Effect of the androgen receptor CAG repeat polymorphism on transcriptional activity: specificity in prostate and non-prostate cell lines

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ABSTRACT

The action of androgens is essential for the development of benign prostatic hyperplasia and carcinoma of the prostate. The androgen receptor is a ligand-dependent nuclear transcription factor. The transcriptional activation domain of the androgen receptor gene contains a polymorphic CAG repeat sequence. A shorter CAG repeat sequence within the normal range has been reported to be associated with increased risk of prostate cancer and symptomatic benign prostatic hyperplasia. Here, we examine the in vitro transcriptional activity of the androgen receptor (AR) with different numbers of CAG repeats within the normal range in a number of different cell lines of prostatic (LNCaP, PC3) and non-prostatic (COS-1, MCF7) origin. We utilize a luciferase reporter driven by the rat probasin promoter (−286/+28) containing two androgen receptor binding sites. Transcriptional activation of the androgen responsive reporter was observed to be greater with the AR containing 15 vs 31 CAG repeats in COS-1 cells (123.2 ± 16.6 vs 78.2 ± 10.9, P value 0.01) and the well differentiated prostate cancer cell line LNCaP (103.4 ± 17.7 vs 81.4 ± 7.7, P value 0.045). No difference was observed in the poorly differentiated prostate cancer cell line, PC3 (106.9 ± 21.9 vs 109.6 ± 21.4, P value >0.5) or the breast cancer cell line MCF7 (120.4 ± 39.4 vs 103.1 ± 23.1, P value >0.5). Dose–response experiments with varying quantities of ligand (0.01, 0.1, 1 and 10 nM dihydrotestosterone) or AR cDNA did not demonstrate significant differences in transactivation of the androgen responsive reporter in PC3 cells by the different AR constructs. This suggests that the lack of influence of CAG number in this prostatic cell line is not related to dose of ligand or quantity of androgen receptor. Western immunoblot analysis of androgen receptor protein in transiently transfected COS-1 cells did not demonstrate a difference in the expression of the androgen receptor protein with different numbers of CAG repeats following incubation in the presence or absence of androgen. Gel shift assay did not demonstrate increased DNA binding by androgen receptor with a shorter CAG repeat sequence. These experiments using a relatively androgen- and prostate-specific reporter provide evidence for an inverse relationship between androgen receptor transcriptional activity and the number of CAG repeats in the transcriptional activation domain. The effect of CAG repeat number was cell specific suggesting the involvement of accessory factors expressed differentially between different cell lines.

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INTRODUCTION

The action of androgens is critical for the growth and development of the prostate and for the development and progression of both prostate cancer and benign prostatic hyperplasia. Androgen action is mediated via binding to the androgen receptor (AR), a ligand-dependent nuclear transcription factor (Rundlett et al. 1990). The human AR gene contains a polymorphic CAG trinucleotide repeat sequence which encodes a polyglutamine tract in the amino terminal domain of the AR protein (Faber et al. 1989). Case control studies have demonstrated an association between the AR
trinucleotide CAG repeat polymorphism and the risk of prostate cancer, with a shorter number of repeats associated with increased risk of disease (Giovannucci et al. 1997, Hakimi et al. 1997, Ingles et al. 1997) and with earlier age at diagnosis (Hardy et al. 1996). Variability in the AR CAG repeat has also been shown to be associated with the risk of developing symptomatic benign prostatic hyperplasia (Giovannucci et al. 1999). The finding of an association between this polymorphism and the pathogenesis of these common benign and malignant androgen-dependent diseases of the prostate lends support to the hypothesis that androgen receptor transcriptional activity varies according to the length of this trinucleotide repeat sequence within the normal range.

The AR polyglutamine tract resides in the amino-terminal domain of the receptor. Mutational deletion studies have shown this domain to be necessary for full in vivo transcriptional activation activity of the receptor, hence this region is referred to as the transcriptional activation domain (Rundlett et al. 1996, Gao et al. 1996). A number of studies have examined the effect of CAG repeat number on AR transcriptional activity. Some of these have demonstrated reduced in vitro transcriptional activation of an androgen responsive reporter construct by an AR with a pathologically expanded CAG repeat sequence (Mhatre et al. 1993, Chamberlain et al. 1994, Jenster et al. 1994, Kazemi-Esfarjani et al. 1995, Nakajima et al. 1996). One study has demonstrated a reduction in AR mRNA and protein levels with an expanded CAG repeat sequence (Choong et al. 1996). Most of these reports have focussed on the expanded CAG repeat sequence as seen in the rare degenerative neurological syndrome, X-linked spinal bulbar muscular atrophy (Quigley et al. 1995). Only one report, utilising COS-7 cells and a reporter construct derived according to the non-androgen specific mouse mammary tumour virus (MMTV) promoter, has specifically examined the effect of CAG variation within the normal range on androgen receptor transcriptional activity (Tut et al. 1997). This study demonstrated an inverse relation between CAG number and activation of an androgen responsive reporter within a narrow range of androgen concentrations.

The actions of steroid hormone receptors and hormone responsive promoters in androgen responsive genes show both tissue and cellular specificity (Rennie et al. 1993, Cleutjens et al. 1996, Brookes et al. 1998). To examine the effect of the AR CAG repeat polymorphism in vitro in relation to the pathogenesis of androgen-dependent diseases within the prostate we have characterised the transcriptional activity of the AR with variable numbers of CAG repeats within the normal range. We studied AR transcriptional activity in a number of cell lines (prostatic and non-prostatic), utilising an androgen responsive reporter construct containing rat probasin promoter (Kasper et al. 1994). This promoter sequence has previously been shown to be relatively androgen (Rennie et al. 1993, Claessens et al. 1996) and prostate specific (Greenberg et al. 1994, Brookes et al. 1998).

MATERIALS AND METHODS

Plasmid DNA constructs

The human (h) androgen receptor expression vector pCMVhAR (provided by Drs Frank French, Dennis Lubahn and Elizabeth Wilson, The Laboratories for Reproductive Biology, University of North Carolina, Chapel Hill, USA) was used to derive constructs with 15, 24 and 31 CAG repeats within exon A of the hAR. Genomic DNA obtained from human subjects with previously determined AR CAG repeat number was amplified by polymerase chain reaction (PCR) using the high fidelity DNA polymerase enzyme Pfu (Stratagene, La Jolla, CA, USA). The PCR reaction mix contained 50 ng genomic DNA template, 1 µM oligonucleotide primers (5’-TCCAGAATCTGTTCCAGAGCG and 5’-GTATCTTCAGTGCTTCTTG) (Amrad Pharmacia Biotech, Melbourne, Australia), 0·2 mM dNTPs (Promega Corporation, Madison, WI, USA), 2·5 units Pfu DNA polymerase, 1× reaction buffer, 2·5 mM MgCl₂, 5% dimethyl sulphoxide (BDH Chemicals, Victoria, Australia) and Milli-Q H₂O to a volume of 50 µl. The reaction mix was denatured at 95°C for 2 min followed by 35 cycles comprising denaturation for 1 min at 95°C, annealing for 2 min at 63°C and polymerisation for 2 min at 72°C. The PCR product contained recognition sequences for the restriction nucleases Smal and BsmI allowing the ligation of a PCR-generated insert into these unique restriction sites within the human androgen receptor cDNA in pCMVhAR. Following restriction digestion the pCMVhAR, DNA was treated with calf intestinal alkaline phosphatase (Promega Corporation) and the digested PCR product was ligated into the expression vector. The ligated DNA was then transformed into Epicurian coli XL1-Blue MRF’ supercompetent cells (Stratagene) and Miniprep DNA was extracted using the Wizard Plus Miniprep DNA purification system (Promega Corporation). To exclude the presence of mutations or changes in the number of CAG repeats during PCR or plasmid replication, subclones were
sequenced by dye terminator automated sequencing (ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit with Amplitaq DNA Polymerase, FS, Perkin Elmer Applied Biosystems, Norwalk, CT, USA) to determine the DNA sequence of the entire insert spanning the SmaI to BsmI ligation sites. Large scale DNA preparations for transient transfection were performed by alkali lysis followed by equilibrium centrifugation in CsCl-ethidium bromide gradient to obtain high purity plasmid DNA suitable for efficient and reproducible transient transfection. Plasmid DNA was quantitated by UV spectrophotometry (Beckman DU-64, Beckman, Palo Alto, CA, USA).

To determine androgen receptor transcriptional activity, a reporter construct containing the firefly luciferase cDNA (pXP2 vector) (Nordeen 1988) downstream of the androgen responsive probasin promoter (−286/+28) was utilised (−286/+28PB-Luc, kindly donated by Professor R J Matusik, University of Manitoba, Canada). An internal control reporter construct expressing the Renilla reniformis luciferase protein under the control of the cytomegalovirus immediate early promoter/enhancer (pRL-CMV, Promega Corporation) was co-transfected to minimise experimental variability introduced by variation in transfection efficiency, cellular viability and cell lysis efficiency.

Cell culture, plasmid DNA transfection and luciferase assay

The cell lines LNCaP, PC3, COS-1 and MCF7 were maintained in Dulbecco’s modification of eagle medium (DMEM) (Trace Scientific Ltd, NSW, Australia) or RPMI (PC3 cells) supplemented with 10% or 5% (PC3 cells) fetal calf serum (CSL, Victoria, Australia), 50 IU/ml penicillin G and 5 µg/ml streptomycin. Cells were incubated at 37 °C in 5% CO2. Charcoal-stripped fetal calf serum was used during all experiments. Cells were passaged at subconfluency with 0-25% trypsin, 1 mM EDTA (Gibco-BRL, Grand Island, NY, USA). Transient transfection was performed by electroporation in a BioRad Gene Pulser with capacitance extender (BioRad, Richmond, CA, USA). Optimal voltage to maximise transfection efficiency with minimal cell death was determined empirically for each cell line with a capacitance setting of 960 µF. For each AR expression construct and experimental control, cells were transfected by electroporation in duplicate cuvettes for each vector combination, pooled, then divided into 6-well culture vessels containing pre-warmed media with charcoal-striped serum. Cells were divided between triplicate wells for each experimental condition. The transiently transfected cells were incubated for a period sufficient to adhere to the culture vessel (6 h for PC3, MCF7, COS-1 cells and 24 h for LNCaP cells) prior to a change of media containing androgen (10 nM dihydrotestosterone (DHT) unless otherwise stated, Sigma-Aldrich Pty Ltd, Castle Hill, NSW, Australia) or an equal volume of ethanol vehicle. After a 24-h incubation, the cells were gently washed with phosphate-buffered saline and harvested with luciferase lysis buffer (Promega Corporation). Luciferase activity was determined using a commercially available assay system (Promega Corporation). Firefly and Renilla luciferase activity were measured sequentially from a single sample by addition of their respective substrates. The Renilla luciferase substrate contains a reagent (Stop & Glo) which quenches the firefly luciferase luminescence thus allowing determination of Renilla luciferase activity. Luminescence was measured using an ML 3000 Microtiter Plate Luminometer (Dynatech Laboratories, Chantilly, VA, USA) with a 20 µl aliquot of cell lysate and 100 µl luciferin reagent according to the manufacturer’s protocol.

Luminescence was measured over a 10-s period following a 3-s delay after the addition of luciferin substrate, from which an integrated sum of detected luminescence was computed. Renilla luciferase background may arise from the instrument and plate as with firefly luciferase, but autoluminescence may also arise by nonenzymatic oxidation of the Renilla luciferin. The background luminescence contributed by these factors was determined in mock transfected cells and was less than 0-1% of Renilla luminescence in transfected cells. The total protein concentration in cell lysate was determined by the Bradford method (Bradford 1976) against a standard curve utilising BSA.

Androgen receptor immunoblot

Three separate electroporations of COS-1 cells were performed (as described above) for the 15 and 31 CAG repeat AR. Transfected cells were pooled, divided between four 10-cm plates for each AR and incubated in charcoal-striped media for 6 h prior to a media change containing either 10 nM dihydrotestosterone or ethanol vehicle. Following incubation of duplicate plates for 24 h in the presence or absence of hormone, cells were washed, then scraped after the addition of lysis buffer (150 mM NaCl, 50 mM Tris, pH 7.5, 1% Triton X, 0-25% deoxycholic acid, 2 mM EGTA, 1 mM vanadate and protease inhibitors leupeptin, phenyl-methylsulfonyl fluoride (PMSF) and aprotinin). Cell lysates were centrifuged for 10 min at 4 °C. The total protein content in cell lysates was
determined in duplicate on each sample using the Bradford method with a standard curve from bovine serum albumin. Lysates (50 µg total protein) were added to 5 x sample buffer (0.475 M Tris–Cl, pH 6–8, 250 mM dithiothreitol, 15% SDS, 50% glycerol and 0.025% bromophenol blue), heated to 100 °C for 2 min and applied to a 7.5% acrylamide gel containing 0.18% Bis, 0.1% SDS and 0.4 M Tris–HCl, pH 8.8. Molecular weight markers were run in the same gel. Following electrophoresis, the protein samples were transferred to Polyscreen polyvinylidene fluoride transfer membrane (NEN Life Science Product Inc, Boston, MA, USA). The membranes were blocked with 5% skimmed milk then incubated sequentially with rabbit polyclonal antibody PG-21 (Prins et al. 1991) (raised against a peptide corresponding to amino acids 1–21 of the human androgen receptor) at a concentration of 1 µg/ml and peroxidase-conjugated swine anti-rabbit immunoglobulins (Dako, CA, USA) at a dilution of 1:1000. Chemoluminescence reaction mixtures were electrophoresed in a pre-run 4% polyacrylamide (19:1 acrylamide:bisacrylamide) gel with 0.5 × TBE buffer (1 × TBE=0.089 M Tris, 0.089 M boric acid and 2 mM EDTA, pH 8.0) at 100 V for 45 min at room temperature. Gels were dried and autoradiographed with BioMax MS film with intensifying screen at −70 °C. Western analysis was performed (as described above) on the same whole cell extracts in duplicate to quantitate AR protein. Quantitation of gel shift bands and Western analysis was performed by densitometry (BioRad GS-710 Calibrated Imaging Densitometer; BioRad). The mean value of the gel shift for each AR was divided by the mean AR value from Western analysis to give a corrected gel shift value (in arbitrary units).

Statistical analysis
Comparison of means was performed by Student’s t-test (two-tailed) where two means were compared. For the analysis of data from experiments comparing the three androgen receptor constructs, the luciferase activity of the 15 and 31 CAG repeat constructs was expressed as a proportion of the 24 CAG repeat construct luciferase activity within each experiment. Two-way analysis of variance was used for multiple comparisons in dose–response experiments. A significance value of P<0.05 was specified for all analyses. Statistical analysis was carried out using the SPSS computer package (SPSS Inc., Chicago, IL, USA).

RESULTS
AR transcriptional activity
Four cell lines were chosen to evaluate the transcriptional activity of the human AR carrying

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different numbers of exon 1 CAG repeats. PC3 cells have the functional and morphological characteristics of a poorly differentiated adenocarcinoma (Kaighn et al. 1979). They do not express endogenous androgen receptor at a level sufficient to induce transcriptional activation of an androgen responsive reporter. The prostate cancer cell line, LNCaP, is a more highly differentiated cell line (Horoszewicz et al. 1983, Quigley et al. 1995) which expresses a mutated AR with broadened ligand specificity. MCF7 is a human mammary adenocarcinoma cell line which retains several characteristics of differentiated mammary epithelium including oestrogen responsiveness (Brandes & Hermonat 1983). It has previously been shown to express low levels of androgen receptor mRNA (Hall et al. 1992), and has been used in order to evaluate the human AR in a non-prostatic sex hormone responsive cell line. COS-1 cells (Gluzman 1981) (African green monkey renal fibroblasts transformed by the SV40 large T antigen) were utilised to obtain high levels of expression of transfected human AR cDNA. The four cell lines studied were transiently co-transfected with human androgen receptor cDNA under the control of the CMV early promoter/enhancer and the androgen responsive probasin firefly luciferase reporter vector (PB-286/ +28 Luc) which contains two well characterised androgen receptor-binding sites (Rennie et al. 1993). Transcriptional activation of the luciferase reporter in the presence and absence of a saturating dose of dihydrotestosterone (10 nM) was determined for AR constructs with CAG repeat numbers spanning the normal range (15, 24 and 31) (Fig. 1). COS-1 cells showed 36·5% less transactivation by the 31 CAG hAR compared with the 15 CAG repeat hAR constructs relative to the value for the 24 CAG repeat hAR construct in each experiment. Within each experiment triplicate luciferase assays were performed for each experimental condition. Results are expressed as the mean ± s.d. from a minimum of 3 independent experiments. *P<0·05, **P≤0·01.

**FIGURE 1.** COS-1, PC3, LNCaP and MCF-7 cells were transiently co-transfected by electroporation with 10 µg pCMVhAR, 10 µg probasin (286/+29) luciferase reporter and 1 µg pCMVRL vector, incubated in the presence (solid bars) or absence (open bars) of 10 nM DHT for 24 h and then harvested in lysis buffer. Relative luciferase activity is calculated from the mean firefly divided by mean *Renilla* luciferase activity of the 15 and 31 CAG repeat hAR constructs relative to the value for the 24 CAG repeat hAR construct in each experiment. Within each experiment triplicate luciferase assays were performed for each experimental condition. Results are expressed as the mean ± s.d. from a minimum of 3 independent experiments. *P<0·05, **P≤0·01.
difference in activity between the 15 and 31 CAG repeat constructs was observed in the breast cancer cell line, MCF-7 (120·9 ± 39·4 vs 103·1 ± 23·1, \( P \) value 0·27). No cryptic promoter activity of the pXP2 firefly luciferase vector was seen in experimental controls in which the promoterless pXP2 vector was co-transfected with pCMVhAR into COS cells and incubated in the presence of 10 nM DHT (data not shown).

No evidence of endogenous androgen receptor transcriptional activity was observed in COS-1, PC3 or MCF-7 cells in control transfections with nil AR cDNA (data not shown). This is consistent with the previously reported absence of androgen receptor activity in COS-1 and PC3 cells. In LNCaP cells, endogenous androgen receptor activity was apparent, as evidenced by an average 8·65-fold induction of the probasin luciferase reporter in the absence of transfected androgen receptor cDNA compared with 70·7-fold luciferase induction following overexpression of the AR by transient transfection (data not shown). Therefore, a nil AR control was performed in each LNCaP cell line experiment and the reported normalised luciferase values were calculated by subtracting the mean luciferase value obtained in the nil AR control from the total luciferase value following incubation in the presence of androgens.

In view of the absence of any difference in androgen-induced transcriptional activation by the hAR constructs in PC3 cells, the response to sub-saturating doses of DHT was studied. No significant difference between the 15 and 31 CAG ARs was evident across a range (0·01, 0·1, 1, 10 nM DHT) of ligand concentrations (\( P=0·29 \), two-way ANOVA). The effect of different quantities of transfected AR vector was also studied in PC3 cells. Using androgen receptor vector quantities of 0·1, 1·0, 10, 20 and 50 µg per electroporation, no significant differences between the 15 and 31 CAG repeat hAR constructs were observed at any of these AR doses.

To determine whether the reduced transactivation of the probasin luciferase reporter by AR with a greater CAG number within the normal range in COS-1 cells is dependent on androgen concentration, the response to increasing doses of DHT up to a saturating dose of 10 nM was studied (Fig. 2A). Maximal luciferase induction was observed at a dose of 1 nM DHT. The luciferase activity for the 15 CAG hAR vector was greater than for the 31 CAG hAR vector across the range of DHT concentrations studied (\( P=0·001 \), two-way ANOVA). The effect of different quantities of transfected AR vector was studied in COS-1 cells to determine whether the difference between AR polymorphic variants was influenced by the level of AR protein. Androgen receptor vector quantities of 0·5, 2·5 and 5 µg per electroporation were transfected (Fig. 2B). Greater transactivation of the reporter was observed with 0·5 µg (\( P<0·05 \)) and 5 µg (\( P<0·05 \)) of transfected 15 CAG vs 31 CAG repeat AR.

**Androgen receptor protein levels**

COS-1 cells were transiently transfected with hAR cDNA containing 15 or 31 exon A CAG repeats and incubated for 24 h in the presence or absence of 10 nM DHT in duplicate prior to harvest of cell lysate for quantitation of AR protein by immunoblot. No reduction in the level of hAR protein was
demonstrated with the higher number of CAG repeats (Fig. 3). As opposed to some prior reports (Choong et al. 1996), this suggests the influence of CAG repeat number on transactivation is not mediated via alteration in the level of androgen receptor protein.

**DNA binding of the androgen receptor**

Gel shift assay was utilised to quantitate binding of the AR CAG repeat polymorphic variants to hormone response elements previously shown to exhibit androgen receptor binding in vitro (Gast et al. 1995, 1998). Whole cell extracts prepared from COS-1 cells transiently transfected with androgen receptor expression vector and incubated in the presence of 100 nM DHT contained 20 µg protein was incubated in binding buffer with 2 µg poly(dI-dC)-poly(dI-dC) and α32P-labelled oligonucleotide. The upper band represents AR specific binding. (B) Western immunoblot of 50 µg total protein of the same cell extracts. Band intensities were quantitated by densitometry and gel shift and subsequently corrected for AR content. Samples were assayed in duplicate. ARE, androgen response element.

The upper gel shift band in the nil AR control is consistent with the upper band being AR specific. Incubation with an excess of unlabelled oligonucleotide resulted in quenching of the band shift whereas incubation with an excess of nonspecific oligonucleotide was associated with persistence of the shifted band, indicating specificity of DNA binding (data not shown).

**Response to different androgenic ligands**

The effect of four different androgenic ligands: DHT, testosterone, R1881 and dehydroepiandrosterone (DHEA), at saturating and a subsaturating
concentration, on induction of the probasin luciferase reporter by the AR polymorphic variants was determined (Fig. 5). COS-1 cells were studied because the greatest difference in transcriptional activation by different AR constructs was observed in this cell line. DHT at a concentration of 10 nM showed significantly greater induction of the reporter by the 15 than the 31 CAG repeat AR ($P = 0.004$). All other androgens at both doses tested showed a trend to greater transactivation by the 15 CAG repeat AR but this did not reach statistical significance, with greater variance between replicate samples. DHEA did not result in significantly greater transactivation of the reporter construct than the nil androgen receptor control.

**DISCUSSION**

The results of these studies demonstrate cell-specific differences in transactivation of an androgen responsive reporter by the human androgen receptor dependent on the length of the exon 1 CAG repeat sequence. The inverse relationship between CAG repeat number and activation of an androgen-responsive reporter was observed in the androgen-dependent human prostate carcinoma cell line, LNCaP, and in COS-1 cells. In a second (androgen independent) prostate cancer cell line, PC3, and the breast cancer cell line, MCF7, no such effect was observed. The finding of reduced transcriptional activity of human AR with a greater number of CAG repeats within the normal range is in keeping with the clinical associations observed in studies of the AR CAG repeat polymorphism and several androgen-dependent diseases of the male reproductive tract including prostate cancer, benign prostatic hypertrophy and impaired sperm production in infertile males (Tut et al. 1997).

Ross (1992) predicted that a 13% difference in androgen-stimulated mitotic activity would result in a 2.8-fold difference in prostate cancer incidence. Based on this estimate, the 21.6% difference in transcriptional activity we have observed in LNCaP cells across the studied normal range of CAG repeats would translate into a greater than fourfold difference in the incidence of prostate cancer if these in vitro results are an accurate reflection of in vivo prostatic AR transcriptional activity. Giovannucci et al. (1997) showed a relative risk of 1.52 for the presence of prostate cancer for men with fewer than 19 CAG repeats compared with greater than 25 repeats. The association was greater for individuals with high grade and high stage prostate cancer and a
shorter CAG repeat sequence was associated with metastasis and mortality from prostate cancer. Ingles et al. (1997) also demonstrated an association between CAG repeat number and prostate cancer risk. The increased risk was shown to be statistically significant only in the subgroup of patients with advanced disease. An increased incidence of lymph node-positive cancer was reported by Hakimi et al. (1997) in men with 16 or 17 CAG repeats in a cohort of men with clinically localised prostate cancer. An association between shorter AR CAG repeat number and earlier age at diagnosis was found by Hardy et al. (1996) in a cohort of patients with advanced prostate cancer. The finding of such an association between a polymorphic marker and a disease characteristic does not necessarily imply a direct biological effect of the allelic variation on the disease process. Such an association could reflect linkage disequilibrium of the marker to another gene locus of biological importance in the development of prostate cancer. Alternatively, the association could reflect co-segregation of the allele with other important genetic or environmental factors involved in the causation of prostate cancer. The finding of a relationship between AR CAG alleles within the normal range and in vitro transcriptional activity of the receptor in a human prostate cancer cell line is evidence to support a direct biological association between this polymorphism and the development of prostate cancer.

The androgen receptor CAG repeat sequence is pathologically expanded to greater than 40 repeats in the rare degenerative neurological disease, X-linked spinal bulbar muscular atrophy (Kennedy’s disease) (La-Spada et al. 1991). A number of studies have examined the in vitro transcriptional activity of the Kennedy’s androgen receptor in comparison with the wild type receptor. An inverse relationship between the size of the repeat sequence and transactivation of an androgen responsive reporter has been reported by a number of groups (Mhatre et al. 1993, Chamberlain et al. 1994, Jenster et al. 1994, Kazemi-Esfarjani et al. 1995, Nakajima et al. 1996). However, not all studies have demonstrated reduced transactivation by the expanded CAG AR compared with the wild type. Choong et al. (1996) observed a reduction in AR mRNA and protein expression with a pathologically expanded CAG repeat sequence. Despite this, they found no difference in inherent AR transcriptional activity using the MMTV promoter in CV1 cells. Neuschmid-Kaspar et al. (1996) reported no difference in transactivation function between the Kennedy’s mutant and the wild type androgen receptor utilising reporters with a number of different androgen responsive promoters. Brooks et al. (1997) showed no reduction in transcriptional activation of an MMTV.LTR reporter by the Kennedy’s AR in a stably transfected motor neuron hybrid cell line. The results of these prior studies are, therefore, conflicting; however the bulk of the evidence supports a reduction in AR transcriptional activity with a pathologically expanded AR CAG repeat sequence. Whether this is due to a reduction in AR expression or due to an inherent reduction in the transcriptional activity of the receptor is unclear. A number of these studies have used semi-quantitative Western immunoblot to determine AR protein levels, and most have been unable to show a difference in the expressed level of AR protein carrying CAG repeat expansions within the Kennedy’s disease range. The possibility of altered mRNA stability of AR with an expanded CAG repeat sequence contributing to the observed differences in AR protein levels seen in some reports has not been addressed in any of these studies.

The above mentioned studies have compared wild type with the Kennedy’s mutant AR. They have not examined the effect of variation in AR CAG repeat number within the normal range on AR transcriptional activity. It is possible that a reduction in transcriptional activity seen when the CAG repeat is pathologically expanded does not extend to an association with variation in the repeat length within the polymorphic range. This hypothesis has only been examined in one prior report. Tut et al. (1997) utilised an MMTV.LTR luciferase reporter in COS-7 cells to study the relationship between AR CAG length and AR transcriptional activity. They showed an AR with 31 CAG repeats to have lower transcriptional activity than a 15 CAG repeat AR in the presence of 30 or 100 nM DHT and 100 nM testosterone. No difference between the transcriptional activity of the two constructs was observed at lower doses of DHT. No difference in AR protein level was observed by immunoblot of AR protein. This constituted the first in vitro evidence for variation in androgen receptor function dependent upon the CAG repeat number within the normal range. In the light of the cell-specific nature of steroid hormone transcriptional activity and the disparate results obtained in different in vitro model systems studying the function of the Kennedy’s AR, the relevance of these findings to disease processes of the male reproductive tract, such as carcinoma of the prostate and benign prostatic hyperplasia, were unclear.

We undertook to examine the effect of variation in AR CAG repeat number in a number of cell lines, including two of prostatic origin, utilising a reporter construct with an androgen responsive promoter.
that has previously been shown to be relatively androgen (Rennie et al. 1993, Claessens et al. 1996) and prostate specific (Greenberg et al. 1994, Brookes et al. 1998). Our findings in COS-1 cells using the probasin luciferase reporter are consistent with those reported by Tut et al. (1997) with an MMTV.LTR reporter construct, showing an inverse relationship between CAG repeat number and AR transcriptional activity. We then examined the effect of variation in CAG repeat number in three other cell lines and demonstrated this relationship in LNCaP cells but not in the less well differentiated prostate cancer cell line PC3 and MCF7 cells. The mechanism underlying the observed reduction in transactivation of the luciferase reporter is unclear. The results reported here do not support the association between CAG number and transactivation of the androgen responsive reporter being mediated by either reduced AR protein levels or an alteration in AR-DNA binding. This suggests reduced transactivational ability of the receptor with a longer polyglutamine tract within the normal range. The cell-specific nature of this association in an otherwise identical experimental system suggests the presence of factors involved in either AR trafficking within the cell, AR turnover or intrinsic AR transcriptional activity being differentially expressed in a manner that influences the impact of the AR CAG repeat polymorphism. Steroid receptor coactivator and co-repressor proteins have recently been demonstrated to play an important role in mediating ligand-dependent receptor transcriptional activity (Glass et al. 1997) and have been proposed to play a role in the cell specificity of steroid receptor function. The AR binding protein, Ras-related nuclear protein/ARA24, displays reduced binding to the AR N-terminal domain and reduced enhancement of AR transactivation of the Kennedy’s expanded CAG AR compared with the wild type receptor (Hsiao et al. 1999). This and other AR binding proteins may play a role in the cell specificity reported here. The difference observed between the two cell lines of malignant prostate origin may have implications for the effect of this polymorphism on prostate tumour specimens at different stages of disease progression. However, until the factors underlying these cell-specific results are better understood, their implications for androgen receptor transcriptional activity in tumours in vivo at different levels of differentiation are purely speculative. The finding of a greater effect of CAG repeat number on AR transcriptional activity in COS cells than in LNCaP cells may be explained by higher levels of plasmid DNA expression in these cells (by virtue of the expression of the SV40 large T antigen) altering the relative level of expression of the transfected AR to endogenously expressed factors involved in AR-mediated transcriptional activity.

The implications of these findings for androgen-dependent disease processes require further evaluation in case control studies with clinically important end points in order to determine whether the measurement of AR CAG allelic status will be of clinical use. An understanding of the factors involved in the interaction of this allele with other important components in androgen receptor transactivational activity may shed further light on the cell-specific nature of the effects reported here and the previous findings of an association with only the risk of advanced or high grade prostate cancer. Through an improved understanding of such mechanisms we may acquire the tools better to predict both risk and progression of androgen-dependent disease processes of the prostate.

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