2 The Androgen Receptor Co-regulator, ARA_{70}

H. Miyamoto, S. Yeh, S.-B. Hwang, N. Fujimoto, P. Hsiao,
H. Ting, K. Nishimura, C. Wang, S. Inui, H. Uemura, H. Kang,
and C. Chang

2.1 Cloning of ARA_{70} 43
2.2 DU145 Cell Lines with Distinct Morphology 45
2.3 AR Vectors and ARA_{70} Vectors 46
2.4 Different ARE Reporters 46
2.5 Mutated ARs 48
2.6 ARA_{70} and Prostate Cancer 50
2.7 Conclusion 50
References 51

2.1 Cloning of ARA_{70}

Androgens play an important role in the process of male sexual differentiation and development. Their actions are mediated by the androgen receptor (AR), which is a member of a large family of ligand-dependent transregulators known as the steroid receptor superfamily (Chang et al. 1988; Evans 1988; Lubahn et al. 1988). To further understand the mechanism of androgen-AR action, we have applied a yeast two-hybrid system to identify the AR-associated proteins. An AR-associated protein, ARA_{70}, was isolated from a brain cDNA library using the GAL4 DNA binding domain (DBD) fused with human AR peptide (amino acids 595–918) as bait (Yeh and Chang 1996). The β-galactosidase liquid assay in the yeast showed that ARA_{70} interacted with AR, but not

with other nuclear receptors, such as retinoic acid receptor (RXR) and TR4 orphan receptor.

To test whether ARA70 can affect the transcriptional activity of AR, AR and ARA70 were co-transfected into human prostate cancer DU145 cells under eukaryotic promoter control. Ligand-free AR has a minimal reporter gene activity of mouse mammary tumor virus-androgen response element-chloramphenicol acethyltransferase (MMTV-ARE-CAT) with or without co-transfection of ARA70. Addition of dihydrotestosterone (DHT) results in a between five and six-fold increase of AR activity. Furthermore, this transcriptional activity can be increased 40- to 60-fold by co-transfection of ARA70 with 1.5 μ g of AR in a dose-dependent manner, reaching a plateau at 4.5 μ g of ARA70 in DU145 cells. To rule out any indirect effects on the basal activity of the MMTV-ARE CAT reporter, the ARE DNA fragment was removed from the reporter plasmid. The results showed that ARA70 induced no activity on this reporter in the presence or absence of androgen. These data suggested that stimulation of AR transcriptional activity by ARA70 may occur through a ligand-bound AR. On the other hand, the transcriptional activity of other steroid receptors, such as the glucocorticoid receptor, progesterone receptor, and estrogen receptor cannot be induced significantly (near two-fold) by ARA70 in the presence of their own ligands. These findings clearly indicated that ARA70 is the first identified ligand-dependent associated protein that might function as a relatively specific co-activator for AR.

To further confirm the interaction between AR and ARA70, we then applied an in vitro immunoprecipitation assay with an AR antibody (CW2). We demonstrated that CW2 can co-precipitate AR and ARA70 when incubated with *in vitro* transcribed/translated full-length human AR and ARA70. This precipitation was specific, as CW2 did not precipitate two other proteins (RXR and TR4 orphan receptor) incubated with AR. A Far-Western assay also demonstrated that ARA70 can bind to the immobilized ligand-binding domain of AR but not other control proteins. Together, these data suggest that an increase in the transcriptional activity by ARA70 is due to a direct interaction between AR and ARA70 (Yeh and Chang 1996).

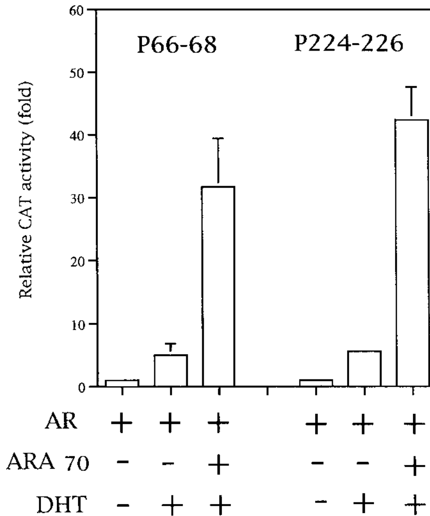

Fig. 1. ARA_{70} can enhance the transcriptional activity of the androgen receptor (*AR*) in different passage numbers of DU145 cells. Fixed amounts of pSG5-AR (1.5 μg) and pSG5-ARA70 (4.5 μg) were transfected into DU145 cells with passage numbers 66–68 and 224–226 in the presence of 10^{-9} *M* dihydrotestosterone (*DHT*) for a chloramphenicol acetyltransferase (*CAT*) assay. In each transfection, 3.5 μg of reporter [mouse mammary tumor virus chloramphenicol acetyltransferase (MMTV-ARE-CAT)] was co-transfected. A β-galactosidase expression plasmid, pCMV-β-gal, was used as an internal control for transfection efficiency. The total amount of DNA was adjusted to 10.5 μg with pSG5 in all experiments. The mock treatment was set as one-fold. All data were the average results ± S.D. of three independent experiments

2.2 DU145 Cell Lines with Distinct Morphology

Two DU145 cell lines (passage numbers 59–68 from American Type Culture Collection and passage numbers 205–226 from Dr. G. Wilding, University of Wisconsin-Madison) were used for our current ARA_{70} studies. Interestingly, we found differences in the morphology of these two DU145 cell lines. Using a Northern blot analysis of these two DU145 cell lines, we detected the expression of ARA_{70} mRNA contrary to our previous report (Yeh and Chang 1996) that suggested ARA_{70} mRNA levels were undetectable. The difference could be due to differ-

ent passage numbers of DU145 cell lines with different morphology. It was reported that the different passage numbers of prostate cancer cell lines may have different characteristics. For example, Northern blot analysis detected prostatic acid phosphatase in prostate cancer LNCaP cell line with the passage numbers from 25 to 44, but not with the passage numbers higher than 100 (Garcia-Arenas et al. 1995). In addition to morphological differences, we found transfection efficiency (using the calcium phosphate method; Mizokami et al. 1994) was also different between these two cell lines, with much lower β-galactosidase activity in DU145 with passage numbers from 59 to 68. In spite of the above differences, the induction of AR transcriptional activity by ARA_{70} was reproducible in these two DU145 cell lines. The average induction in these cells was approximately five- to eight-fold in the presence of 10^{-9} *M* DHT (Fig. 1).

2.3 AR Vectors and ARA_{70} Vectors

To rule out the possibility that the effect of ARA_{70} resulted from the plasmid backbone sequence, the pSG5-AR expression vector was replaced with the pCMV-AR or pCMVneo-AR expression vector. As shown in Fig. 2a, ARA_{70} can induce the transcriptional activity of AR with different expression vectors in DU145 cells. Also, AR transcriptional activity was induced both with pSG5-ARA_{70} and pCMV-ARA_{70} (Fig. 2b). For a control, a parent expression vector was always used to obtain equal amounts of plasmids in each transfection. Together, these data indicate that DHT-dependent induction of the transcriptional activity of AR by ARA_{70} is not due to the backbone of the vectors.

2.4 Different ARE Reporters

We replaced MMTV-ARE CAT with PSA-ARE CAT, which encompasses the promoter of the prostate-specific antigen (PSA) gene from –540 bp to the translational start site. PSA is a classic androgen target gene and is widely used as a tumor marker for patients with prostate cancer. As shown in Fig. 3, ARA_{70} enhanced AR transcriptional activity on the PSA receptor gene five-fold in the presence of 10^{-9} *M* DHT. We

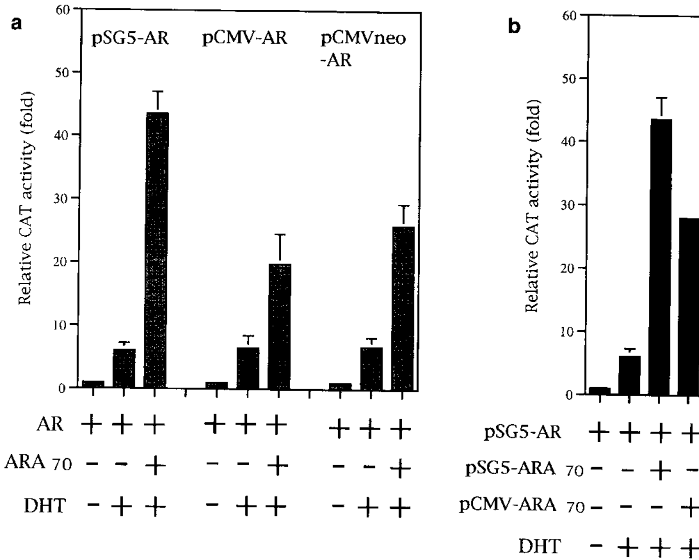

Fig. 2. Effects of ARA_{70} with different backbone plasmids of the androgen receptor (*AR*) (**a**) and *ARA_{70}* (**b**). Chloramphenicol acetyltransferase (*CAT*) activity was determined in DU145 cells co-transfected with (**a**) *pSG5-AR* (1.5 μg), *pCMV-AR* (1.5 μg), or *pCMVneo-AR* (1.5 μg), and *pSG5-ARA_{70}* (4.5 μg), or (**b**) *pSG5-AR* (1.5 μg) and *pSG5-ARA_{70}* (4.5 μg), or *pCMV-ARA_{70}* (4.5 μg) in the absence or presence of 10^{-9} *M* dihydrotestosterone (*DHT*). Mouse mammary tumor virus chloramphenicol acetyltransferase was used as a reporter for the assay of transcriptional activity. All data were the average results ± S.D. of three independent experiments

further applied two copies of ARE oligomers (tyrosine aminotransferase; TAT-ARE) linked to the CAT reporter to test ARA_{70} effect. While the CAT conversion rate was low, ARA_{70} still showed an induction effect (Fig. 3). These results suggest that ARA_{70} may be able to induce general AR target genes which may play an important role in the development of normal prostate and prostate cancer.

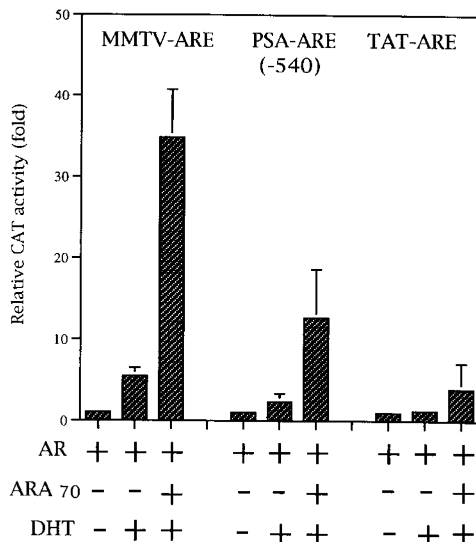

Fig. 3. Effects of ARA_{70} with different ARE reporters. Chloramphenicol acethyltransferase (*CAT*) activity was determined in DU145 cells co-transfected with pSG5-AR (1.5 μg), pSG5-ARA_{70} (4.5 μg), and 3.5 μg of mouse mammary tumor virus chloramphenicol acetyltransferase (*MMTV-ARE*) CAT, prostate-specific antigen ARE (*PSA-ARE*) CAT, or tyrosine aminotransferase ARE (*TAT-ARE*) CAT in the absence or presence of 10^{-9} *M* dihydrotestosterone (*DHT*). All data were the average results ± S.D. of three independent experiments

2.5 Mutated ARs

The effect of ARA_{70} on the transcriptional activity of mutant AR was also examined. It has been reported that some human prostate tumors were found to have mutations of the AR gene, and that a number of mutations in the hormone-binding domain can alter the specificity of AR (Taplin et al. 1995). Therefore, it was of great interest to examine whether ARA_{70} interacted with mutant ARs to understand any association between ARA_{70} and prostate cancer. In this study, three mutant ARs from human prostate tumors (AR715, AR874, and AR877) were tested. As shown in Fig. 4, the induction of transcriptional activity of these mutated ARs by ARA_{70} was similar to that of wild-type AR in the

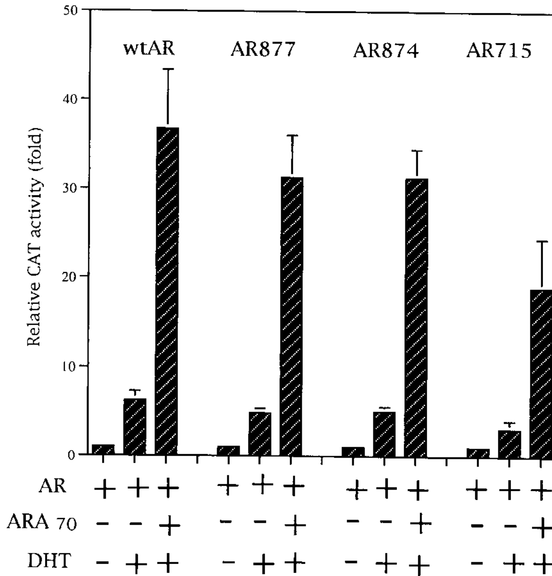

Fig. 4. Effects of ARA_{70} on the mutated androgen receptor (*AR*) transcriptional activity. Chloramphenicol acetyltransferase (*CAT*) activity was determined in DU145 cells co-transfected with pSG5-wild-type-AR (*wtAR*) (1.5 μg), pSVL-AR877 (*AR877*) (1.5 μg), pSVL-AR874 (*AR874*) (1.5 μg), or pSVL-AR715 (*AR715*) (1.5 μg), and pSG5-ARA_{70} (*ARA_{70}*) (4.5 μg) in the absence or presence of 10^{-9} *M* dihydrotestosterone (*DHT*). All data were the average results ± S.D. of three independent experiments

presence of 10^{-9} *M* DHT in DU145 cells (between six- and seven-fold). Another AR mutation with one amino acid substitution at the second zinc finger of DBD (Arg614 to His614) that proved to be insensitve to androgen action was demonstrated to be insensitive to ARA_{70} induction (data not shown). These results suggest that functional AR and ARA_{70} may be needed for the maximal androgen action in DU145 cells.

2.6 ARA_{70} and Prostate Cancer

We have recently reported that higher concentrations of hydroxyflutamide (HF), one of the antiandrogens, may become an agonist to androgens and that ARA_{70} may be able to enhance this agonistic activity (Yeh et al. 1997). We are also testing other antiandrogens, and the results indicate that agonist effect of these antiandrogens may be enhanced in the presence of AR and ARA_{70} in DU145 cells. These data suggest that agonist activity of the antiandrogens may require some co-activators, such as ARA_{70}, for their maximal action. Our preliminary data also demonstrated the expression of ARA_{70} in human prostate cancer. *In situ* hybridization revealed that the signals of ARA_{70} mRNA expression were found in most prostate cancer tissues, and that expression levels of ARA_{70} were widely distributed (unpublished data). Together, these data suggest that ARA_{70} expression may be involved in the response to antiandrogen therapy for prostate cancer.

2.7 Conclusion

Using several different controls, our data clearly demonstrate that ARA_{70} can increase the transcriptional activity of AR in DU145 cells by between three- and eight-fold. Since the induction can be detected only when AR and ARA_{70} in a ratio above 1:3 were co-transfected, it may occur in a very tightly controlled fashion. The correct conditions of transfection and ratios of AR and ARA_{70} may play a critical role in the induction of AR transcriptional activity. Further studies with antisense ARA_{70} or so-called dominant negative experiments, as well as knock-out ARA_{70} gene may prove that these *in vitro* effects of ARA_{70} can also occur *in vivo*. Until then, *in vitro* transfection data may only suggest a potential role of ARA_{70} in androgen-AR function.

In conclusion, ARA_{70} can induce the transcriptional activity of both wild-type and several mutant ARs in DU145 cells in a ligand-dependent manner. The induction occurs irrespective of plasmid backbone of AR or ARA_{70} vector, or reporter of the androgen target gene. Our data suggest that ARA_{70} may function as a relatively specific co-activator for AR. Although several co-factors have been demonstrated to interact with steroid receptors (review Horwitz et al. 1996), none of these co-factors

have been reported to specifically enhance AR-mediated transcriptional activity. Therefore, it is likely that ARA_{70} has a different mechanism for interacting with AR. Further studies of the potential role of ARA_{70} are required to better understand the molecular mechanism of androgen action.

Acknowledgments. The plasmids of ARs with mutations in codons 715, 874, and 877 were kindly provided by Dr. Steven Balk.
This work was supported by NIH grants CA71570 and CA68568.
Authors H. Miyamoto and S. Yeh contributed equally to this work and should both be considered as first author of this chapter.

References

Chang C, Kokontis J, Liao ST (1988) Molecular cloning of human and rat complementary DNA encoding androgen receptors. Science 240:324–326

Evans RM (1988) The steroid and thyroid hormone receptor superfamily. Science 240:889–895

Garcia-Arenas R, Lin F-F, Lin D, Jin L-P, Shih CC-Y, Chang C, Lin M-F (1995) The expression of prostatic acid phosphate is transcriptionally regulated in human prostate carcinoma cells. Mol Cell Endocrinol 111:29–37

Horwitz KB, Jackson TA, Bain DL, Richer JK, Takimoto GS, Tung L (1996) Nuclear receptor coactivators and corepressors. Mol Endocrinol 10:1167–1177

Lubahn DB, Joseph DR, Sullivan PM, Willard HF, French FS, Wilson EM (1988) Cloning of human androgen receptor complementary DNA and localization to the X chromosome. Science 240:327–330

Mizokami A, Yeh S, Chang C (1994) Identification of 3',5'-cyclic adenosine monophosphate response element and other *cis*-acting elements in the human androgen receptor gene promoter. Mol Endocrinol 8:77–88

Taplin M-E, Bubley GJ, Shuster TD, Frantz ME, Spooner AM, Ogata GK, Keer HN, Balk SP (1995) Mutation of the androgen-receptor gene in metastatic androgen-independent prostate cancer. N Engl J Med 332:1393–1398

Yeh S, Chang C (1996) Cloning and characterization of a specific coactivator, ARA_{70}, for the androgen receptor in human prostate cells. Proc Natl Acad Sci USA 93:5517–5521

Yeh S, Miyamoto H, Chang C (1997) Hydroxyflutamide may not always be a pure antiandrogen. Lancet 349:852–853