Replacing the Mouse Androgen Receptor with Human Alleles Demonstrates Glutamine Tract Length-Dependent Effects on Physiology and Tumorigenesis in Mice

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Polymorphism in the length of the N-terminal glutamine (Q) tract in the human androgen receptor (AR) has been implicated in affecting aspects of male health ranging from fertility to cancer. Extreme expansion of the tract underlies Kennedy disease, and in vitro the AR Q tract length correlates inversely with transactivation capacity. However, whether normal variation influences physiology or the etiology of disease has been controversial. To assess directly the functional significance of Q tract variation, we converted the mouse AR to the human sequence by germline gene targeting, introducing alleles with 12, 21, or 48 glutamines. These three “humanized” AR (h/mAR) mouse lines were grossly normal in growth, behavior, fertility, and reproductive tract morphology. Phenotypic analysis revealed traits that varied subtly with Q tract length, including body fat amount and, more notably, seminal vesicle weight. Upon molecular analysis, tissue-specific differences in AR levels and target gene expression were detected between mouse lines. In the prostate, probasin, Nkx3.1, and clusterin mRNAs trended in directions predicted for inverse correlation of Q tract length with AR activation. Remarkably, when crossed with transgenic adenocarcinoma of mouse prostate (TRAMP) mice, striking genotype-dependent differences in prostate cancer initiation and progression were revealed. This link between Q tract length and prostate cancer, likely due to differential activation of AR targets, corroborates human epidemiological studies. This h/mAR allelic series in a homogeneous mouse genetic background allows examination of numerous physiological traits for Q tract influences and provides an animal model to test novel drugs targeted specifically to human AR. (Molecular Endocrinology 20: 1248–1260, 2006)

The Androgen Receptor (AR) is a recently evolved, highly conserved member of the superfamily of nuclear receptor transcription factors (1). In mammals, AR is responsible for male primary and secondary sexual differentiation and also influences numerous physiological processes not directly linked to reproduction. Moreover, development, homeostasis, and tumorigenesis of the prostate depend on androgen acting via its receptor. Like the other nuclear receptors, AR function relies on three major structural regions: the most conserved central DNA binding domain, the moderately conserved C-terminal ligand binding domain, and the N-terminal transactivation domain (NTD) that bears little similarity among receptors. Because the AR gene (Ar) is located on the X chromosome, males are hemizygous, and mutations are phenotypically evident. This allows extensive receptor structure/function correlations to be drawn from cases of partial to complete androgen insensitivity (2). Polymorphisms of Ar that influence its function within a nonpathological range may underlie phenotypic variation in male traits and could modify susceptibility to androgen-dependent diseases.

The most studied polymorphisms in the NTD of the AR are variations in the length of two polyamino acid tracts comprised of glutamine (Q) or glycine (G) residues (3, 4). Glutamine-rich regions are functionally important domains in a number of transcription factors, including Sp1, TATA binding protein, and glucocorticoid receptor (5). The human AR (hAR) polyglutamine tract, encoded by a CAG trinucleotide repeat, has 9–37 residues in the normal population, with the majority between 15 and 30, whereas the G tract, encoded by GGN repeats, varies between 8 and 18 residues (6). The Q tract has received significant attention, in part because expansion of the CAG repeat beyond 40 residues is associated with the late-onset
neurodegenerative disease spinal and bulbar muscular atrophy (SBMA, Kennedy disease) (7).

The mechanisms by which Q tract length affects AR function have been well explored experimentally. Expanded Q tract lengths result in decreased mRNA and protein levels, both in vivo and in vitro (8). Transfection studies that take into account differences in AR levels have shown an inverse relationship between Q tract length and AR transactivation (9–14), with one study linking maximum AR activity to a tract length of 15 or 17 Q repeats (15). The greater transcriptional efficacy for shorter Q tract lengths may encompass several activities, including increased interaction between the AR N-terminal and ligand binding domains, and increased association with p160 coactivators or SWI/SNF chromatin remodeling components (9, 16, 17). The presence of Q tracts in numerous transcription factors also suggests they may be sites for cofactor interactions. A candidate coactivator for AR, the Ras-related nuclear protein (RAN)/ARA24, interacts directly with the Q tract, with both interaction and consequent AR activation diminished with increasing Q tract length (18).

Although extensive expansion of the Q tract in man results in SBMA, it is debated whether variation within the normal range has a detectable physiological outcome or influences androgen-dependent disease. Longer Q tract length has been associated with defective spermatogenesis and male infertility in some populations (19, 20). Associations have been found between Q tract length and body fat mass (21), testosterone levels (22), and high-density lipoprotein cholesterol levels (23). Greater effort has focused on discerning an effect in prostate cancer, especially because shorter tracts occur in the higher risk African-American population (24). Although several studies link shorter Q tract length with increased risk, earlier age of onset, or greater aggressivity of prostate cancer (24–28), other studies have found no association (29–37). These conflicting results may be due to patient selection bias, small sample size, genetic heterogeneity of the human population, or confounding gene-gene or gene-environment interactions.

To address whether the Q tract plays a role in male phenotypic variation or disease origins, we created a mouse model containing hAR alleles varying in Q tract length. mAR has a Q tract disrupted by several histidines and displaced 120 amino acids toward the C terminus, a greatly abbreviated G tract, and 15% divergence in N-terminal amino acid sequence compared with hAR (38). Because of these differences, we swapped the entire human and murine AR NTDs to introduce Q tract variants and further enhance relevance to human studies. In contrast to the N terminus, the DNA and ligand binding domains are identical between man and mouse, except for a few amino acid differences in the hinge region. Reduced transcriptional activity of rodent relative to hAR in vitro has been mapped to the NTD (39), suggesting that sequence differences therein are functionally significant. The mouse NTD was replaced by homologous recombinant

RESULTS

Generation of Humanized AR (h/mAR) Mice

h/mAR mice were created by recombining a DNA fragment encompassing most of human Ar exon 1 (from amino acid 31 to 484) with the mouse Ar gene (Fig. 1). Targeting vectors to accomplish this included a selectable marker for neomycin resistance (neo*) flanked by loxP sites (40). A fragment of mouse chromosomal Ar, containing 4.3 kb of 5′ flanking sequence, the entire exon 1 coding region, and 120 bp of intron 1, was cloned on one side, and 2 kb of the contiguous intron 1 sequence on the other side of the oppositely oriented neo expression cassette. Human AR cDNA sequences [kindly provided by M. McPhaul and M. Marcelli (41)] were exchanged via conserved restriction sites, resulting in h/mAR exon 1 embedded within mouse chromosomal (regulatory) sequence and differing from hAR in only four codons (Fig. 1A). Three targeting vectors were created containing 12, 21, or 48 Q residues in the N-terminal tract, because the median human Q tract has 21 residues, whereas tract lengths of 12 and 48 Qs represent extremes. Although 50 Qs can result in SBMA in man, introduction of transgenic ARs with as many as 65 Qs has not led to detectable phenotypes in mice, where gene defects often must be more severe to model a human syndrome (42).

Since the mouse AR (mAR) fragment was obtained from a J1 embryonic stem (ES) cell DNA library, CJ7 ES cells that derive from the same 129/Sv mouse substrain were selected to optimize recombination (43). Correctly targeted clones were identified by Southern blot (Fig. 1B), examined for euploidy, and transiently transfected with cre recombinase to remove the neo cassette. Two independent ES cell clones per Q tract allele were injected into C57BL/6J blastocysts to create chimeric animals. Male chimeras were bred to C57BL/6J females, and daughters carrying the targeted allele were bred to C57BL/6J males. Progeny from this cross were genotyped (Fig. 1, C and D), and h/mAR males were bred to heterozygous females to generate the h/mAR males, mAR males, and homozygous h/mAR female mice used in this study.
h/mAR Mice are Grossly Normal and Fertile

Neither sex of h/mAR mice showed any remarkable differences from mAR littermates in appearance or behavior, despite a reported 2-fold greater transcriptional activity of the human receptor (Ref. 39 and our unpublished data). h/mAR males were fertile, as were homozygous females, and had grossly normal reproductive tract anatomy, indicating full functionality of the h/mAR gene in the mouse. Because Q tract length may affect fertility in men (14, 44, 45), h/mAR alleles were compared by breeding homozygous h/mAR females to males with the same AR genotype. Each h/mAR allelic variant produced similar numbers of pups per litter (Table 1) at similar frequency (data not shown), with average numbers similar to those reported for the C57BL/6J and 129 parental strains (46).

Body mass and composition in men have been reported to be affected by Q tract length (21, 47). Therefore, body weights of male h/mAR mice and wild-type littermates were measured at 3 and 9 wk and 6, 18, and 24 months of age. No statistically significant differences were found at any time point, although both 12Q-h/mAR and 48Q-h/mAR mice consistently

Table 1. Normal Fertility, Body Weight (BW), and Body Fat of h/mAR Mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Litter Size</th>
<th>BW at 6 Months</th>
<th>BW at 24 Months</th>
<th>Body Fat at 24 Months</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n Pups/Litter</td>
<td>n g</td>
<td>n g</td>
<td>n %</td>
</tr>
<tr>
<td>mAR</td>
<td>n.d.</td>
<td>10 29.1 ± 1.4</td>
<td>7 29.6 ± 0.5</td>
<td>5 13.51 ± 4.7</td>
</tr>
<tr>
<td>12Q-hAR</td>
<td>10 7.1 ± 0.7</td>
<td>11 31.7 ± 0.7</td>
<td>5 34.0 ± 2.5</td>
<td>5 20.34 ± 2.7</td>
</tr>
<tr>
<td>21Q-hAR</td>
<td>15 6.0 ± 0.7</td>
<td>6 29.5 ± 1.0</td>
<td>4 30.1 ± 1.5</td>
<td>8 9.95 ± 2.2</td>
</tr>
<tr>
<td>48Q-hAR</td>
<td>10 7.1 ± 1.0</td>
<td>10 31.9 ± 1.0</td>
<td>9 32.0 ± 1.2</td>
<td>9 14.85 ± 2.5</td>
</tr>
</tbody>
</table>

n.d., Not done.
weighed more than 21Q-h/mAR mice (Table 1). Interestingly, body composition analysis performed by dual-energy X-ray absorptiometry of 24-month-old males showed a trend toward higher percentage body fat in 12Q-h/mAR and 48Q-h/mAR mice, whereas total body weight remained similar (Table 1).

To determine whether hormone levels were affected, either directly or by feedback mechanisms, testosterone was measured in serum of male h/mAR mice and mAR littermates at 6 months of age. Testosterone levels varied widely between individuals and for the same individuals tested at different times, as reported previously for mice (48), masking an ability to detect statistically significant differences (Table 2). Therefore, as a more direct physiological marker of testosterone activity (49, 50), seminal vesicle weight was measured at 6, 18, and 24 months of age (Table 2). Increasingly with age, 12Q-h/mAR mice had higher mean seminal vesicle weights, reaching statistical significance at 2 yr, and 48Q-h/mAR mice had lower mean seminal vesicle weights. This could indicate increased AR activity in the short Q tract mice and reduced AR activity in the long Q tract mice, in accord with in vitro analysis. The increasing differential in seminal vesicle weights with age may reflect cumulative lifetime effects of androgen via ARs of differing efficacy.

**Testis Physiology of h/mAR Mice Is Normal, but Variations Occur at the Molecular Level**

h/mAR activity in the testis was examined because morphology and function of this organ, in both spermatogenesis and androgen synthesis, is highly sensitive to androgen regulation. Testes from 6-month-old h/mAR mice and mAR littermates (n = 6 per genotype) were fixed, sectioned, stained with hematoxylin and eosin (H&E), and examined by light microscopy. Morphology was normal for all h/mAR alleles, with seminiferous tubules similar in size and density to those in mAR mice, normal interstitial cells, and all stages of spermatogenesis appearing normal. Sertoli and interstitial cells showed nuclear staining in all genotypes.

![Fig. 2. Testis Morphology, Spermatogenesis and AR Cellular Localization Appear Normal in h/mAR Mice](https://example.com/fig2)

**Table 2. Testosterone Levels and Seminal Vesicle Weights with Age in h/mAR Mice**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Testosterone</th>
<th>Seminal Vesicle Weight</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>6 Months</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>ng/ml</td>
</tr>
<tr>
<td>mAR</td>
<td>17</td>
<td>1.59 ± 0.66</td>
</tr>
<tr>
<td>12Q-hAR</td>
<td>9</td>
<td>1.91 ± 1.11</td>
</tr>
<tr>
<td>21Q-hAR</td>
<td>10</td>
<td>1.41 ± 0.54</td>
</tr>
<tr>
<td>48Q-hAR</td>
<td>9</td>
<td>2.45 ± 0.99</td>
</tr>
</tbody>
</table>

BW, Body weight; n.d., not done.
spermatogenesis present (Fig. 2A). Upon immunohis-
tological examination for AR, strong nuclear staining
was present in interstitial and Sertoli cells in mAR and
h/mAR mice (Fig. 2B).

Because AR expression is affected by Q tract length
in vitro (8), AR mRNA levels were measured by real-
time PCR. In 6-month-old mice, testis AR mRNA was
1.3- to 1.6-fold higher in all h/mAR compared with
mAR mice, with the most statistically significant dif-
ference occurring between mAR and 21Q-h/mAR mice
($P < 0.05$) (Fig. 3A). Testis AR protein levels, deter-
mined by Western blotting of tissue homogenates,
were also somewhat higher in h/mAR than mAR mice
(Fig. 3B). Because the expanded Q tract in SBMA
leads to AR aggregation, the pellet fraction of the total
protein isolate was examined to assess levels of de-
natured protein or protein complexes, which has been
done previously as a rapid means of testing for aggre-
gation of proteins with expanded Q tracts (51). 48Q-
h/mAR was more abundant in the pellet than super-
natant fraction, suggesting possibly problematic
protein folding. However, cytoplasmic particles or in-
tranuclear aggregates were not detected in testis by
immunohistochemistry (Fig. 2B) or in muscle, although
an equivalent 113Q-h/mAR knock-in allele produced
substantial aggregation (51). Thus, the 48Q-h/mAR
may have a capacity for increased aggregation, per-
haps exacerbated during cell lysis and sample
preparation.

To pursue further a basis for an apparent differential
androgen sensitivity in these mice, we compared Leydig
cell function to that in testicular feminized (tfm) animals
that lack functional AR. Leydig cells in tfm mice fail to
differentiate fully and so are deficient in adult androgen
synthesis (52). Testis RNA was analyzed by semiquanti-
tative RT-PCR for expression of the testosterone syn-
thetic enzymes $Hsd3b1$, a marker of fetal Leydig cell
function, and $Hsd17b3$, characteristic of mature Leydig

![Fig. 3. Testis AR Levels Are Slightly Higher in h/mAR Mice](image)

A, Real-time RT-PCR was used to quantitate AR mRNA
levels in testes of five to six mice per genotype, and results
were calibrated relative to mAR. Groups designated “a” or
“b” are significantly different ($P < 0.05$), whereas “ab” is not
significantly different from “a” or “b”. B, Total protein samples
testes from three mice per genotype were separated into
supernatant and pellet fractions and analyzed by Western
blot; a representative of each genotype is shown. C, Western
blots were scanned by densitometry, and AR levels in the
pellet were normalized to the tubulin signal. The histograms
indicate the mean values for three mice per genotype. AR
levels are higher in h/mAR mice, most notably in the 48Q-h/
mAR pellet fraction, indicating a greater potential for aggre-
gation in ARs with longer Q tracts.

![Fig. 4. Leydig Cell Maturation Is Normal in h/mAR Mice](image)

Semiquantitative RT-PCR for Leydig cell markers was per-
formed with testis RNA from three mice per genotype. Testis
RNA from tfm, a mouse model of androgen insensitivity with
impaired Leydig cell maturation, was used as a control. Top,
$Hsd3b1$, a marker of fetal Leydig cells, is not significantly
different in all groups. Bottom, $Hsd17b3$, a marker of mature
Leydig cells, is normal in 12Q-h/mAR and 21Q-h/mAR mice
as compared with mAR littermates, while 48Q-h/mAR have
levels between tfm and mAR mice. Groups with different
letter superscripts are significantly different ($P < 0.05$).
cells (53) (Fig. 4). Although Hsd3b1 mRNA did not vary significantly with AR allele, Hsd17b3 levels did. mAR, 12Q-h/mAR, and 21Q-h/mAR mice expressed similar levels of the adult Hsd17b3, which were significantly higher than tfm levels (P < 0.05), as expected for normal adult Leydig cell populations. The 48Q-h/mAR mice had levels of Hsd17b3 intermediate to the tfm and other mice, although the difference from any other genotype did not reach statistical significance, similar to trends in other traits noted above.

**h/mAR Prostates Appear Normal, but Expression of AR and Target Genes Is Affected**

Prostates of h/mAR mice were examined because AR plays a critical role in the development of this organ and the Q tract has been implicated in its tumorigenesis. At the light microscopy level, prostates from 6-month-old h/mAR mice and mAR littermates (n = 6 per genotype) appeared normal in all lobes (Fig. 5A). Low levels of hyperplasia and mouse prostatic intraepithelial neoplasia (PIN) increase with age (54), but in a preliminary scan of prostates from 12-, 18-, and 24-month-old males (n = 3 per genotype) the levels of hyperplasia and PIN were equivalent in mAR and h/mAR mice (data not shown). When stained for AR by immunohistochemistry, mAR and h/mAR prostates both showed strong nuclear staining in epithelia and stroma, as expected (Fig. 5B, upper panels). Similarly, levels and localization of the steroid receptor coactivator-1 (SRC-1) did not appear to differ between strains (Fig. 5B, lower panels).

To probe molecular regulation in the prostate, expression of mRNAs for AR and several of its direct as well as indirect target genes was measured by real-time RT-PCR in 6-month-old h/mAR mice and mAR littermates. Foxa1 is not directly dependent on androgen for expression but interacts with AR in transcriptional regulation of numerous other prostate genes (55, 56). Nkx3.1, a prostate-specific homeobox gene critical in differentiation of the gland (57), and probasin, a protein secreted from mature prostatic epithelium (58), are both directly regulated by AR at the transcriptional level. Clusterin, also known as testosterone-repressed prostate message-2, encodes an antiapoptotic protein that is up-regulated after androgen ablation (59).

In contrast to testis, h/mAR mice had somewhat lower levels of AR mRNA in prostate compared with mAR mice, and levels of each allele appeared to correlate inversely with Q tract length, but again this trend was below the level of statistical significance (Fig. 6). Foxa1 expression was uniform across genotypes, suggesting little sensitivity to AR levels or activity. Despite h/mAR levels being somewhat less than mAR, Nkx3.1 expression was similar in mAR, 12Q-h/mAR, and 21Q-h/mAR mice. 48Q-h/mAR mice, however, had significantly lower Nkx3.1 expression compared with mAR mice (P < 0.05), suggesting in accord with in vitro data that the longer Q tract led to decreased AR activation. There was more variability in Nkx3.1 expression for the short Q tract allele, with most 12Q mice expressing at or significantly above mean levels, whereas a subset was consistently lower; the basis of this variability is not clear. Probasin expression was highest in mAR mice and in those with the 12Q-h/mAR allele among the h/mAR mice. Clusterin mRNA, which is negatively regulated by AR, was significantly higher in the 48Q-h/mAR mice. Overall, the AR alleles did not

**Fig. 5.** Prostate Morphology and AR Subcellular Localization Is Normal in h/mAR Mice

A, H&E staining of prostates from 6-month-old mice shows normal multilobular structure in mAR, 12Q-h/mAR, 21Q-h/mAR, and 48Q-h/mAR mice at ×100 original magnification. VP, Ventral prostate; LP, lateral prostate; DP, dorsal prostate. B, Six-month-old mice show nuclear immunohistological stain for AR (upper panels) and SRC-1 (lower panels) in epithelium and stroma of mouse prostates at ×1000 original magnification.
produce large differences in expression across promoters, but trends were consistent with greater transcriptional activation by the 12Q allele (e.g. for probasin and to a lesser extent Nkx3.1) and weaker transcriptional efficacy of the 48Q allele (both in reduced activation of Nkx3.1 and reduced repression of clusterin).

**Q Tract Length Affects Prostate Tumor Initiation in a Transgenic Model**

Prostate cancer does not occur spontaneously at an appreciable level in mice, even with h/mAR alleles. Therefore, to investigate the effect of Q tract length in oncogenesis, h/mAR females were crossed with a transgenic model of prostate adenocarcinoma (TRAMP) (60). TRAMP mice carry the SV40 T antigen (Tag) driven by a prostate epithelium-specific probasin promoter. Males develop PIN by 12 wk of age and die of prostate cancer within 4 months to 1 yr. h/mAR-TRAMP mice were followed for tumor development by abdominal palpation performed weekly by two independent observers. Mice were euthanized when moribund, and prostate tumors and metastases were preserved for analysis. We compared disease status at 29 wk of age, because at this time about half of the mice had evident disease. At 29 wk, 65% of wild-type mAR mice and 52% of 21Q-h/mAR littermates had a palpable tumor or had died due to prostate cancer. In contrast, overt disease was present in 85% of 12Q-h/mAR mice and only 28% of 48Q-h/mAR mice (Fig. 7). Because there were generally only a few weeks from palpation of tumor until death, tumorigenesis can be represented as the sum of mice with palpable cancer plus those with cancer death compared with those without palpable tumor. The number of mice that died per group additionally reflects differences in length of disease, as well as differences in tumor initiation, that are affected by AR allele. Although cancer in the mice with an average human Q tract length AR progressed similarly as in wild-type mice, the short Q tract AR resulted in significantly earlier tumor development, whereas the long Q tract appeared to be protective. This striking dependence of disease progression on Q tract length corroborates several epidemiological

**Fig. 6. Prostate Gene Expression Indicates Some Sensitivity to AR Q Tract Length**

Real-time RT-PCR was used to quantitate AR, Foxa1, Nkx3.1, probasin, and clusterin mRNA levels in prostates of three to eight mice per genotype. Results were calibrated relative to mAR levels. Differences in AR and Foxa1 mRNA levels between genotypes did not reach statistical significance. Nkx3.1, a direct AR target, showed a significant decrease in 48Q-h/mAR mice.

**Fig. 7. h/mAR Q Tract Length Affects Initiation of Prostate Tumors**

Intact or castrated h/mAR-TRAMP mice were abdominally palpated weekly to track tumor initiation and necropsied at death to confirm the presence of a tumor. The status of each genotype at 29 wk of age is shown, with those mice already dead represented by the black portion of the bar, those with tumor but alive represented in stripes, and those with no palpable tumor in white. Longer Q tract length is protective in prostate tumor initiation, whereas shorter Q tract length promotes earlier disease.
studies and discerns a difference not detected in others, probably accentuated by the homogeneous mouse genetic background. Furthermore, modest AR-dependent gene expression differences in the prostate, such as those detected for Nkx3.1, probasin, and perhaps AR itself, likely contribute to these Q tract effects in oncogenesis.

DISCUSSION

Whether AR Q tract length variation in a nonpathogenic range influences androgen-dependent traits ranging from fertility to prostate cancer has been difficult to resolve due to conflicting epidemiological data and lack of experimental models. To test Q tract length effects directly, we “humanized” the murine AR by replacing exon 1 with the equivalent human region, including 12, 21, or 48 CAG repeats. The resulting AR variant strains support proper anatomy, reproduction, and behavior, indicating that these h/mARs are fully functional in mice, regardless of differences in transcriptional capacity \textit{in vitro} (39, 41). However, detailed analyses reveal allele-specific variance, albeit within a normal range, in some physiological indicators. Moreover, when homeostasis is perturbed by activation of an oncogenic pathway, remarkable Q tract length-dependent disease progression is evident.

In creating these mice, we chose Q tract lengths at the extremes of those found in the normal human population to optimize the ability to obtain informative phenotypes. Furthermore, in models of pathology, mice often require more severe alleles than those that cause the disease in man. A relevant example is Kennedy disease, which in man is caused by expansion of the AR Q tract beyond 40 residues, but in mice an AR cDNA transgene containing 65 CAG repeats produces no abnormal phenotype (42). Other polyglutamine expansion disorders such as Huntington disease are also not obtained in mice by modest Q tract expansions expressed at endogenous levels (61, 62). Absence of severe phenotypes, as well as stability of CAG repeat number over time in mice, unlike in man, may be augmented by the short lifespan of the mouse. The 48Q-h/mAR mice show no muscle weakness or wasting with age and have morphologically normal testes with no Sertoli cell pathology or disruption of spermatogenesis. In contrast, these traits are exhibited by 113Q-h/mAR mice created in parallel, which prove to be an effective model of Kennedy disease (Ref. 51; and Lieberman, A., unpublished observations).

Although the 48Q-h/mAR mice show no evidence of Kennedy disease, they are at the low end of the normal range (compared with 21Q-h/mAR mice and mAR littermates) for some androgen-dependent traits, such as seminal vesicle weight. By comparison, the 12Q-h/mAR mice exhibit somewhat higher than normal seminal vesicle weight. Body fat percentage is slightly increased in 48Q-h/mAR mice, similar to men with longer Q tracts (21), but this does not develop into late-onset obesity as observed in AR null male mice (63). These traits likely stem, directly or indirectly, from modest alterations in transcriptional activity of the variant Q tract ARs, which are cumulative over lifetime and thus lead to more pronounced phenotypes with age. Nevertheless, the 48Q-h/mAR still has sufficient potency to produce healthy fertile males essentially indistinguishable from the other Q tract variant mice or their wild-type littermates. The altered activity may be compensated for by numerous factors responsive to hormone levels and to feedback mechanisms within the organism.

At the molecular level, AR expression and downstream regulation differ somewhat between these mouse lines, dependent on organ site. In testis, there is slightly more AR mRNA with h/mAR alleles than for wild-type mouse littermates, corresponding to somewhat higher AR protein levels. In prostate, the inverse difference appears, with mAR mRNA levels somewhat higher than for the humanized alleles. Because the genes differ only within exon 1, this may reflect regulatory elements that differ between man and mouse within the coding region, operating at transcriptional or posttranscriptional levels in cell-specific manners. Furthermore, mouse cofactors that interact with the NTD may have differential interactions with hAR, affecting autogenous as well as downstream differences. In the prostate, where the AR mRNA levels are nearly equivalent among mAR and h/mAR alleles, species-specific cofactor preferences may account for somewhat higher probasin expression in mAR than h/mAR mice. Within the h/mAR mice, however, probasin expression reveals sensitivity to AR Q tract length, most notable in increased expression with the short Q tract AR. Transcriptional weakness of the long Q tract allele is revealed both in reduced expression of Nkx3.1 and reduced repression of clusterin. Q tract length effects are likely to vary between tissues as they do between cell lines, suggesting involvement of differentially expressed accessory factors (10). Again, however, these differences are not sufficient to manifest at the light microscope level, and the prostates appear normal.

The clinically important question of whether Q tract length influences prostate cancer risk has been difficult to confirm in man due to factors including genetic heterogeneity, limited statistical power, and bias to earlier detection of pathology by increasing use of prostate-specific antigen testing (34, 64). To test Q tract association experimentally, a prostate-targeted transgenic oncogene was introduced by crossing the h/mAR mice to TRAMP (60). In contrast to physiological traits of h/mAR mice that have intriguing variations but rarely reach statistical significance, the Q tract length dramatically affects prostate tumor initiation or early stage growth. At a time point of 29 wk, when about half of the mAR and 21Q-h/mAR mice have palpable tumors or have died, nearly all of the short Q tract mice have tumors, but only one fourth of the long...
Q tract mice do. This striking inverse correlation between Q tract length and disease becomes more pronounced over time, because 48Q-h/mAR mice can live longer than 1 yr before tumor development (Albertelli, M. A., and D. M. Robins, unpublished observations). This result, in a genetically simplified model, corroborates the notion that Q tract length impacts initiation and/or progression of prostate cancer.

Existing evidence is consistent with the idea that differences in receptor transcriptional activity mediate the effect of AR Q tract length variation. These differences could be modest but additive over time, because the increased risk to prostate cancer caused by androgen exposure is cumulative over the lifespan. In the TRAMP model, an obvious candidate for a critical sensor of differential AR strength is the androgen-dependent probasin promoter driving the T-antigen oncogene. Activation of this promoter should parallel expression of the endogenous probasin gene, which is sensitive to Q tract differences in AR (Fig. 6). The 12Q-h/mAR might produce critical levels of the oncogene transcript earlier, and conversely the 48Q-h/mAR might lag in this process, relative to the other alleles. Although this differential gene activation can be shown in vitro, it appears less significant when tested in vivo, as we have done. However, the critical measurement may be at a particularly early time point and in a subset of cells, when the oncogenic event must actually occur. At later times, T-antigen levels in tumors are similar among strains of h/mAR mice (Albertelli, M. A., and D. M. Robins, unpublished observations), in agreement with other studies in mouse cancer models that find little correlation between level of expression of transgenic oncogene and tumor progression (65). Furthermore, T-antigen expression is unlikely to be the sole determinant of cancer progression, or one might anticipate more rapid initiation in mAR mice, paralleling their greater probasin expression.

Although prostate cancer in man does not initiate with T-antigen, early disease is androgen-dependent, and downstream events are similar for both. Thus, differential activation of probasin-driven T-antigen by Q tract length variants of AR may not be simply a trivial explanation of the effects we see but instead analogous to differential oncogenic activation in man, where the androgen-dependent causative events are still unknown. A recent candidate for such an event comes from finding common translocations in prostate cancer that place members of the ETS transcription factor family under the control of androgen-dependent promoters (66). Furthermore, additional stochastic androgen-dependent events are required for tumorigenesis, and differential activation of multiple genes is likely involved. We have shown that Q tract length inversely correlates with differential activation of Nkx3.1, a factor critical for both prostate differentiation and oncogenesis. Finding reduced Nkx3.1 expression in the 48Q-h/mAR mice that are more resistant to tumorigenesis may be somewhat paradoxical because loss of Nkx3.1 function is frequently associated with prostate cancer (57). However, both tumorigenesis and activation of Nkx3.1 downstream target genes are exquisitely sensitive to gene dosage in mice, and it is not clear when these differences in Nkx3.1 levels exert an effect or in which of many interacting pathways. It may be that in our model, Nkx3.1 levels accurately indicate AR transcriptional strength but are less critical for TRAMP tumorigenesis where potent T-antigen dominance may supplant usual cooperative mechanisms. Identification of additional androgen-dependent targets sensitive to Q tract length by expression array analysis may shed light on this question.

For the most part, the h/mAR Q tract variant mice show only subtle differences in physiological or molecular characteristics, within the range of normal phenotypic variation. Yet, when the homeostatic balance is upset by the stress of cancer, this variation is amplified, and distinct patterns of disease progression are produced. It may be that mechanisms that limit the effects of genetic variation (e.g. in AR activity) and buffer the organism from environmental change (e.g. in androgen levels) are abrogated by the global dysregulation that accompanies oncogenesis. One such compensatory mechanism is provided by chaperone molecules. Intriguingly, heat shock protein 90 deficiency in Drosophila is known to reveal otherwise cryptic genetic variation (67). Because heat shock protein 90 interacts with AR, as well as most components of signal transduction cascades involved in development and cancer, it may play a role in amplifying subclinical phenotypes that lead to dramatic stress-response differences in man. These mice demonstrate that a functional difference in AR activity within the normal range of phenotypic variation can affect prostate cancer biology. Whether one or a few CAGs more or less would have a detectable effect in this model is impractical to test. In man, there is evidence that receptors with Q tracts outside a critical range of 16–29 residues may mediate disease phenotype more significantly than length differences across the entire range (16). More important than the absolute length is determining the downstream effects of Q tract variation, which is possible in the experimental system of the h/mAR mice. On a broader level than just Q tract association, these mice represent an allelic series of ARs titrated for transactivation strength, and thus may provide clues to the endocrine basis of infertility and cardiovascular disease, as well as prostate cancer. Finally, these h/mAR mice provide a preclinical model for testing essential new androgen therapies designed to overcome the critical problem of androgen-independent but AR-dependent prostate tumor growth.
**MATERIALS AND METHODS**

**Construction of Targeting Vector and Generation of h/mAR Mice**

Targeting vectors were constructed using the backbone vector pGEM5zf(-)LPL1 (40), a mouse AR genomic clone isolated from a J1 mouse ES cell library in phage EM6L3 (the library was kindly obtained from Tom Glaser, University of Michigan), and hAR cDNAs obtained from M. J. McPhaul and M. Marcelli (41). In the vector, a BamHI site within the loxP-PGKneo-loxP cassette was eliminated, a HindIII site in the polylinker was converted to an NheI site to one side of the neo cassette, and BamHI and HindIII sites were introduced into the polylinker on the other side. This allowed insertion 3’ to the neo’ cassette of a 5.8-kb SacI-SpeI fragment, extending from upstream of mAR to 120 bp into intron 1; the contiguous 2-kb BamHI-HindIII fragment of intron 1 was placed 5’ to the cassette (Fig. 1A). hAR sequences were introduced in place of mouse exon 1 coding information by exchanging homologous Smal-Bsu36I fragments (nt 93-nt 1453 of 21Q-hAR, nt 93-nt 1433 of mAR). This fragment includes 88 amino acids that differ between man and mouse, not counting the polymorphic Q and G tracts, leaving one different mouse residue near the N terminus and three near the end of exon 1 in the now humanized AR exon 1. In the vector, there are 4 kb of homology upstream and 2 kb downstream for recombination with the neo’ cassette oppositely oriented to Ar. Three vectors were created, with ARs containing 12, 21, or 48 glutamines in the Q tract.

C57 ES cells, derived from the same mouse substrain as provided by the genomic library, were electroporated with vectors linearized at the MluI polylinker site. Over 100 C54-18-selected ES cell colonies were screened for recombination by Southern blot hybridization relying on diagnostic SphiI fragments created by an additional SphiI site in the vector, visualized by 5’ and 3’ probes to genomic sequences outside the extent of the vector (Fig. 1B). For the 21Q-h/mAR allele, for example, 22 colonies were correctly recombined, and of these, two colonies had more than 80% euploid cells as determined by analysis of chromosomes spreads. These two clones underwent transient transfection with a cre recombinase expression plasmid (pMCG-Cre, Ref. 69). The majority of colonies showed correct excision of the neo’ cassette, first by PCR analysis, and confirmed by Southern blotting for correct AR gene structure as above. Two independent targeted clones per h/mAR allele were injected into C57BL/6J blastocysts by the University of Michigan Transgenic Animal Models Core.

**Mouse Breeding and Care**

Male chimeric mice that had significant agouti contribution were crossed with C57BL/6J females. Resulting agouti female progeny, indicating transmission of the targeted paternal X chromosome, were bred to C57BL/6J males. Offspring from this cross (genotyped as in Fig. 1, C and D) were then interbred to create homozygous female and hemizygous male mice used in this study. Homozygous TRAMP mice on the C57BL/6J background (a gift of Martin Sanda) were crossed to heterozygous h/mAR female mice (generation F4) to obtain male h/mAR-TRAMP and mAR-TRAMP mice used in this study. All mouse procedures were approved by the University of Michigan Committee on Use and Care of Animals, in accord with the NIH Guidelines for the Care and Use of Experimental Animals.

**Genotyping**

DNA was isolated from tail biopsies by standard procedure and analyzed by PCR using primers that amplify both mouse and h/mAR regions encompassing the G tract (forward primer, 5’-CCACGTGTTCCTGCTGGGCCCCAC-3’; reverse primer, 5’-GACACTGTTACACAACTTCTGG-3’ in a 10-μl reaction containing 1.25 mm MgCl2, 0.2 mm deoxynucleotide triphosphates, 0.5 μM each primer, and 1 U Taq polymerase (Invitrogen, Carlsbad, CA). Cycling conditions consisted of 40 cycles of 94°C for 25 sec, 67°C for 30 sec, and 72°C for 30 sec. PCR products were visualized on 2.5% agarose gels with ethidium bromide. Product lengths were 289 bp for mAR and 247 bp for h/mAR (Fig. 1C). Additional genotyping of Q tract length was performed by PCR using primers that amplified the h/mAR but not mAR Q tract region (forward primer, 5’-ACCAGAGGCCGC- GGC-3’; reverse primer, 5’-GACCTCAGGGCGGAC- TGGC-3’) in a 20-μl reaction similar to above but containing 1.5 mm MgCl2. PCR products were visualized on 4% agarose gels, and product lengths were 221 bp for 12Q-h/mAR, 248 bp for 21Q-h/mAR, and 329 bp for 48Q-h/mAR. TRAMP genotyping was performed according to instructions (The Jackson Laboratory, Bar Harbor, ME).

**Serum Hormone Levels**

Serum testosterone levels were determined by RIA (Diagnostic Systems Laboratories, Inc., Webster, TX), following the manufacturer’s instructions.

**Real-Time PCR Analysis**

Total RNA was isolated from tissues using RNaseasy columns (QIAGEN, Hilden, Germany) and reverse transcribed using the High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA). Taqman assays were performed using 5 ng cDNA and FAM-TAMRA labeled primers and probes designed by Applied Biosystems (AR, Foxa1, Nkx3.1, clusterin) or SYBR Green (probasin, forward primer, 5’-TAGGCTCTCAAGTGTTAGTG-3’; reverse primer, 5’-ACGGAAG- TAGGTTCTCAAAGG-3’) in a 20-μl reaction containing 2X Taq polymerase (Invitrogen, Carlsbad, CA). Cycling conditions consisted of 40 cycles of 94°C for 25 sec, 67°C for 30 sec, and 72°C for 30 sec. PCR products were visualized on 2.5% agarose gels and product lengths were 289 bp for mAR and 247 bp for h/mAR (Fig. 1C).

**Leydig Cell Marker Semi-Quantitative RT-PCR**

Total testis RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen), and cDNA was amplified using primers and conditions as reported previously (53). Amplification products were visualized on agarose gels stained with ethidium bromide and quantified by densitometry.

**Western Blotting**

Tissues were homogenized in radioimmunoprecipitation assay (RIPA) buffer containing complete protease inhibitors (Roche, Basel, Switzerland) and centrifuged at 15,000 × g for 15 min to obtain supernatant and pellet fractions. Pellet fractions were resuspended in the same volume of RIPA buffer as original homogenate and rehomogenized for further analysis. Protein concentrations were determined by the Bradford method (Bio-Rad Dc Protein Assay, Bio-Rad Laboratories, Hercules, CA). Thirty micrograms of testis protein (for both supernatant and pellet fractions) were boiled in RIPA buffer for 10 min and examined by 7% SDS-PAGE. Protein was transferred to polyvinylidene difluoride membrane using a tank transfer system. Nonspecific sites were blocked with 5% nonfat dry milk in PBS/0.05% Tween 20 for 1 h at room temperature. Membranes were probed with AR (N-20; Santa Cruz Biotechnology, Santa Cruz, CA) or β-tubulin (H-235,
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Statistical Analysis

Data are presented as mean ± SEM. Statistical analysis was performed by ANOVA and Tukey’s studentized range (honestly significant difference) test for multiple comparisons. P < 0.05 was considered significant.

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