Reduced Androgen Receptor Gene Expression with First Exon CAG Repeat Expansion

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The molecular basis for partial androgen insensitivity associated with adult onset spinal/bulbar muscular atrophy was investigated by transient transfection of human androgen receptor (AR) expression vectors containing increasing CAG repeat lengths in the first exon. An inverse relationship was observed between CAG repeat length and AR mRNA and protein levels. Trinucleotide repeat lengths of 43 and 65 associated with spinal/bulbar muscular atrophy decreased AR mRNA and protein levels but did not alter equilibrium binding affinity for [^{3}H]R1881 or inherent transcriptional activity of AR, expressed as androgen-dependent fold induction of a mouse mammary tumor virus promoter-luciferase reporter vector. The findings indicate that glutamine expansion up to 66 residues in the NH_2-terminal domain of AR does not alter AR functional activity. Rather, CAG repeat expansion in the region of the first exon reduces AR mRNA and protein expression. The study reveals a previously unrecognized effect of CAG repeat length on AR mRNA expression and a novel molecular mechanism for androgen resistance. (Molecular Endocrinology 10: 1527–1535, 1996)

INTRODUCTION

Neurodegenerative diseases caused by trinucleotide repeat expansions include adult onset spinal/bulbar muscular atrophy (SBMA) (1), Huntington's disease (2), spinocerebellar ataxia type 1 (3), dentatorubral pallidoluysian atrophy (4), and Machado-Joseph disease (5). Each is caused by a CAG expansion that codes for glutamine (Gln) when present in the coding region. Fragile X syndrome is caused by CGG expansion (6–8), myotonic dystrophy by CTG expansion (9, 10), and as recently reported, Friedreich's ataxia (11) by GAA expansion (for reviews, Refs. 12–15). Triplet repeat expansion and neurodegeneration are shared features of these diseases, but differences occur in repeat length and stability, degree of anticipation (16), and location of the expansion within coding or noncoding regions. Expansion of the androgen receptor (AR) CAG repeat in the first exon of the coding region for the NH_2-terminal domain to greater than 40 Gln residues results in X-linked SBMA or Kennedy's disease (1). Clinical features of SBMA include progressive muscle weakness and atrophy (17–20) associated with loss of lower motor and primary sensory neurons (21) and partial androgen insensitivity as evidenced by testicular atrophy, impotence, gynecomastia, oligo- or azoospermia, and elevated serum gonadotrophin levels (19, 22, 23). Earlier onset, severity of muscle weakness (24–26), and degree of androgen resistance (27) correlate with longer repeats. A similar correlation between repeat length and age of onset of disease symptoms has been reported in dentatorubral pallidoluysian atrophy (4, 28), spinocerebellar ataxia type 1 (3), and Huntington's disease (29). A tendency toward increased repeats with transmission from parent to child is observed in families with SBMA (30), being more pronounced when there is paternal transmission (27). Instability of triplet repeats associated with human hereditary disease may result from errors in DNA mismatch repair through formation of single-stranded hairpin structures and DNA polymerase slippage during replication (31, 32). Repeat length instability noted during sperm typing reveals that contractions are 9 times more frequent than expansions in the normal allele, likely contributing to the rare occurrence of SBMA (33). Polymorphisms of the AR CAG repeat are stable in the normal population and have become useful genetic markers in clonality analysis (34–36) and forensic science (37).

Several previous reports indicate that expansion of the Gln repeat in the AR NH_2-terminal region results in a structurally altered protein with reduced transcriptional capacity (38–40). Normal [^{3}H]R1881 equilibrium binding affinity and protein expression by immunoblot support Gln tract inhibition of the transactivation func-

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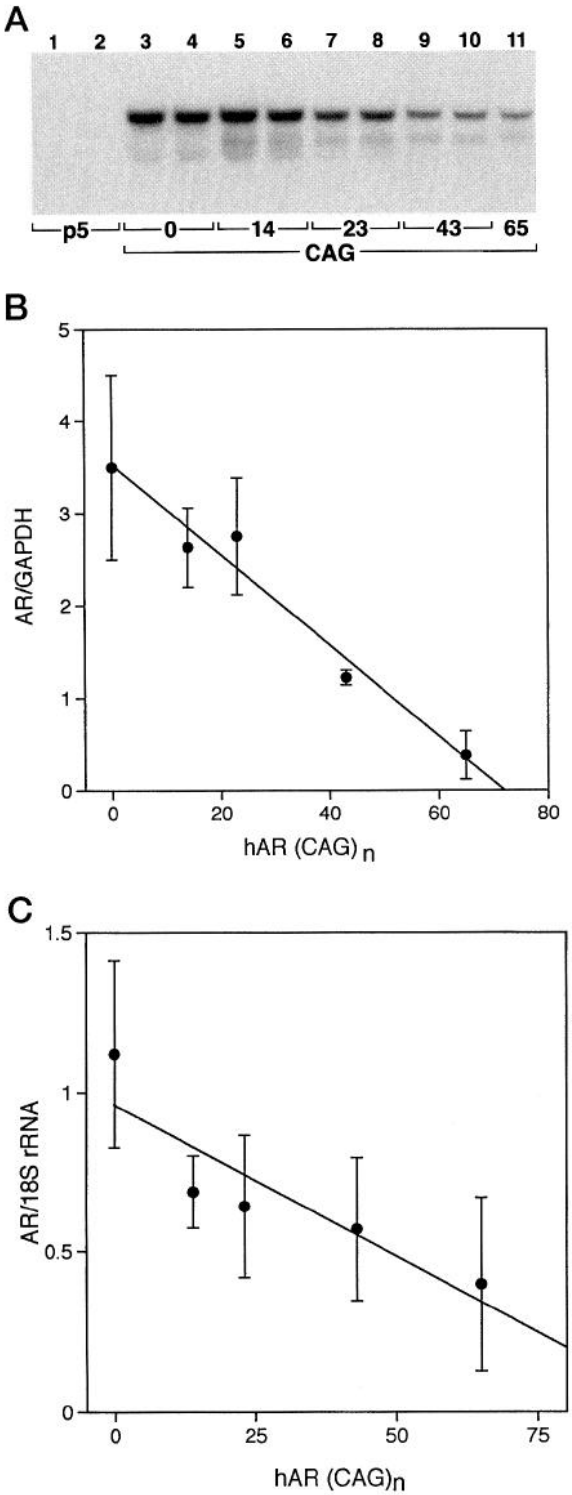

Fig. 1. Inverse Relationship between AR mRNA Expression and CAG Repeat Length in Transiently Transfected COS Cells

Total RNA was extracted as described from COS cells transfected in duplicate plates at 1 μg/4 $\times$ 10^5 COS cells per 6-cm dish with parent expression vector pCMV5 (p5) (lanes 1 and 2) or pCMVhAR containing the indicated CAG repeat lengths in the region encoding the AR NH_2-terminal domain (lanes 3–11). RNA was denatured using glyoxal and dimethylsulfoxide and fractionated on 1% agarose gels containing 10 mM NaH_2PO_4, pH 6.8. A, Representative Northern blot of total RNA (10 μg/lane) extracted from COS cells and probed with a [^{32}P]dCTP (Amersham, Arlington Heights, IL; 3000 Ci/mmol), random primed labeled 335-bp human AR PCR fragment (nucleotides 2559–2894) (44) (specific activity > 10^7 cpm/μg DNA). Hybridization was carried out overnight at 68 C in 0.75 M NaCl, 0.075 sodium citrate, pH 7.0, 5 $\times$ Denhardts (89), 0.1% SDS, 100 μg/ml sheared salmon sperm DNA. Filters were washed at high stringency using 0.015 M NaCl, 1.5 mM sodium citrate, pH 7.0, 0.1% SDS at 50 C for 1 h. After AR Northern analysis, filters were washed by boiling in 0.5% SDS for 5 min and rehybridized with a GAPDH probe to normalize for RNA loading. B, Ratio of densitometric scanning units of Northern blots for AR and GAPDH mRNA (mean ± SE, n = 3) relative to CAG repeat length in COS cells. Shown are the combined data from three independent experiments. C, Inverse relationship between CAG repeat number and AR mRNA levels in CV1 cells transfected in duplicate as described above with 10 μg/10^6 cells per 10-cm dish (mean ± SE, n = 2).

tion (40). Other reports indicate reduced equilibrium binding affinity of [^{3}H]R1881 in suprapubic skin fibroblasts from a normal equilibrium dissociation constant (K_d) of 0.19 nM to 0.24–11.7 nM in SBMA patients, a change that correlated with the severity of testicular atrophy and gynecomastia (41, 42). Our analysis using AR with an expansion of CAG repeats in the range associated with SBMA raises the alternative hypothesis of reduced AR mRNA and protein expression.

RESULTS

AR mRNA Expression

Equivalent amounts of the full-length human AR expression vector DNA with 0, 14, 23, 43, and 65 CAG repeats in the region of exon 1 that codes for the NH_2-terminal domain were transiently expressed in monkey kidney COS cells, and RNA was extracted for Northern blot analysis. As shown in Fig. 1A, AR mRNA levels were inversely related to CAG repeat length. CAG repeats of 43 and 65, associated with SBMA, resulted in lower amounts of AR mRNA than did wild type AR with 23 repeats or 0 or 14 repeats. A 4- to 5-fold reduction was observed between 0 and 65 CAG repeats and a 2- to 3-fold reduction between wild type 23 and 65 CAG repeats. When the data from several experiments were normalized by rehybridization with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe, there was a linear inverse relationship between CAG repeat length and levels of AR mRNA expression (Fig. 1B). Similarly, levels of AR mRNA expression in CV1 cells used in the transcription assays described below were inversely correlated with CAG repeat length, although absolute levels were reduced relative to COS cells (Fig. 1C).

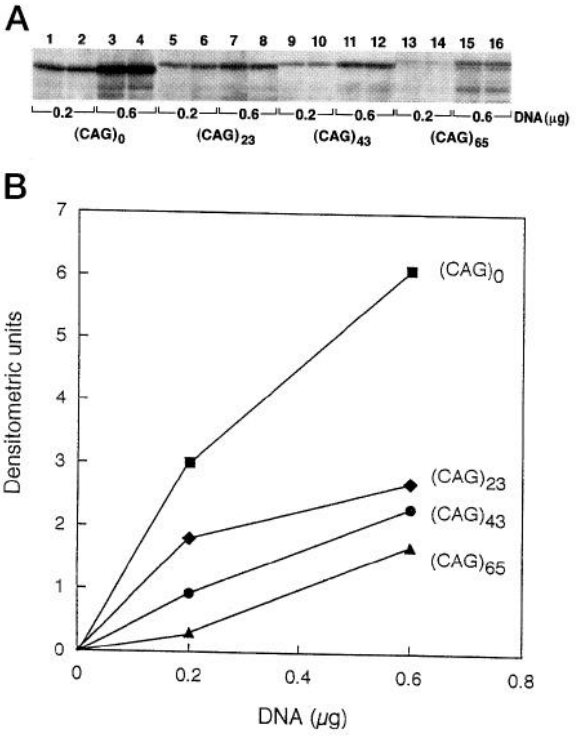

Fig. 2. Immunoblot Analysis of COS Cell Extracts after Transfection of AR Expression Vector DNA Containing Increasing CAG Repeat Lengths

COS cells were transiently transfected using calcium phosphate DNA precipitation in duplicate with human AR expression vector DNA containing increasing CAG repeat lengths (0.2 or 0.6 μg DNA/5 $\times$ 10^5 cells per 6-cm dish). Forty-eight hours after transfection, cells were harvested as described, analyzed on 9% acrylamide gels containing SDS, and probed with rabbit antipeptide AR IgG AR52 and visualized using fluorescein-tagged goat anti-rabbit IgG. A, A representative immunoblot of COS cell extracts transfected with pCMVhAR containing the indicated number of CAG repeats using 0.2 or 0.6 μg DNA. Note that AR migration is progressively slower reflecting the increased molecular mass due to Gln expansion. B, Densitometric analysis of the immunoblot shown in panel A where the points represent the mean of AR protein levels expressed at each concentration of transfected AR DNA.

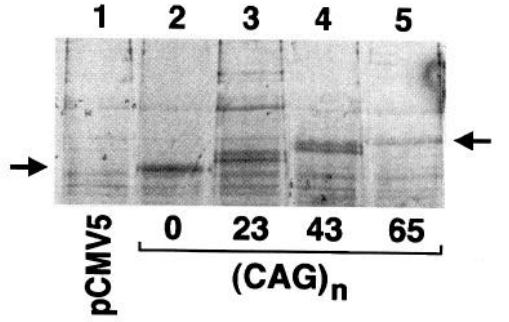

Fig. 3. [^{35}S]Methionine Incorporation into AR Protein after Transfection with Expression Vector DNA Containing Increasing Number of CAG Repeats

COS cells plated at 1.2 $\times$ 10^6 cells per 10-cm dish were transfected as previously described (43, 90) except using calcium phosphate with 2 μg parent vector pCMV5 lacking AR sequence (lane 1) or with AR expression vector DNA pCMVhAR with increasing CAG repeat length as indicated (lanes 2–5). Forty-eight hours after transfection, the cells were labeled with 100 μCi/dish [^{35}S]methionine/cysteine Trans Label (ICN Biomedical, Inc., Irvine, CA; 1000 Ci/mmol) for 1 h at 37 C and harvested as previously described (90). AR52 antipeptide AR antibody was used to immunoprecipitate radiolabeled AR. The *arrows* delineate the position of migration of AR whose DNA has 0 (*left*) and 65 (*right*) CAG repeats where AR 23 and 43 CAG have intermediate migration. The single band observed with deletion of the Gln repeat contrasts the characteristic double bands of wild type AR with Gln 24 or AR with Gln 44 and 66, suggesting a disturbance in phosphorylation at residue Ser 94 (91).

Immunoblot Analysis of AR Protein

Transfection of increasing amounts of AR expression vector DNA using 0.2, 0.6 μg (Fig. 2), or 1 μg (not shown)/5 $\times$ 10^5 COS cells resulted in a dose-dependent increase in AR protein expression (Fig. 2A). As the CAG repeat expanded to 43 and 65, AR protein signal intensity decreased over the DNA concentration range (Fig. 2) suggesting a direct relationship between reduced AR mRNA and protein levels. Using 0.2 and 0.6 μg AR DNA, the 65-CAG repeat was associated with 10- and 4-fold reductions, respectively, in AR protein signal intensity compared with 0 CAG repeats. At 0.2 μg, AR DNA with 65 CAG repeats resulted in an AR protein signal intensity that was 6-fold less than wild type AR with 23 CAG repeats. Using [^{35}S]methionine incorporation into expressed protein, an inverse relationship was again observed between the amount of AR protein synthesized and CAG repeat length (Fig. 3). Both methods of AR protein detection reflect the decreased AR mRNA levels resulting from expansion of the CAG repeat. AR protein degradation analyzed as previously described using pulse/chase labeling with [^{35}S]methionine (43) was not altered relative to wild type AR by expansion of the CAG repeat (data not shown).

Androgen Binding

COS cells transfected with increasing CAG repeat-length AR expression vector DNA using calcium phosphate precipitation were analyzed for AR binding affinity and capacity for [^{3}H]R1881 (a radiolabeled synthetic androgen). Scatchard plot analysis revealed equivalent equilibrium binding affinity, 0.24 $\pm$ 0.05 nM (K_d), for AR encoded by sequence containing 0–65 CAG repeats, but binding capacity was reduced for AR encoded by sequence containing 43 or 65 CAG repeats (Fig. 4). The half-time of [^{3}H]R1881 dissociation, determined at 37 C as previously described (43), of 3.0 $\pm$ 0.5 h was not influenced by the 44- or 66-Gln repeat length (data not shown). The CAG repeat in human AR immediately precedes a single CAA codon

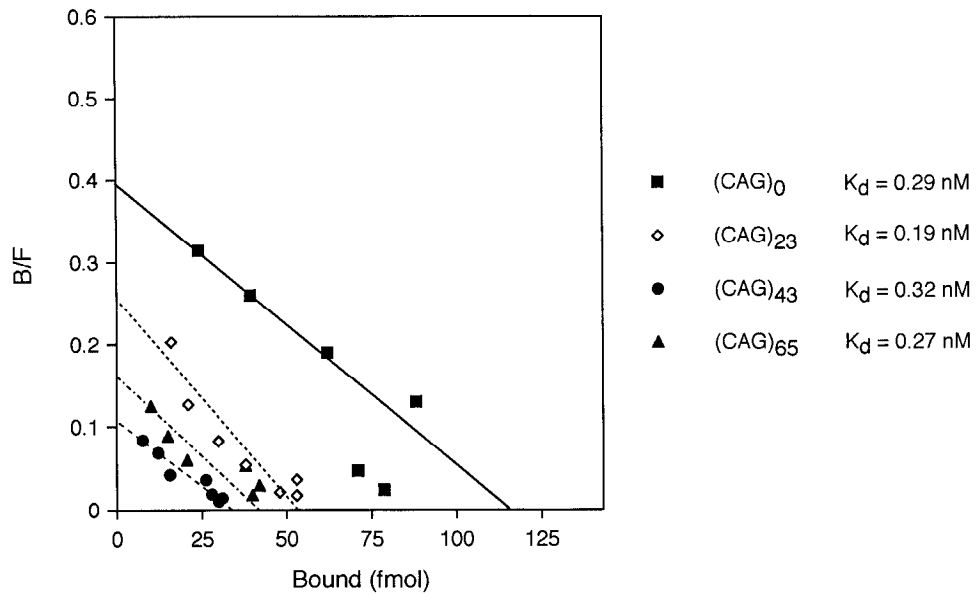

Fig. 4. Scatchard Plot Analysis of Equilibrium Binding of [^{3}H]R1881 Binding to AR Expressed from DNA Containing Increasing Number of CAG Repeats

Binding assays were performed as described in *Materials and Methods* using increasing concentrations of [^{3}H]R1881 between 0.25–8 nM. Shown are representative data of four experiments.

for Gln (44); hence the number of Gln residues is CAG_{n+1}. Binding capacity was the only parameter of ligand binding that was altered by expansion of the Gln repeat and reflected decreased AR mRNA and protein expression with CAG expansion.

Transcriptional Activity

Androgen-induced transcriptional activity by AR with increasing CAG repeats was tested by transient cotransfection in monkey kidney CV1 cells using a luciferase reporter vector containing the androgen-responsive mouse mammary tumor virus promoter. As shown in Fig. 5, luciferase activities induced by 0.01 and 0.1 nM dihydrotestosterone were indistinguishable with ARs of increasing Gln repeat lengths when expressed as fold induction relative to background activity determined in the absence of dihydrotestosterone. Reduction in total luciferase activity sometimes observed with Gln repeat expansion in our studies and those of others (38, 40) likely results from reduced AR protein levels. Because the fold induction was similar to that of wild type AR, the results suggest that increased Gln repeat lengths do not alter the inherent transcriptional activity of AR.

DISCUSSION

Results presented in this report indicate that CAG expansion within the AR first exon region coding for the NH_2-terminal transcriptional activation domain correlates with decreased AR mRNA and protein levels but not with loss of inherent AR functional activity. These observations may account for the symptoms of androgen resistance observed in SBMA. They may also explain recent observations correlating prostate cancer severity with CAG repeat length. Short AR CAG repeats correlate with high-grade prostate cancer, and the development of metastatic disease (45) is observed with higher frequency in populations susceptible to prostate cancer (46, 47) and have been reported as somatic deletions (48). As prostate cancer is initially androgen dependent, it is possible that higher AR expression enhances tumor progression.

The AR NH_2-terminal domain is essential for androgen-mediated transcriptional activation (49, 50). However, transcriptional activity does not require the Gln repeat that begins at residue 58 or the 24 glycine repeat beginning at residue 449. A Gln repeat polymorphism of 11–31 residues (37) occurs in the normal population and reflects a degree of sequence tolerance within the NH_2-terminal domain that could not be tolerated in the DNA- or ligand-binding domains. Remarkably, natural mutations that cause androgen insensitivity rarely occur in the NH_2-terminal region (51) even though there is less sequence conservation in this region among ARs from different species (52, 53). The glycine repeat is absent in rat AR, and the position of a 22-Gln repeat begins at residue 174 in rat AR rather than 58 in human AR, indicating position independence within the NH_2-terminal domain. In some nonhuman primates, the Gln repeat is reduced to four residues (54). Expansion to 66 Gln, as observed in some cases of SBMA, does not interfere with the androgen-induced NH_2-/carboxyl-terminal interaction

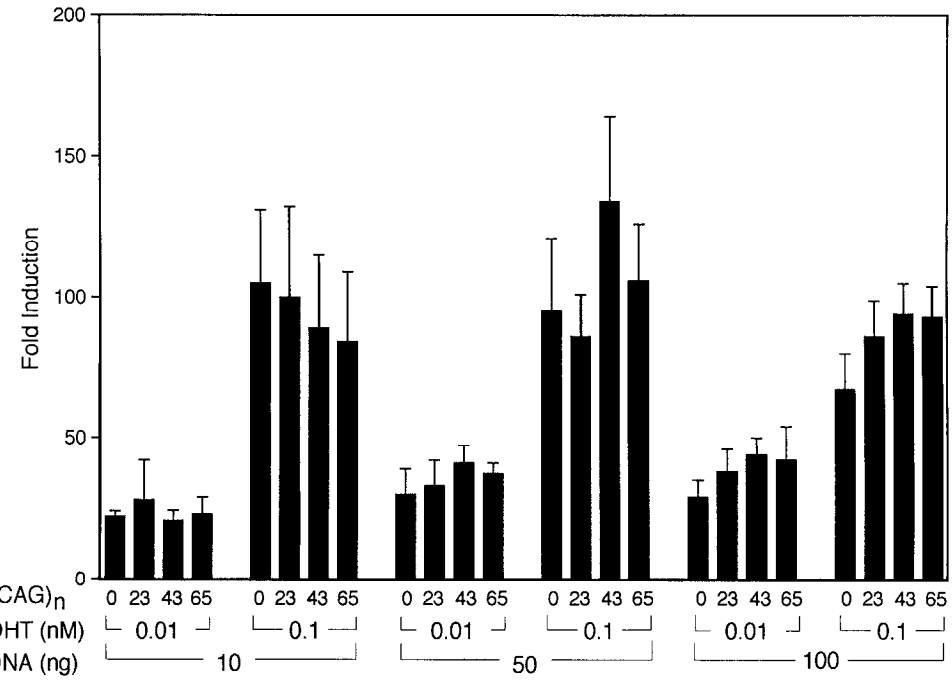

Fig. 5. Influence of CAG Repeat Length on AR Transcriptional Activity

CV1 cells were transiently transfected with 10, 50, and 100 ng pCMVhAR expression vector DNA containing increasing numbers of CAG repeats. Transient cotransfections were performed in duplicate as described using calcium phosphate precipitation with the mouse mammary tumor virus-luciferase reporter vector. Cells were incubated with 0.01 and 0.1 nM dihydrotestosterone (DHT) for 30 h and harvested in lysis buffer as described. Shown is the fold induction, which represents the ratio of luciferase activity induced in DHT- treated cells to that of untreated cells transfected with respective vectors. The *bars* represent the mean of five experiments ± SE.

required during AR dimerization and transcriptional activation (55). Nor, as shown here, does Gln expansion or deletion influence equilibrium androgen-binding affinity, kinetics of androgen binding, or transcriptional activation.

Disruption of AR functional activity results in the syndrome of androgen insensitivity which, in its complete form, is characterized by a female external phenotype at birth and loss of normal male sexual development in 46XY genetic males (51). Partial androgen insensitivity is characterized by incomplete masculinization and results from gene mutations that cause less severe disruption of AR function. AR missense mutations usually interfere with androgen or DNA binding (43, 51, 56). SBMA patients undergo normal prenatal and pubertal development and are fertile, indicating the presence of a functional AR. As adults, usually after the age of 30 yr (57) and in proportion to CAG repeat length, features of partial androgen resistance develop, including gynecomastia and elevated serum gonadotropin levels (9, 27). Gynecomastia in the adult male is a sensitive indicator of disturbed androgen-estrogen ratios or of impaired androgen action mediated by AR (58). The concentration of AR begins to decrease in the normal male between the ages of 20–30 yr, as shown strikingly in human foreskin fibroblasts (59). Our findings suggest that adult-onset androgen insensitivity observed in SBMA results from reduced AR mRNA and protein production compounded by normal AR attrition and the normal age-dependent decline in androgen levels (60, 61). During embryonic development and puberty, higher AR expression and androgen production may compensate for the inhibitory effects of the first exon-expanded CAG repeat on AR mRNA expression, allowing normal male sexual development to occur.

Previous results using tissue from affected individuals support the hypothesis that reduced AR mRNA and protein expression is the molecular basis for androgen insensitivity in SBMA patients. Equilibrium androgen binding affinity was unchanged, but B_{max} was reduced in SBMA patients with gynecomastia (62). Absence of AR immunostaining in scrotal skin from affected subjects (63) contrasted with strong immunostaining in age-matched controls, an indication of reduced AR protein expression in affected subjects. RT-PCR analysis of spinal cord showed 4- to 7-fold reduced AR mRNA levels compared with normal controls and undetectable AR protein by immunoblot analysis (64). Reduced androgen binding capacity with normal affinity was reported in SBMA cultured genital skin fibroblasts (65). Several clones of a stable neuronal cell line prepared with a 65-CAG repeat expansion had reduced AR protein levels compared with wild type controls (66).

Subjects with complete androgen insensitivity have normal muscle strength, indicating that reduced AR

expression cannot account for the progressive muscle weakness of SBMA. Localization of [^{3}H]dihydrotestosterone by autoradiography in motor neurons of the cranial nerves and spinal cord in normal male rats (67) reveals AR expression in the same motor neurons that are affected in SBMA (21, 23). A role for the Gln repeat expansion in this disease cannot, therefore, be excluded. Gln repeats form polar zipper β-sheets associated through hydrogen bonds that assist in protein-protein interactions (68). Inappropriate protein-protein interactions may contribute to motor neuron loss and muscle wasting through a gain in function related to specific neuron groups as proposed for the huntingtin-associated protein in Huntington's disease (69) and GAPDH in Huntington's disease and dentatorubral-pallidoluysian atrophy (70, 71).

Decreased expression of affected alleles in triplet-repeat expansion diseases may result from a common mechanism that involves transcriptional interference. Expanded CGG repeats in fragile X reduce FMR-1 mRNA levels (72), and the expanded CTG repeat in myotonic dystrophy reduces mRNA and protein levels of myotonin-protein kinase (73). The CTG triplet repeat in the mouse growth-inhibitory factor/metallothionein III gene promoter represses transcriptional activity in a direction- and position-independent manner (74). In the *cyt-5* gene of *Neurospora crassa*, a poly Gln region results in low mRNA transcript levels (75). One possible mechanism for transcriptional interference by trinucleotide repeat expansion is preferential nucleosome assembly. In myotonic dystrophy and Huntington's disease, electron microscopy revealed increased nucleosome formation at the expanded CTG (CAG) triplet repeat that could interfere with transcription complex progression (76). Although CTG repeat expansion failed to enhance nucleosome formation, preferential nucleosome assembly in the repeat region was thought to contribute to repeat expansion (77). The repeat length associated with most triplet-repeat disease phenotypes correlates with the length of DNA required for nucleosome formation (146 bp). Pertinent to the present study, transient expression vectors form nucleosome structures as mammalian cells rapidly assemble nonintegrated circular plasmids into minichromosomes (78–80). Other mechanisms for transcriptional interference by expanded triplet-repeat sequences include hypermethylation of CGG triplet repeats causing reduced expression of the fragile X mental retardation gene (72), RNA polymerase pausing proportional to repeat length (16, 81), the formation of hairpin structures by triplet repeats (16, 82), and intermolecular DNA triplex formation (83).

MATERIALS AND METHODS

Construction of AR Expression Vectors

Blood samples from two patients with SBMA were provided by Kenneth H. Fischbeck (University of Pennsylvania, Philadelphia, PA). Genomic DNA was amplified using *Taq* polymerase and primers flanking the NH_2-terminal region containing the CAG repeat. Amplified DNA was cloned into the wild type AR expression vector, pCMVhAR, described previously (49). CAG repeat length of 14 was obtained during cloning of the 43-CAG repeat by deletion in *Escherichia coli* during plasmid amplification. Deletion of the CAG repeat from wild type AR was constructed using a single mutagenic primer as previously described (84). All PCR-amplified regions were verified by sequencing using the dideoxy method and Sequenase (USB, Cleveland, Ohio).

Cell Culture and Transfection

Transient transfection assays were optimized for linear expression relative to transfected DNA concentration for monkey kidney COS-1 and CV1 cells. Cells were maintained and transfected in DMEM supplemented with 10% bovine calf serum, 20 mM HEPES, pH 7.2, and antibiotics in humidified 5% CO_2 as described previously (85) with some modifications. For Northern and immunoblot analysis, COS cells plated at 4–5 $\times$ 10^5/6-cm dish were transfected using calcium phosphate with 0.1–1 μg DNA/plate as indicated. For AR mRNA analysis in CV1 cells, cells were plated at a density of 1 $\times$ 10^6/10-cm dish and transfected with 10 μg DNA/plate using calcium phosphate precipitation. A 10-min precipitation reaction was followed by media addition for the formation of uniformly small DNA precipitates. Forty-eight hours after transfection, RNA was isolated by the acid guanidinium thiocyanate phenol method (86) using TRIzol reagent (GIBCO BRL, Gaithersburg, MD). Northern blot analysis was performed as described previously (87) and, after analysis for AR mRNA, the filters were stripped and rehybridized with an 18S ribosomal RNA cDNA probe pTZRNA18S (Ambion, Inc., Austin, TX) to standardize for RNA loading. For immunoblots, transfected COS cells were harvested in 100 μl SDS sample buffer and analyzed on 9% acrylamide gels containing SDS as described previously (85). AR bands were quantitated by lazer densitometry absorbance scanning using an LKB Ultrascanner (LKB Instruments, Rockville, MD) and analyzed using Gelscan XL version 2.1 (Pharmacia, Piscataway, NJ). [^{3}H]R1881 binding studies were performed in COS cells after transfection of 1 μg DNA/2 $\times$ 10^5 cells per well of 12-well tissue culture plates. Cells were transfected using calcium phosphate and, 48 h after transfection, were incubated with increasing concentrations of [^{3}H]R1881 from 0.25–8 nM in the presence and absence of 100-fold excess unlabeled R1881 as described previously (88). Androgen-dependent transcription assays were performed using calcium phosphate by plating 3 $\times$ 10^5 CV1 cells/6-cm dish and were transfected with the indicated concentrations of AR expression vector DNA and 5 μg luciferase reporter vector as previously described (85).

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REFERENCES

1. La Spada AR, Wilson EM, Lubahn DB, Harding AE, Fischbeck KH 1991 Androgen receptor gene mutations in X-linked spinal and bulbar muscular atrophy. Nature 352:77–79
2. The Huntington's Disease Collaborative Research Group 1993 A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. Cell 72:971–983
3. Orr HT, Chung MY, Banfi S, Kwiatkowski TJ, Servadio A, Beaudet AL, McCall AE, Duvick LA, Ranum LPW, Zoghbi HY 1993 Expansion of an unstable trinucleotide CAG repeat in spinocerebellar ataxia type 1. Nat Genet 4:221–226
4. Koide R, Ikeuchi T, Onodera O, Tanaka H, Igarashi S, Endo K, Takahashi H, Kondo R, Ishikawa A, Hayashi T, Saito M, Tamoda A, Miike T, Naito H, Ikuta F, Tsuji S 1994 Unstable expansion of CAG repeat in hereditary dentatorubral-pallidoluysian atrophy (DRPLA). Nat Genet 6:9–13
5. Kawaguchi Y, Okamoto T, Taniwaki M, Aizawa M, Inoue M, Katayama S, Kawakami H, Nakamura S, Nishimura M, Akiguchi I, Kimura J, Narumiya S, Kakizuka A 1994 CAG expansions in a novel gene for Machado-Joseph disease at chromosome 14q32.1. Nat Genet 8:221–228
6. Kremer EJ, Pritchard M, Lynch M, Yu S, Holman K, Baker E, Warren ST, Schlessinger D, Sutherland GR, Richards RI 1991 Mapping of DNA instability at the fragile X to a trinucleotide repeat sequence p(CCG)*n*. Science 252:1711–1714
7. Heitz D, Rousseau F, Devys D, Saccone S, Abderrahim H, Le Paslier D, Cohen D, Vincent A, Toniolo D, Valle GD, Johnson S, Schlessinger D, Oberlé I, Mandel JL 1991 Isolation of sequences that span the fragile X and identification of a fragile X-related CpG island. Science 251:1236–1239
8. Verkerk AJMH, Pieretti M, Sutcliffe JS, Fu YH, Kuhl DPA, Pizzuti A, Reiner O, Richards S, Victoria MF, Zhang F, Eussen BE, van Ommen GJB, Blonden LAJ, Riggins GJ, Chastain JL, Kunst CB, Galjaard H, Caskey CT, Nelson DL, Oostra BA, Warren ST 1991 Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. Cell 65:905–914
9. Brook JD, McCurrach ME, Harley HG, Buckler AJ, Church D, Aburatani H, Hunter K, Stanton VP, Thirion JP, Hudson T, Sohn R, Zemelman B, Snell RG, Rundle SA, Crow S, Davies J, Shelbourne P, Buxton J, Jones C, Juvonen V, Johnson K, Harper PS, Shaw DJ, Housman DE 1992 Molecular basis of myotonic dystrophy: expansion of a trinucleotide (CTG) repeat at the 3′ end of a transcript encoding a protein kinase family member. Cell 68:799–808
10. Harley HG, Brook JD, Rundle SA, Crow S, Reardon W, Buckler AJ, Harper PS, Housman DE, Shaw DJ 1992 Expansion of an unstable DNA region and phenotypic variation in myotonic dystrophy. Nature 355:545–546
11. Campuzano V, Montermini L, Molto MD, Pianese L, Cossée M, Cavalcanti F, Monros E, Rodius F, Duclos F, Monticelli A, Zara F, Canizares J, Koutnikova H, Bidichandani SI, Gellera C, Brice A, Trouillas P, De Michele G, Filla A, De Frutos R, Palau F, Patel PI, Di Donato S, Mandel JL, Cocozza S, Koenig M, Pandolfo M 1996 Friedreich's ataxia: autosomal recessive disease caused by an intronic GAA triplet repeat expansion. Science 271:1423–1427
12. Sutherland GR, Richards RI 1995 Simple tandem DNA repeats and human genetic disease. Proc Natl Acad Sci USA 92:3636–3641
13. Warren ST 1996 The expanding world of trinucleotide repeats. Science 271:1374–1375
14. Caskey CT, Pizzuti A, Fu YH, Fenwick RG, Nelson DL 1992 Triplet repeat mutations in human disease. Science 256:784–788
15. Nelson DL 1996 Allelic expansion underlies many genetic diseases. Growth Genet Horm 12:1–4
16. Wells RD 1996 Molecular basis of genetic instability of triplet repeats. J Biol Chem 271:2875–2878
17. Kennedy WR, Alter M, Sung JH 1968 Progressive proximal spinal and bulbar muscular atrophy of late onset: a sex-linked recessive trait. Neurology 18:671–680
18. Harding AE, Thomas PK, Baraitser M, Bradbury PG, Morgan-Hughes JA, Ponsford JR 1982 X-linked recessive bulbospinal neuronopathy: a report of ten cases. J Neurol Neurosurg Psychiat 45:1012–1019
19. Arbizu T, Santamaria J, Gomez JM, Quilez A, Serra JP 1983 A family with adult spinal and bulbar muscular atrophy, X-linked inheritance and associated testicular failure. J Neurol Sci 59:371–382
20. Fischbeck KH, Ionasescu V, Ritter AW, Ionasescu R, Davies K, Ball S, Bosch P, Burns T, Hausmanowa-Petrusewicz I, Borkowska J, Ringel SP, Stern LZ 1986 Localization of the gene for X-linked spinal muscular atrophy. Neurology 36:1595–1598
21. Sobue G, Hashizume Y, Mukai E, Hirayama M, Mitsuma T, Takahashi A 1989 X-linked recessive bulbospinal neuronopathy: a clinicopathological study. Brain 112:209–232
22. Amato AA, Prior TW, Barohn RJ, Snyder P, Papp A, Mendell JR 1993 Kennedy's disease: a clinicopathologic correlation with mutations in the androgen receptor gene. Neurology 43:791–794
23. Nagashima T, Seko K, Hirose K, Mannen T, Yoshimura S, Arima R, Nagashima K, Morimatu Y 1988 Familial bulbospinal muscular atrophy associated with testicular atrophy and sensory neuropathy (Kennedy-Alter-Sung syndrome): autopsy case report of two brothers. J Neurol Sci 87:141–152
24. La Spada AR, Roling DB, Harding AE, Warner CL, Speigel R, Hausmanowa-Petrusewicz I, Yee WC, Fischbeck KH 1992 Meiotic stability and genotype-phenotype correlation of the trinucleotide repeat in X-linked spinal and bulbar muscular atrophy. Nat Genet 2:301–304
25. Doyu M, Sobue G, Mukai E, Kachi T, Yasuda T, Mitsuma T, Takahashi A 1992 Severity of X-linked recessive bulbospinal neuronopathy correlates with size of the tandem CAG repeat in androgen receptor gene. Ann Neurol 32:707–710
26. Igarashi S, Tanno Y, Onodera O, Yamazaki M, Sato S, Ishikawa A, Miyatani N, Nagashima M, Ishikawa Y, Sahashi K, Ibi T, Miyatake T, Tsuji S 1992 Strong correlation between the number of CAG repeats in androgen receptor genes and the clinical onset of features of spinal and bulbar muscular atrophy. Neurology 42:2300–2302
27. Shimada N, Sobue G, Doyu M, Yamamoto K, Yasuda T, Mukai E, Kachi T, Mitsuma T 1995 X-linked recessive bulbospinal neuronopathy: clinical phenotypes and CAG repeat size in androgen receptor gene. Muscle Nerve 18:1378–1384
28. Nagafuchi S, Yanagisawa H, Sato K, Shirayama T, Ohsaki E, Bundo M, Takeda T, Tadokoro K, Kondo I, Murayama N, Tanaka Y, Kikushima H, Umino K, Kurosawa H, Furukawa T, Nihei K, Inoue T, Sano A, Komure O, Takahashi M, Yoshizawa T, Kanazawa I, Yamada M 1994 Dentatorubral and pallidoluysian atrophy expansion of an unstable CAG trinucleotide on chromosome 12p. Nat Genet 6:14–18
29. Andrew SE, Goldberg YP, Kremer B, Telenius H, Theilmann J, Adam S, Starr E, Squitieri F, Lin B, Kalchman MA, Graham RK, Hayden MR 1993 The relationship be-

tween trinucleotide (CAG) repeat length and clinical features of Huntington's disease. Nat Genet 4:398–403
30. Biancalana V, Serville F, Pommier J, Julien J, Hanauer A, Mandel JL 1992 Moderate instability of the trinucleotide repeat in spino bulbar muscular atrophy. Hum Mol Genet 1:255–258
31. Strand M, Prolla TA, Liskay RM, Petes TD 1993 Destabilization of tracts of simple repetitive DNA in yeast by mutations affecting DNA mismatch repair. Nature 365:274–276
32. Jaworski A, Rosche WA, Gellibolian R, Kang S, Shimizu M, Bowater RP, Sinden RR, Wells RD 1995 Mismatch repair in *Escherichia coli* enhances instability of $(CTG)_n$ triplet repeats from human hereditary diseases. Proc Natl Acad Sci USA 92:11019–11023
33. Zhang L, Leeflang EP, Yu J, Arnheim N 1994 Studying human mutations by sperm typing: instability of CAG trinucleotide repeats in the human androgen receptor gene. Nat Genet 7:531–535
34. Delabesse E, Aral S, Kamoun P, Varet B, Turhan AG 1995 Quantitative non-radioactive clonality analysis of human leukemic cells and progenitors using the human androgen receptor (AR) gene. Leukemia 9:1578–1582
35. Park TW, Felix JC, Wright TC 1995 X chromosome inactivation and microsatellite instability in early and advanced bilateral ovarian carcinomas. Cancer Res 55:4793–4796
36. Mutter GL, Boynton KA 1995 PCR bias in amplification of androgen receptor alleles, a trinucleotide repeat marker used in clonality studies. Nucleic Acids Res 23:1411–1418
37. Edwards A, Hammond HA, Jin L, Caskey CT, Chakraborty R 1992 Genetic variation at five trimeric and tetrameric tandem repeat loci in four human population groups. Genomics 12:241–253
38. Mhatre AN, Trifiro MA, Kaufman M, Kazemi-Esfarjani P, Figlewicz D, Rouleau G, Pinsky L 1993 Reduced transcriptional regulatory competence of the androgen receptor in X-linked spinal and bulbar muscular atrophy. Nat Genet 5:184–187
39. Trifiro MA, Kazemi-Esfarjani P, Pinsky L 1994 X-linked muscular atrophy and the androgen receptor. Trends Endocrinol Metab 5:416–421
40. Chamberlain NL, Driver ED, Miesfeld RL 1994 The length and location of CAG trinucleotide repeats in the androgen receptor N-terminal domain affect transactivation function. Nucleic Acids Res 22:3181–3186
41. MacLean HE, Choi WT, Rekaris G, Warne GL, Zajac JD 1995 Abnormal androgen receptor binding affinity in subjects with Kennedy's disease (spinal and bulbar muscular atrophy). J Clin Endocrinol Metab 80:508–516
42. MacLean HE, Warne GL, Zajac JD 1995 Defects of androgen receptor function: from sex reversal to motor neurone disease. Mol Cell Endocrinol 112:133–141
43. Zhou ZX, Lane MV, Kemppainen JA, French FS, Wilson EM 1995 Specificity of ligand dependent androgen receptor stabilization: receptor domain interactions influence ligand dissociation and receptor stability. Mol Endocrinol 9:208–218
44. Lubahn DB, Joseph DR, Sar M, Tan JA, Higgs HN, Larson RE, French FS, Wilson EM 1988 The human androgen receptor: complementary DNA cloning, sequence analysis and gene expression in prostate. Mol Endocrinol 2:1265–1275
45. Giovannucci E, Stampfer M, Krithivas K, Brown M, Talcott J, Hennekens C, Kantoff P 1996 The CAG repeat contained within the androgen receptor is a heritable factor that influences the clinical behavior of prostate cancer. Proc Am Soc Clin Oncol 15:252 (Abstract 646)
46. Coetzee GA, Ross RK 1994 Re: prostate cancer and the androgen receptor. J Natl Cancer Inst 86:872–873
47. Irvine RA, Yu MC, Ross RK, Coetzee GA 1995 The CAG and GGC microsatellites of the androgen receptor gene are in linkage disequilibrium in men with prostate cancer. Cancer Res 55:1937–1940
48. Schoenberg MP, Hakimi JM, Wang S, Bova GS, Epstein JI, Fischbeck KH, Isaacs WB, Walsh PC, Barrack ER 1994 Microsatellite mutation ($CAG_{24\rightarrow18}$) in the androgen receptor gene in human prostate cancer. Biochem Biophys Res Commun 198:74–80
49. Simental JA, Sar M, Lane MV, French FS, Wilson EM 1991 Transcriptional activation and nuclear targeting signals of the human androgen receptor. J Biol Chem 266:510–518
50. Jenster G, van der Korput HAGM, Trapman J, Brinkmann AO 1995 Identification of two transcription activation units in the N-terminal domain of the human androgen receptor. J Biol Chem 270:7341–7346
51. Quigley CA, De Bellis A, Marschke KB, El-Awady MK, Wilson EM, French FS 1995 Androgen receptor defects: historical, clinical and molecular perspectives. Endocr Rev 16:271–321
52. Charest NJ, Zhou ZX, Lubahn DG, Olsen KL, Wilson EM, French FS 1991 A frameshift mutation destabilizes androgen receptor messenger RNA in the Tfm mouse. Mol Endocrinol 5:573–581
53. He WW, Fischer LM, Sun S, Bilhartz DL, Zhu X, Young CYF, Kelley DB, Tindall DJ 1990 Molecular cloning of androgen receptors from divergent species with a polymerase chain reaction technique: complete cDNA sequence of the mouse androgen receptor and isolation of androgen receptor cDNA probes from dog, guinea pig and clawed frog. Biochem Biophys Res Commun 171:697–704
54. Djian P, Hancock JM, Chana HS 1996 Codon repeats in genes associated with human diseases: fewer repeats in the genes of nonhuman primates and nucleotide substitutions concentrated at the sites of reiteration. Proc Natl Acad Sci USA 93:417–421
55. Langley E, Zhou ZX, Wilson EM 1995 Evidence for an antiparallel orientation of the ligand activated human androgen receptor dimer. J Biol Chem 270:29983–29990
56. French FS, Lubahn DB, Brown TR, Simental JA, Quigley CA, Yarbrough WG, Tan JA, Sar M, Joseph DR, Evans BAJ, Hughes IA, Migeon CJ, Wilson EM 1990 Molecular basis of androgen insensitivity. Recent Prog Horm Res 46:1–42
57. Brooks BP, Fischbeck KH 1995 Spinal and bulbar muscular atrophy: a trinucleotide-repeat expansion neurodegenerative disease. Trends Neurosci 18:459–461
58. Wilson JD, Aiman J, MacDonald PC 1980 The pathogenesis of gynecomastia. Adv Intern Med 25:1–32
59. Roehrborn CG, Lange JL, George FW, Wilson JD 1987 Changes in amount and intracellular distribution of androgen receptor in human foreskin as a function of age. J Clin Invest 79:44–47
60. Nahoul K, Roger M 1990 Age-related decline in plasma bioavailable testosterone in adult men. J Steroid Biochem 35:293–299
61. Zumoff B, Strain GW, Kream J, O'Connor J, Rosenfeld RS, Levin J, Fukushima DK 1982 Age variation of the 24-hour mean plasma concentrations of androgens, estrogens, and gonadotropins in normal adult men. J Clin Endocrinol Metab 54:534–538
62. Warner CL, Griffin JE, Wilson JD, Jacobs LD, Murray KR, Fischbeck KH, Dickoff D, Griggs RC 1992 X-linked spinomuscular atrophy: a kindred with associated abnormal androgen receptor binding. Neurology 42:2181–2184
63. Matsuura T, Demura T, Aimoto Y, Mizuno T, Moriwaka F, Tashiro K 1992 Androgen receptor abnormality in X-linked spinal and bulbar muscular atrophy. Neurology 42:1724–1726
64. Nakamura M, Mita S, Murakami T, Uchino M, Watanabe S, Tokunaga M, Kumamoto T, Ando M 1994 Exonic trinucleotide repeats and expression of androgen receptor gene in spinal cord from X-linked spinal and bulbar

muscular atrophy. J Neurol Sci 122:74–79
65. Danek A, Witt TN, Mann K, Schweikert HU, Romalo G, La Spada AR, Fischbeck KH 1994 Decrease in androgen binding and effect of androgen treatment in a case of X-linked bulbospinal neuronopathy. Clin Invest 72:892–897
66. Brooks BP, Paulson HL, Merry DE, Salazar-Greuso EF, Wilson EM, Fischbeck KH 1996 Characterization of the spinal-and-bulbar muscular atrophy gene in a neuronal cell culture system. Am J Hum Genet [Suppl 59], Abstract 1442, p A250
67. Sar M, Stumpf WE 1977 Androgen concentration in motor neurons of cranial nerves and spinal cord. Science 197:77–79
68. Perutz MF, Johnson T, Suzuki M, Finch JT 1994 Glutamine repeats as polar zippers: their possible role in inherited neurodegenerative diseases. Proc Natl Acad Sci USA 91:5355–5358
69. Li XJ, Li SH, Sharp AH, Nucifora FC, Schilling G, Lanahan A, Worley P, Snyder SH, Ross CA 1995 A huntingtin-associated protein enriched in brain with implications for pathology. Nature 378:398–402
70. Burke JR, Enghild JJ, Martin ME, Jou YS, Myers RM, Roses AD, Vance JM, Strittmatter WJ 1996 Huntington and DRPLA proteins selectively interact with the enzyme GAPDH. Nature Med 2:347–350
71. Roses AD 1996 From genes to mechanisms to therapies: lessons to be learned from neurological disorders. Nature Med 2:267–269
72. Pieretti M, Zhang F, Fu YH, Warren ST, Oostra BA, Caskey CT, Nelson DL 1991 Absence of expression of the FMR-1 gene in fragile X syndrome. Cell 66:817–822
73. Fu YH, Friedman DL, Richards S, Pearlman JA, Gibbs RA, Pizzuti A, Ashizawa T, Perryman MB, Scarlato G, Fenwick RG, Caskey CT 1993 Decreased expression of myotonin-protein kinase messenger RNA and protein in adult form of myotonic dystrophy. Science 260:235–238
74. Imagawa M, Ishikawa Y, Shimano H, Osada S, Nishihara T 1995 CTG triplet repeat in mouse growth inhibitory factor/metallothionein III gene promoter represses the transcriptional activity of the heterologous promoters. J Biol Chem 270:20898–20900
75. Chen B, Kubelik AR, Mohr S, Breitenberger CA 1996 Cloning and characterization of the *Neurospora crassa cyt-5* gene: a nuclear-coded mitochondrial RNA polymerase with a polyglutamine repeat. J Biol Chem 271:6537–6544
76. Wang YH, Amirhaeri S, Kang S, Wells RD, Griffith JD 1994 Preferential nucleosome assembly at DNA triplet repeats from the myotonic dystrophy gene. Science 265:669–671
77. Godde JS, Wolffe AP 1996 Nucleosome assembly on CTG triplet repeats. J Biol Chem 271:15222–15229
78. Reeves R, Gorman CM, Howard B 1985 Minichromosome assembly of non-integrated plasmid DNA transfected into mammalian cells. Nucleic Acids Res 13:3599–3615
79. Cereghini S, Yaniv M 1984 Assembly of transfected DNA into chromatin: structural changes in the origin-promoter-enhancer region upon replication. EMBO J 3:1243–1253
80. Beato M, Candau R, Chávez S, Möws C, Truss M 1996 Interaction of steroid hormone receptors with transcription factors involves chromatin remodelling. J Steroid Biochem Mol Biol 56:47–59
81. Kang S, Ohshima K, Shimizu M, Amirhaeri S, Wells RD 1995 Pausing of DNA synthesis *in vitro* at specific loci in CTG and CGG triplet repeats from human hereditary disease genes. J Biol Chem 270:27014–27021
82. Nadel Y, Weisman-Shomer P, Fry M 1995 The fragile X syndrome single strand d(CGG)*n* nucleotide repeats readily fold back to form unimolecular hairpin structures. J Biol Chem 270:28970–28977
83. Ohshima K, Kang S, Larson JE, Wells RD 1996 Cloning, characterization and properties of seven triplet repeat DNA sequences. J Biol Chem 271:16773–16783
84. Choong CS, Sturm MJ, Strophair JA, McCulloch RK, Tilley WD, Leedman PJ, Hurley DM 1996 Partial androgen insensitivity caused by an androgen receptor mutation at amino acid 907 (Gly→Arg) that results in decreased ligand binding affinity and reduced androgen receptor messenger ribonucleic acid levels. J Clin Endocrinol Metab 81:236–243
85. Zhou ZX, Sar M, Simental JA, Lane MV, Wilson EM 1994 A ligand dependent bipartite nuclear targeting signal in the human androgen receptor: requirement for the DNA binding domain and modulation by the NH_2-terminal and carboxyl-terminal sequences. J Biol Chem 269: 13115–13123
86. Chomczynski P, Sacchi N 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159
87. Quarmby VE, Yarbrough WG, Lubahn DB, French FS, Wilson EM 1990 Autologous down regulation of androgen receptor messenger ribonucleic acid. Mol Endocrinol 4:22–28
88. Yarbrough WG, Quarmby VE, Simental JA, Joseph DR, Sar M, Lubahn DB, Olsen KL, French FS, Wilson EM 1990 A single base mutation in the androgen receptor gene causes androgen insensitivity in the Tfm rat. J Biol Chem 265:8893–8900
89. Denhardt DT 1966 A membrane-filter technique for the detection of complementary DNA. Biochem Biophys Res Commun 23:641–646
90. Kemppainen JA, Lane MV, Sar M, Wilson EM 1992 Androgen receptor phosphorylation, turnover, nuclear transport and transcriptional activation: specificity for steroids and antihormones. J Biol Chem 267:968–974
91. Zhou ZX, Kemppainen JA, Wilson EM 1995 Identification of three proline-directed phosphorylation sites in the human androgen receptor. Mol Endocrinol 9:605–615