀ value of ~ 0.6 . The cells were lysed in Z buffer (60 mM

Kennedy's disease (SBMA)¹ is a motor neuron disease characterized by progressive muscle weakness and atrophy (1). Over 50% of the affected males may also have gynecomastia and reduced fertility, suggesting a defect in AR function (2). This hypothesis was confirmed by finding an expansion of poly-Q in SBMA AR (3). Several hypotheses have been proposed to explain how changes in poly-Q length within AR contribute to the disease. 1) SBMA AR may reduce binding affinity to androgen (4); 2) SBMA AR may become more unstable and can degrade to small toxic AR (5); and 3) SBMA AR may aggregate in cytoplasm by cross-linking with itself or other proteins (6). The last hypothesis inspired us to find the poten-

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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) AF052578.

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¹ The abbreviations used are: SBMA, X-linked spinal and bulbar muscular atrophy; AR, androgen receptor; poly-Q, polyglutamine; ARA24, AR-associated protein 24; Ran, Ras-related nuclear protein; RCC1, regulator of chromosome condensation 1; ARA24as, antisense ARA24; ARA160, AR-associated protein 160; Luc, luciferase; DHT, 5 α -dihydrotestosterone; CAT, chloramphenicol acetyltransferase; GBD, GAL4 DNA binding domain; WT, wild type.

Na_2HPO_4 , 40 mM NaH_2PO_4 , 10 mM KCl, 1 mM MgSO_4 , 50 mM 2-mercaptoethanol, pH 7.0) by five freeze-thaw cycles in liquid nitrogen and at 37 °C. The A_{420} value was measured following incubation with *O*-Nitrophenol β -galactopyranoside. The relative β -galactosidase value was determined with normalization of the sample A_{600} value.

Plasmids—pCMX-ARA24 and pCMX-ARA24 C-del were constructed by ligating full-length and C-del mutant (amino acids 1–210, Fig. 1A) ARA24, respectively, into pCMX expression vector. pCMV-antisense ARA24 (ARA24as) was constructed by inserting a 334-base pair *Bgl*II fragment of Ran/ARA24, which spans the 5'-untranslated region and the translation start codon of ARA24 (nucleotides 1–334, Fig. 1A), into pCMV vector in the antisense orientation. pCMX-ARA160 was constructed by inserting the full-length ARA160 into pCMX expression vector. pG5E1b-Luc was constructed by ligating 5 \times GAL4 binding sites and E1b TATA core promoter into pGL3 (Promega). pMMTV-Luc is driven by mouse mammary tumor virus-long terminal repeat promoter, which was provided by B. O'Malley at Baylor University. p6R-ARQ1, p6R-ARQ25, p6R-ARQ35, and p6R-ARQ49, which express AR of different poly-Q lengths under the control of Rous sarcoma virus promoter, were provided by R. L. Miesfeld from the University of Arizona, Tucson. pM-ARA24 was generated by inserting the full-length ARA24 into pM vector (CLONTECH). pVP16-ARN-Q1, pVP16-ARN-Q25, and pVP16-ARN-Q49 were generated by inserting each poly-Q AR N-terminal domain (AR amino acids 38–500) (11) into pVP16 vector (CLONTECH).

Transfection and Reporter Gene Assays—Human prostate cancer PC-3 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. $\sim 4 \times 10^5$ cells (50–70% confluency in 35-mm Petri dishes) growing in Dulbecco's modified Eagle's medium supplemented with 10% charcoal-dextran stripped fetal bovine serum were transfected by SuperFect transfection reagent (QIAGEN) with DNA as described in each figure legend. As a control of each expression plasmid, the same amount of parental expression plasmid was used and indicated as a minus in each figures 1, 2, 4, and 5. 24 h after transfection, the medium was changed to fresh medium containing different hormones as indicated in each figure legend for another 24 h. Luciferase activities were measured using the Dual-LuciferaseTM reporter assay system and Turner Designs luminometer TD-20/20 (Promega). Relative luciferase activities were plotted using the activity of DHT-AR in the absence of coactivator as 1. The results were summarized from at least three sets of transfection and presented as mean \pm S.E. For antisense ARA24 experiments, human androgen non-responsive NCI H1299 cells were transfected by the calcium phosphate method, and CAT assays were performed as described previously (18–22). Each final assessment represents the mean \pm S.E. of five independent experiments. For detecting the elimination of endogenous ARA24 protein levels by the introduction of antisense ARA24 (ARA2400as) into cells, H1299 cells were electroporated by Gene Pulser (Bio-Rad) with 10 μg of either parental vector (pCMV) or pCMV-ARA24as. The cells were harvested 48 h posttransfection.

RESULTS

Cloning of ARA24 by Yeast Two-hybrid System—A hybrid protein bait, GAL4 DNA binding domain (GBD) fused with the human AR poly-Q region (amino acids 11–208), was expressed in the yeast strain Y190 to screen the human brain cDNA library. A clone that showed strong interaction with the AR poly-Q region was identified. This clone has an insert of 1566 base pairs with an open reading frame encoding a peptide of 216 amino acids. Based on the calculated molecular mass (24 kDa) and its ability to bind to AR, we named this clone ARA24. GenBankTM sequence comparison found ARA24 has the same amino acid sequence as Ran, a Ras-like small nuclear GTPase. The nucleotide and deduced amino acid sequences of the ARA24 clone from yeast two-hybrid screening are shown in Fig. 1A.

We used coimmunoprecipitation followed by Western blot analysis to confirm the interaction between ARA24 and AR. In the presence of AR, ARA24 can be coimmunoprecipitated by anti-AR antibody (NH27) conjugated to protein A-agarose beads (Fig. 1B, lanes 1–3), and neither AR nor ARA24 alone (Fig. 1B, lanes 4 and 5) showed a comparable level of coimmunoprecipitation. Binding of GTP or GDP to ARA24 did not cause substantial differences of ARA24 protein to interact with

AR in the coimmunoprecipitation. These results strengthen the interaction of AR and ARA24 found in yeast screening. Our results also suggest that various guanine nucleotide binding states of ARA24 may not affect its interaction with AR *in vitro*. Furthermore, one-hybrid interaction in mammalian cells was applied to demonstrate the functional interaction of ARA24 with AR. Expression of GBD-fused ARA24 and AR in the presence of androgen forms a transactive complex that can activate the transcription of pG5-E1b-Luc. As shown in Fig. 1C, coexpression of GBD-fused ARA24 and AR in the presence of 1 nM DHT induces the luciferase activity. Taken together, results from each of the three experiments described in Fig. 1 provide strong evidence that AR can interact well with ARA24, the first identified AR poly-Q region-associated protein.

Expression of Either Wild Type ARA24 or Mutant ARA24 (C-del) Enhances AR Transactivation—It has been demonstrated that WT Ran/ARA24 or mutant Ran/ARA24, ARA24 C-del, with C-terminal hexapeptide deletion can be overexpressed up to 7–8-fold above endogenous background in transient transfection without affecting cell cycle progression (23). It was also suggested that the conserved acidic hexapeptide sequence, DEDDDL at the C-terminal end of Ran/ARA24, is not required for Ran/ARA24 to stay in the nucleus but might be required for interaction with an effector (23). We therefore examined the role of both WT ARA24 and ARA24 C-del in AR-mediated transactivation. Cotransfection of WT ARA24 enhanced AR-mediated transactivation ~ 4 -fold in PC-3 cells at a transfection ratio of AR to WT ARA24 of 1:30 (Fig. 2A). Unexpectedly, we found ARA24 C-del was a more potent AR coactivator. ARA24 C-del enhances AR transactivation up to 18-fold in PC-3 cells at a transfection ratio of AR to ARA24 C-del of 1:30 (Fig. 2B). Using antisense ARA24 expression plasmid (ARA24as), we also found that AR transactivation was significantly decreased by cotransfecting ARA24as (Fig. 2C). Western blot analysis data showed that the cotransfection of ARA24as could decrease the cellular ARA24 protein significantly 48 h after transfection, therefore confirming the reduced AR transactivation could be due to the reduced ARA24 protein caused by expression of ARA24as (Fig. 2D). Together, our results indicate that AR transactivation can be either enhanced or repressed by increasing or decreasing the cellular ARA24 protein amount.

Expression of ARA24 and ARA160 Increases DHT Activity Over 10-fold—Because ARA160, another identified AR N-terminal coactivator,² can also enhance AR transactivation, it is important to know the profile of DHT-induced AR transactivation in the presence or absence of these two AR N-terminal ARAs. In the absence of ARA160 and ARA24, DHT starts to induce AR-mediated transactivation between 10^{-9} and 10^{-10} M (Fig. 3, lanes 1–4). In the presence of ARA24 or ARA160, DHT significantly induces AR-mediated transactivation from 10^{-10} to 10^{-11} M (Fig. 3, lanes 5–12). These data suggest that these AR N-terminal coactivators can increase DHT sensitivity over 10-fold. This 10-fold increase may allow the low concentration of androgen (10^{-10} – 10^{-11} M) that exists in the serum of prostate cancer patients undergoing the androgen ablation therapy to induce androgen target genes. This may imply that these AR N-terminal coactivators could play very important roles in the hormone-refractory prostate cancer.

Expansion of AR Poly-Q Length Diminishes AR-ARA24 Interaction—As ARA24 associates to the AR poly-Q region, we then used yeast two-hybrid and mammalian two-hybrid assays to test if the different lengths of poly-Q in AR can influence this interaction. As shown in Fig. 4A, the interaction between

² Hsiao, P.-W., and Chang, C. (1999) *J. Biol. Chem.*, in press.

A

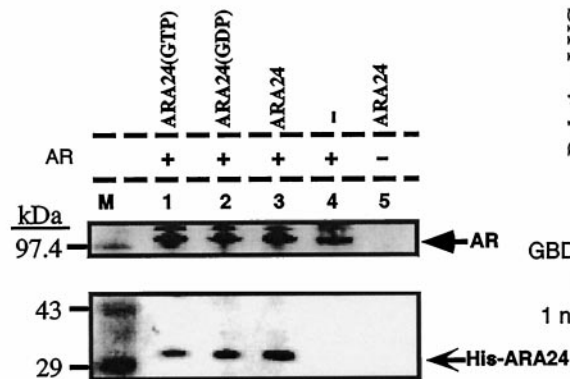
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1  M A A Q G E P Q V Q F K
61  cttgtattgggttggtggtggtgactggaaaagccctctgtaaacgctcatttgact
13  L V L V G D G G T G K T T F V K R H L T
121  ggtgaatttgagaagaagtatgtagccacctgggtgttgaggttccatccccagtggtc
33  G E F E K K Y V A T L G V E V H P L V F
181  caccaacacagaggaaccttaagttcattgtagggacacagccggccaggaagaattc
53  H T N R G P I K F N V W D T A G Q E K F
241  ggtgactgagagatggctattatataccaagccagtggtccatcataatgtttgatgta
73  G G L R D G Y Y I Q A Q C A I I M F D V
301  acatgagaggttacttacaagatgtgctaactggcattagagatctggtagcagtggt
93  T S R V T Y K N V P N W H R D L V R V C
361  gaaaacatccccattggttgggtggcaacaagtgatataaggacaggaagtgag
113  E N I P I V L C G N K V D I K D R K V K
421  gggaaatccattgtctccccaagaagaatctccagctactagacattcttgcacaa
133  A K S I V P H R K K N L Q Y Y D I S A K
481  agtaactacaacttgaagaagccctctctgcttggtaggaagctcattggagaccct
153  S N Y N F E K P F L W L A R K L I G D P
541  aactggaaatgttggcattgctgctctgcccaccagagtgctcagccagcct
173  H L E F V A M P A L A P P E V V H D P A
601  ttggcagcagctatgagcagcagctagaggttctcagcaaacctctctccagatgag
193  L A A Q Y E H D L E V A Q T T A L P D E
661  gatgatgacctgtgagaatgagctgggcccagcgtcagaagcttagttttaggcag
213  D D D L *
721  ctgtctgctgtagtgcagcggcagcgtgtgtgcccacctcattatctagctaaaggg
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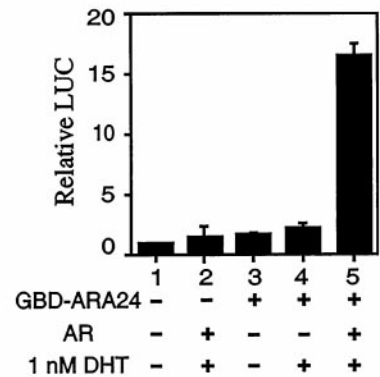
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FIG. 1. Sequences of human ARA24 cDNA and coimmunoprecipitation of ARA24 with AR. A, the 1566-base pair nucleotide sequences in the ARA24 clone contain an open reading frame 651 bases long (position from nucleotide bases 25 to 675) that encodes a peptide of 216 amino acids. The sequences also include a 5'-untranslated region of 24 base pairs and a relatively long 3'-untranslated region of 891 base pairs (GenBank™ accession number AF052578). B, coimmunoprecipitation of AR and ARA24. AR and His-ARA24, either alone (lanes 4 and 5) or combined (lane 1, GTP-bound His-ARA24; lane 2, GDP-bound His-ARA24; lane 3, nucleotide-free His-ARA24) were immunoprecipitated with anti-AR antibody (NH27). After washing, 50% of immunoprecipitates were analyzed by Western blot with NH27 anti-AR monoclonal antibody and anti-Ran/ARA24 antibody (Santa Cruz Biotechnology). M is the high molecular weight pre-stain protein marker (Life Technologies, Inc.). Both AR and His-ARA24 positions are indicated by an arrow. ARA24(GTP), GTP-bound His-ARA24; ARA24(GDP), GDP-bound His-ARA24. C, mammalian one-hybrid CAT assays. H1299 cells were transfected with 4 μ g of reporter construct, pG5CAT (CLONTECH) with or without pM-ARA24 (1 μ g) and pCMV-AR (1 μ g) and with or without addition of 1 nM DHT as depicted in the figure.

B



C



ARA24 and the AR poly-Q region in yeast cells decreased with the increase of AR poly-Q length. This result was further confirmed in mammalian Chinese hamster ovary cells by reciprocal two-hybrid assay with exchanged fusion partners (GBD-fused ARA24 *versus* VP16-ARN in mammalian systems as compared with GAD-ARA24 *versus* GBD-ARN in yeast systems) (Fig. 4B). Together, these results consistently show that the interaction between ARA24 and the AR poly-Q region is inversely correlated with AR poly-Q length in both yeast and mammalian Chinese hamster ovary cells. These data represent the first evidence to show that WT AR (Q-25) and SBMA AR (Q-49) can bind differentially to ARA24, the first identified AR poly-Q region coactivator.

Coactivation of ARA24 to AR Decreases with the Expansion of AR Poly-Q Length—We then compared the coactivator activity of ARA24 to AR with different poly-Q lengths (Q-25 *versus* Q-49). In the absence of ARA24 cotransfection, AR Q-49 shows a little less activity than AR Q-25 as previously reported (Fig. 5, lane 6 *versus* lane 2). Cotransfection of ARA24 enhances AR Q-25 transactivation 4-fold (Fig. 5, lane 4 *versus* lane 2). However, cotransfection of ARA24 enhances AR Q-49 transactivation only 2-fold (Fig. 5, lane 8 *versus* lane 6). Together, results

shown in Figs. 4 and 5 provide strong evidence to suggest that the lower AR transactivation of SBMA AR (Q-49) as compared with normal AR (Q-25) could be because of a weaker interaction with ARA24. The consequence of this weaker interaction could also result in the weaker AR transactivation.

DISCUSSION

Previous studies concerning the transactivation activity of AR with different poly-Q lengths have consistently shown their inverse relationship (21, 25). The present study demonstrates that ARA24 interacts with the AR poly-Q region and functions as an AR coactivator. The interaction between AR and ARA24 decreases with the expansion of AR poly-Q, as well as the coactivation of cotransfected ARA24 to the SBMA AR. These results provide a potential mechanism for the partial androgen-insensitive syndrome in Kennedy's disease.

It is well documented that a continual cycling of binding and hydrolyzing GTP from ARA24 is required in multiple cellular processes (reviewed in Ref. 8). Yeast mutations *prp20* and *rna1*, which are homologues of mammalian Ran-guanine nucleotide exchange factor and Ran-GTPase-activating protein,

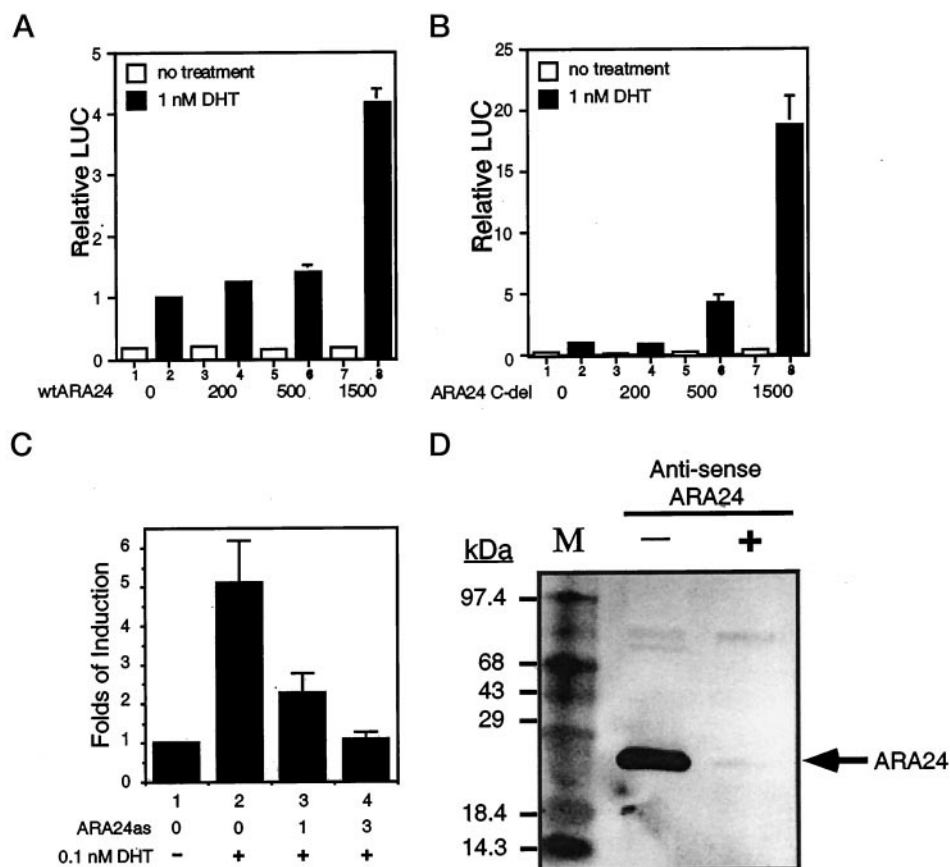


FIG. 2. Cotransfection of wild type ARA24 or mutant ARA24 C-del enhances AR transactivation. *A*, PC-3 cells were transfected with a DNA mixture containing pMMTV-Luc reporter (400 ng), pRL-CMV (50 ng), pCMV-AR (50 ng), and increasing amounts of pCMX-ARA24. DHT treatment, as indicated in the figures, was done 24 h after transfection. Luciferase activity was measured after another 24 h of incubation. *B*, PC-3 cells were transfected with the same DNA mixture as in Fig. 2*A* except pCMX-ARA24 C-del was replaced with pCMX-ARA24 (wild type). *C*, NCI H1299 cells were transfected with a DNA mixture containing pMMTV-CAT reporter (3 μ g), pSV- β -galactosidase (1 μ g), pSG5-AR (1 μ g), and increasing amounts of pCMV-ARA24as as indicated in the figure. DHT treatment was done 24 h after transfection. CAT and β -galactosidase activities were measured after another 24 h of incubation. *D*, the Western blotting of the cellular ARA24 protein. H1299 cells were electroporated either with parental vector, pCMV (indicated by a *minus*) or pCMV-ARA24as construct (indicated by a *plus* sign). Cells were harvested 48 h after transfection, and 15 μ g of total cell lysate was examined by Western blot analysis and by anti-ARA24 antibody (Santa Cruz Biotechnology).

respectively, have been shown to exhibit the phenotype of reduced accuracy in positioning the correct transcription initiation site (26). These phenotypes are readily explained by either a change in chromatin structure or by certain defects in the transcription regulation. The ability to enhance AR transactivation by ARA24 may therefore provide a bridge for ARA24 to link to the transcriptional regulation.

As shown in Fig. 1*B*, AR forms complexes with ARA24 regardless of the bound guanine nucleotide, which seems different from what has been observed for a GTPase effector protein that exhibits selective binding to either a GTPase-GTP or a GTPase-GDP. Upon inactivation of RCC1, ARA24 was shown dispersed in the nucleus and cytosol, suggesting that GTP-bound ARA24 is the major form in the nucleus (27). It seems that the AR-ARA24 interaction is not regulated by the guanine binding status of ARA24 *in vitro*. It is not clear whether the guanine binding status of ARA24 plays any role when mediating AR transactivation in cells.

ARA24 is a very abundant cellular protein. It is estimated that HeLa cells contain about 10^7 ARA24 molecules per cell (7). This high abundance does cause great difficulty when attempting to show its biochemical function by the addition of exogenous ARA24. This is probably the reason we need to use a relatively high dose of exogenous ARA24 to show its coactivator activity (Fig. 2*A*). Nevertheless, the ratios of receptor to coactivator we used here (1:10 or 1:30) are close to the ratios of other steroid receptors to their coactivators. For example, full-

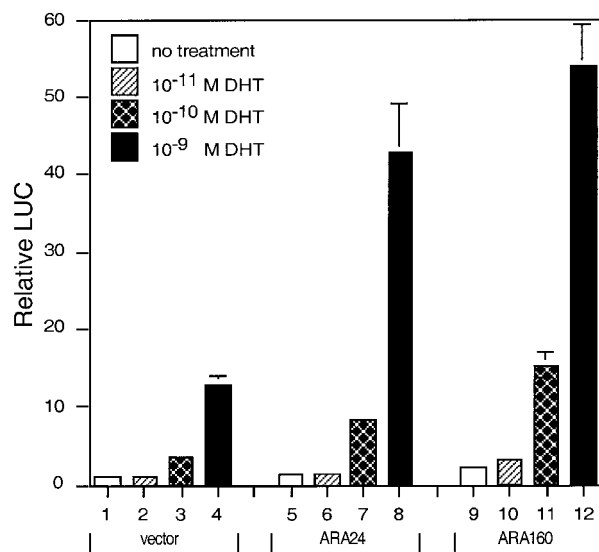


FIG. 3. ARA24 and ARA160 enhance the DHT activity for AR transcriptional activity in human prostate PC-3 cells. PC-3 cells were transfected with a DNA mixture containing pMMTV-Luc reporter (400 ng), pRL-CMV (50 ng), pCMV-AR (50 ng), and equal amounts of pCMX vector, pCMX-ARA24, or pCMX-ARA160 (1500 ng). Cells were treated with increasing concentrations of DHT (10^{-11} , 10^{-10} , and 10^{-9} M as indicated in the figure) 24 h after transfection. Luciferase activity was measured after another 24-h incubation.

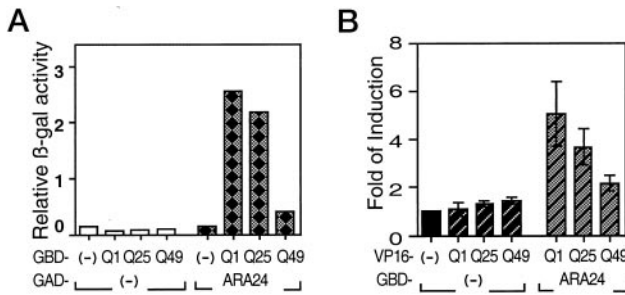


FIG. 4. The affinity of ARA24 to AR decreased by increasing the length of AR poly-Q. *A*, yeast (Y190) was transformed with GAL4AD-ARA24 and GAL4DBD-AR-N1 with altered poly-Q length (Q1, Q25, and Q49). The two-hybrid interaction is determined by β -galactosidase activity expressed in the yeast cells. *B*, the fusion partners of GAL4DBD and VP16AD for the two-hybrid proteins were exchanged and transiently transfected into Chinese hamster ovary cells to assay the reciprocal hybrid interaction. Chinese hamster ovary cells (3×10^5) were transfected with a DNA mixture containing pRL-tk (2 μ g), pG5E1b-Luc (4 μ g), pM-ARA24 (1 μ g), pVP16-ARN (1 μ g), and carrier DNA, pBluescript (3 μ g), by the calcium phosphate precipitation method. The relative two-hybrid interaction was determined by measurement of relative luciferase activity.

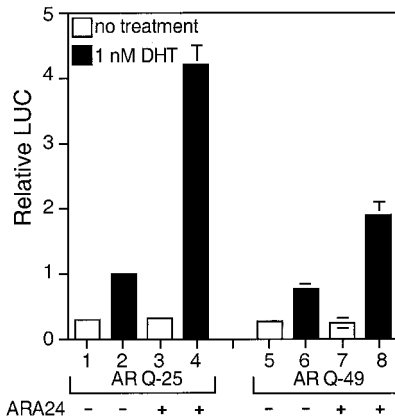


FIG. 5. The coactivation of ARA24 to AR diminished with poly-Q extension. PC-3 cells were transfected with a DNA mixture containing pMMTV-Luc reporter (300 ng), pRL-CMV (50 ng), p6R-AR (500 ng, AR Q-25 in lanes 1–4, AR Q-49 in lanes 5–8), and 1200 ng of either pCMX (lanes 3, 4, 7, and 8) or pCMX-ARA24 (lanes 1, 2, 5, and 6). DHT treatment was done as in Fig. 2.

length SRC-1 requires the addition ratio of 1:20 or 1:40 to see the coactivator effects (28). On the other hand, cotransfection of ARA24as to decrease the cellular ARA24 protein level can also repress the AR transactivation. As shown in Fig. 2B, deleting the six amino acid residues of the acidic C terminus in ARA24 results in a dominant positive AR coactivator. A lower (500 ng) level of ARA24 C-del is enough to exert a coactivation effect to AR compared with 1500 ng of WT ARA24. Moreover, increasing ARA24 C-del to a higher dose (1500 ng) further enhances DHT-induced AR transactivation from 4-fold to 18-fold. As the C-terminal hexapeptide sequence of ARA24 is conserved across species, it is likely that a repressor-like factor may bind to the conserved hexapeptide sequence, and deletion of this hexapeptide may therefore allow ARA24 C-del to function more efficiently as a coactivator to enhance AR transactivation.

The AR coactivator effects of ARA24 and ARA160 are not only enhancement of AR transactivation at the normal physiological concentration of DHT but also at the low DHT level (10^{-10} – 10^{-11} M) that is found in prostate cancer patients undergoing androgen ablation therapy. The increase of androgen

sensitivity by the non-androgen factors could be one of the reasons why the patients' prostate-specific antigen level might increase again even though the patients' serum testosterone is low (10^{-10} M). Our findings that either ARA24 or ARA160 can increase androgen sensitivity so that 10^{-10} M DHT can still efficiently stimulate androgen target genes, including prostate-specific antigen, may provide one molecular mechanism to explain the failure of androgen ablation therapy.

The polymorphic poly-Q stretch of AR has been specially recognized for being clinically associated with Kennedy's disease. Subsequent *in vitro* studies also show that the longer poly-Q stretch may result in weaker AR transactivation (21, 25). Recently, pathological statistics have further correlated the shorter poly-Q AR with higher prostate cancer risk and a higher chance of developing distant metastasis (24, 29). Our results showed that ARA24 can interact differentially with different lengths of poly-Q within AR, and its ability to enhance AR transactivation also decreases with expansion of poly-Q length in AR. A very interesting question to pursue in the future might be whether expression of ARA24 or its ability to interact with the AR poly-Q region could become an indicator to predict prostate cancer risk.

As ARA24 is also involved in nuclear transportation that can promote the nuclear localization of proteins, it is possible that the weaker interaction of ARA24 to longer poly-Q AR (SBMA AR) may result in aggregation of SBMA AR in cytoplasm, instead of translocation into the nucleus to function as a transcriptional activator. A detailed analysis of subcellular distribution of ARA24 and SBMA AR in Kennedy's disease cells should be able to answer such an interesting question.

In summary, our results suggested that ARA24 is the first identified AR N-terminal associated coactivator that can interact differentially with AR bearing different poly-Q lengths. This finding may help us to further understand the potential roles of AR function in Kennedy's disease and progression of prostate cancer.

REFERENCES

- Kennedy, W. R., Alter, M., and Sung, J. H. (1968) *Neurology* **18**, 671–680
- Nagashima, T., Seko, K., Hirose, K., Mannen, T., Yoshimura, R., Arima, R., Nagashima, K., and Morimatsu, Y. (1988) *J. Neurol. Sci.* **87**, 141–152
- La Spada, A. R., Wilson, E. M., Lubahn, D. B., Harding, A. E., and Fischbeck, K. H. (1991) *Nature* **352**, 77–79
- MacLean, H. E., Choi, W. T., Rekaris, G., Warne, G. L., and Zajac, J. D. (1995) *J. Clin. Endocrinol. Metab.* **80**, 508–516
- Choong, C. S., Kempainen, J. A., Zhou, Z. X., and Wilson, E. M. (1996) *Mol. Endocrinol.* **10**, 1527–1535
- Merry, D. E., Kobayashi, Y., Bailey, C. K., Taye, A. A., and Fischbeck, K. H. (1998) *Hum. Mol. Genet.* **7**, 693–701
- Bischoff, F. R., and Pongstingl, H. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 10830–10834
- Rush, M. G., Drivas, G., and D'Eustachio, P. (1996) *Bioessays* **18**, 103–112
- Nishitani, H., Ohtsubo, M., Yamashita, K., Iida, H., Pines, J., Yasudo, H., Shibata, Y., Hunter, T., and Nishimoto, T. (1991) *EMBO J.* **10**, 1555–1564
- Klebe, C., Bischoff, F. R., Pongstingl, H., and Wittinghofer, A. (1995) *Biochemistry* **34**, 639–647
- Chang, C., Kokontis, J., and Liao, S. (1988) *Science* **240**, 324–326
- Yeh, S., and Chang, C. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 5517–5521
- Fugimoto, N., Yeh, S., Kang, H.-Y., Chang, H., Inui, S., Chang, H.-C., Mizokami, A., and Chang, C. (1999) *J. Biol. Chem.* **274**, 8316–8321
- Kang, H.-Y., Yeh, S., Fugimoto, N., and Chang, C. (1999) *J. Biol. Chem.* **274**, 8570–8576
- Chang, C., Wang, C., DeLuca, H. F., Ross, T. K., and Shih, C. C. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 5946–5950
- Yeh, S., Miyamoto, H., Nishimura, K., Kang, H., Ludlow, J., Hsiao, P., Wang, C., Su, C., and Chang, C. (1998) *Biochem. Biophys. Res. Commun.* **248**, 361–367
- Chi, N. C., Adam, E. J., Visser, G. D., and Adam, S. A. (1996) *J. Cell Biol.* **135**, 559–569
- Miyamoto, H., Yeh, S., Wilding, G., and Chang, C. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 7379–7384
- Miyamoto, H., Yeh, S., Lardy, H., Messing, E., and Chang, C. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 11083–11088
- Yeh, S., and Chang, C. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 5527–5532
- Yeh, S., Lin, H., Kang, H.-Y., Lin, M., and Chang, C. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 5458–5463

22. Lin, D.-L., and Chang, C. (1996) *J. Biol. Chem.* **271**, 14649–14652
23. Ren, M., Coutavas, E., D'Eustachio, P., and Rush, M. G. (1994) *Mol. Cell. Biol.* **14**, 4216–4224
24. Stanford, J. L., Just, J. J., Gibbs, M., Wicklund, K. G., Neal, C. L., Blumenstein, B. A., and Ostrander, E. A. (1997) *Cancer Res.* **57**, 1194–1198
25. Kazemi-Esfarjani, P., Trifiro, M. A., and Pinsky, L. (1995) *Hum. Mol. Genet.* **4**, 523–527
26. Forrester, W., Stutz, F., Rosbash, M., and Wickens, M. (1992) *Genes Dev.* **6**, 1914–1926
27. Ren, M. G., Drivas, P., D'Eustachio, P., and Rush, M. G. (1993) *J. Cell Biol.* **120**, 313–323
28. Takeshita, A., Cardona, G. R., Koibuchi, N., Suen, C.-S., and Chin, W. W. (1997) *J. Biol. Chem.* **272**, 27629–27634
29. Giovannucci, E., Stampfer, M. J., Krithivas, K., Brown, M., Dahl, D., Brufsky, A., Talcott, J., Hennekens, C. H., and Kantoff, P. W. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 3320–3323