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Androgens Regulate the Mammalian Homologues of Invertebrate Sex Determination Genes *tra-2* and *fox-1*

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Androgens, like other steroid hormones, exert profound effects on cell growth and survival by modulating the expression of target genes. In vertebrates, androgens play a critical role downstream of the testis determination pathway, influencing the expression of sexually dimorphic traits. Among cells of the nervous system, motor neurons respond to trophic effects of androgen stimulation, with a subpopulation of spinal motor neurons exhibiting sexually dimorphic survival. To study the mechanisms of androgen action in these cells, we performed a subtractive screen for genes upregulated by androgen in a motor neuron cell line. We show androgen-inducible expression of two RNA-binding proteins that are the mammalian homologues of invertebrate sex determination genes. Androgens upregulate the expression of *tra-2α,* an enhancer of RNA splicing homologous to *Drosophila tra-2,* and promote redistribution of the protein from a diffuse to a speckled pattern within the nucleus. Similarly, androgens upregulate the expression of a novel gene homologous to *Caenorhabditis elegans fox-1.* These data indicate that androgens exert their effects, in part, by modulating the expression and function of genes involved in RNA processing, and identify homologues of invertebrate sex determination genes as androgen-responsive genes in mammals.

Key Words: **androgen receptor; motor neuron; RNA-binding proteins; sex determination.**

Androgens are critical mediators of sexual dimorphism in mammals that exert their effects by binding to the androgen receptor, a ligand-activated transcription factor that regulates the expression of target genes. Androgens exert these effects by acting downstream of testis determination, activating genetic pathways that selectively promote survival and proliferation, or induce programmed cell death, in different cell populations. That these androgen-responsive pathways are necessary for the expression of sexually dimorphic traits is supported by the feminization of genetic males with a loss of androgen receptor function (1, 2). While the pathways mediating these effects are not well defined in mammals, genetic analysis has led to the characterization of pathways that determine sexual dimorphism in invertebrates (3, 4). These pathways are composed of a cascade of transcription factors whose expression and splicing are regulated by RNA-binding proteins.

In the mammalian nervous system, androgens exert broad effects both during development and in the mature animal. Androgens are trophic for certain neuronal populations, including sexually dimorphic neurons of the spinal cord and hypothalamus (5). Among these androgen–responsive populations are the motor neurons of the brainstem and spinal cord, for which androgens act as trophic factors and promote survival after injury (6, 7). Motor neurons of the sexually dimorphic spinal nucleus of the bulbocavernosus are exquisitely sensitive to the trophic effects of androgens, which promote their survival during development (8) and increase their dendritic length and soma size in adult animals (9). The androgen receptor gene is mutated in a degenerative disease of motor neurons, spinal and bulbar muscular atrophy (SBMA), in which there is an expansion of a CAG/polyglutamine tract leading to a toxic gain-of-function (10, 11). This mutation also causes a partial loss of androgen receptor function in patients with SBMA (12–14) that may contribute to the cell specificity of the disease by depriving motor neurons of trophic effects. The pathways by which androgens exert these effects are largely unknown.

We used a previously characterized a mouse motor neuron cell line that stably expresses the androgen receptor and responds to androgen (15) as a model

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system to study the mechanisms of androgen action. Here we identify two RNA-binding proteins that are induced by androgen treatment of these cells. Interestingly, these genes encode mammalian homologues of the invertebrate sex determination factors *tra-2* of *Drosophila* and *fox-1* of *C. elegans*. Our data indicate that androgens exert their effects by modulating the expression of genes involved in RNA processing, and identify homologues of invertebrate sex determination genes as androgen-responsive in mammals.

MATERIALS AND METHODS

Subtractive hybridization. Mouse MN-1 cells stably expressing the full-length human androgen receptor (androgen receptor clone 5, abbreviated AR5) were used for induction. The plasmid construct containing the human androgen receptor cDNA used to generate this line was previously described (16). AR5 cells bind androgen with a K_d = 0.53 nM and a B_{max} = 551 fmol/mg (17). Cells were grown with or without ligand, 10 nM R1881 (New England Nuclear) for 24 h in DMEM with 2% charcoal-stripped fetal calf serum, 0.4 μg/ml aphidicolin, and 1 mM sodium butyrate, as described (15). Poly(A) RNA was isolated and used to make cDNA libraries (Life Technologies). Subtractive hybridization was carried out as described (18). Briefly, half of each library was ligated to one of two pairs of oligovectors and then amplified by PCR. A 30-fold excess photobiotinylated uninduced driver was hybridized with non-biotinylated induced tracer. Biotinylated cDNAs were removed with streptavidin. Each round of subtraction was followed by PCR amplification, with the resulting product used as the tracer for the next round of subtraction. Parallel subtraction was carried out using non-biotinylated uninduced tracer as a negative control. Oligovectors were removed from the enriched libraries after three and four rounds of subtraction, and the cDNAs were cloned into pGEM7Z. Differential screening of approximately 10,000 colonies was performed using labeled enriched libraries as probes. Clones that specifically hybridized with probe from androgen-induced libraries were further characterized by Northern blot and sequence analysis.

Rapid amplification of cDNA ends (RACE). Full-length cDNAs for mouse tra-2α and fox-1 homologue (fxh) were isolated from MN-1 cell cDNA using the Marathon cDNA amplification kit (Clontech). Primers for 5′ and 3′ RACE were generated from sequences obtained from the subtractive hybridization. RACE products were isolated and cloned into pCR4-TOPO (Invitrogen) prior to sequencing.

Expression analysis. Total RNA (RNeasy, Qiagen) or poly(A) RNA (FastTrack, Invitrogen) was isolated from MN-1 cells (clone AR5) following treatment with or without R1881, as described (15). R1881 concentration and duration of stimulation varied, and is indicated in figure legends. Total RNA from wild-type and testicular feminized (*tfm*) rat testis was isolated using RNeasy (Qiagen). Mouse multitissue Northern blots containing poly(A) RNA were obtained from Clontech. Equal loading of RNA on these blots was confirmed by probing for actin (data not shown). Domain-specific tra-2α probes were amplified by PCR from plasmids containing tra-2α RACE products, or from the original tra-2α fragment obtained from subtraction (RRM probe). The intron specific probe was generated using primers 5′-GTTCGTGAAGAAATTGAAGAG and 5′-TTCAAGTGCTTCTATCTGACC, and the 3′ RS domain probe using primers 5′-GATAGAGGATATGATCGAGG and 5′-ATCAATAGCGTCTTGGACTA. The 5′ RS domain probe was amplified using primers 5′-TACTCGGGTGTATAGGACCT and 5′-TCTCGATCAAGATCAAAGTCC. The fxh probe was amplified from human placental cDNA using primers 5′-GGTAACTCAGGGTAACCAGGAGC and 5′-ACCCGAATCCCTTAGAGCCACGT.

Western blot and immunofluorescence microscopy. Full-length cDNAs encoding human tra-2α and mouse fxh were cloned into pTracer-EF (Invitrogen) with an in-frame C-terminal V5 epitope tag. Expression of the transgene was driven by the EF-1α promoter, and expression of a GFP-tagged zeocin resistance gene was driven by the CMV promoter. For immunofluorescence microscopy, MN-1 cells (clone AR5) grown on Matrigel-coated chambered slides were transfected using Lipofectamine (Life Technologies). At 24 h posttransfection, the cells were placed in DMEM containing 10% charcoal and dextran-stripped fetal bovine serum (HyClone). At 48 h posttransfection, the cells were treated with 10 nM R1881 or vehicle control for 20 min, fixed with methanol, and stained with an anti-V5 antibody (Invitrogen) diluted 1:125. Staining was visualized using a Texas red conjugated secondary (Jackson ImmunoResearch) by deconvolution microscopy (Delta Vision). For Western blot, protein was obtained from HEK 293T cells 48 h posttransfection, and transgene expression was confirmed using an anti-V5 antibody.

RESULTS

To identify genes upregulated by androgen in motor neurons, we used a previously characterized *in vitro* model consisting of a motor neuron cell line stably expressing the androgen receptor (MN-1 cells, clone AR5) (15). In this model, the androgen receptor binds a non-metabolizable synthetic androgen (R1881) in nanomolar concentrations, translocates from the cytoplasm to the nucleus, and induces increased soma size and promotes cell survival in low serum. A PCR-based subtractive hybridization screen (18) was performed to identify genes with expression that is upregulated by androgen in these cells. Analysis of approximately 10,000 clones from the enriched libraries led to the identification of genes that are upregulated by stimulation with 10 nM R1881 for 24 h. Here we present characterization of two of these genes, both of which are mammalian homologues of invertebrate sex determination genes. Both genes encode RNA binding proteins, and as such, may exert broad effects on protein expression.

tra-2α Is an Androgen-Responsive Gene

tra-2α is the mouse orthologue of a *Drosophila* gene that plays an essential role in fly sex determination (3). Our subtractive screen yielded clones containing two fragments of the mouse *tra-2α* cDNA. These cDNA fragments were used as probes for Northern blotting to confirm androgen-responsiveness, and to determine the time course and extent of tra-2α mRNA induction (Fig. 1a). While flies have a single *tra-2* gene, mammals have two paralogues, designated *tra-2α* and *tra-2β*. The proteins encoded by the human *tra-2* genes function identically in cell free assays as enhancers of RNA splicing (19). We were therefore interested in determining whether both of these genes are androgen-responsive in our system. Tra-2β was not identified as an androgen-responsive transcript in our subtractive screen. Northern blot confirmed that expression of tra-2β mRNA does not change in response to androgen (Fig. 1b), suggesting that these genes may have dis-

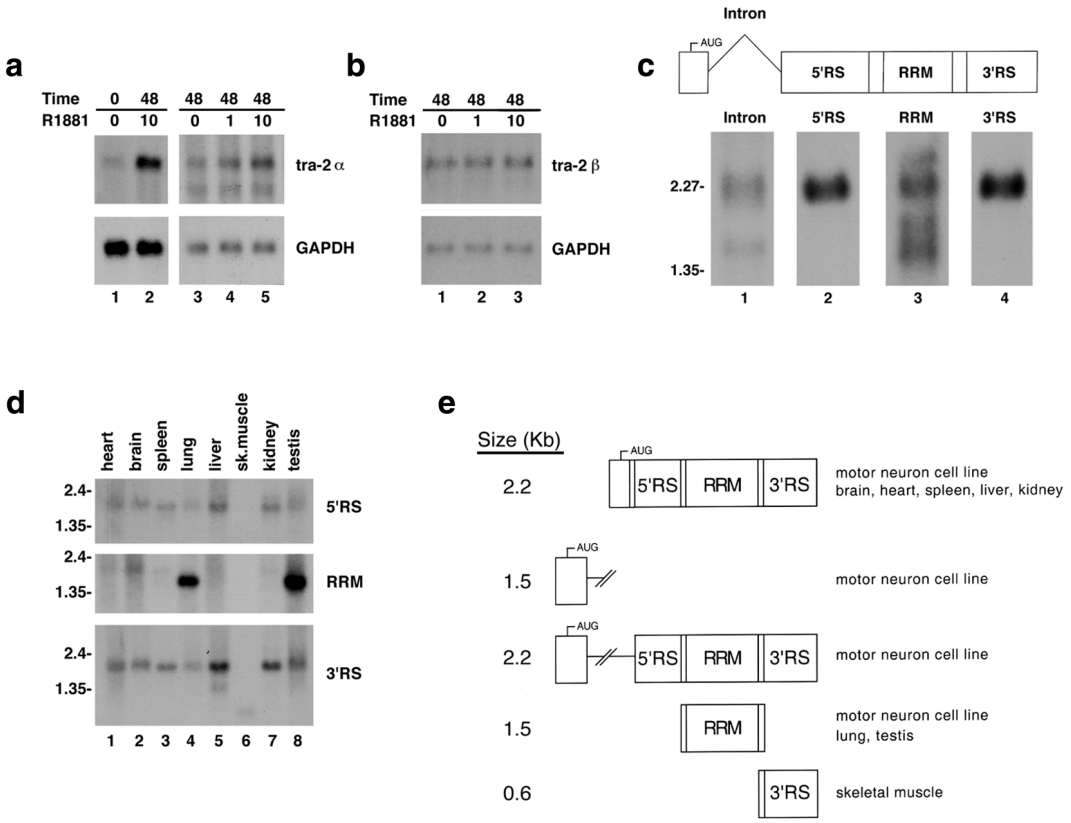

FIG. 1. *In vitro* and *in vivo* expression of *tra-2α*, an androgen-responsive gene. (a) Androgen-dependent induction of tra-2α mRNA. Northern blot of poly(A) RNA (2 μg/lane) from MN-1 clone AR5. Lane 1, time 0; Lane 2, 48 h stimulation with 10 nM R1881; Lane 3, 48 h unstimulated; Lanes 4 and 5, 48 h stimulation with 1 and 10 nM R1881, respectively. Lanes 1, 2, and 3–5 are from separate experiments. (b) Tra-2β mRNA expression in MN-1 clone AR5. Northern blot of total RNA (10 μg/lane) (Lane 1, 48 h unstimulated; Lanes 2 and 3, 48 h stimulation with 1 and 10 nM R1881, respectively). (c) Tra-2α mRNA expression in MN-1 clone AR5 assessed by domain-specific probes. Northern blot of poly(A) RNA (2 μg/lane) probed for a retained intron (lane 1), the 5′ and 3′ arginine and serine-rich domains (lanes 2 and 4), and the RNA recognition motif (lane 3). The translational start codon is indicated by AUG. (d) Tra-2α mRNA expression in tissues of adult male mice, assessed by domain-specific probes. (e) Summary of tra-2α mRNA splice variants detected in MN-1 cells and in mouse tissues. Approximate transcript sizes are indicated at the left. Splice variants are diagrammed in the middle, with open rectangles representing exons, and the horizontal line representing the retained intron. The double hatched diagonal line represents an in-frame stop codon. The tissue or cell line predominantly expressing each splice variant is indicated at the right.

tinct *in vivo* functions as reflected by their differential regulation.

The tra-2α protein contains a central RNA recognition motif (RRM) (20) flanked by two arginine-serine rich domains (RS domains) that mediate protein interaction and contribute to nuclear import (21, 22). The full-length mouse tra-2α cDNA was isolated by 5′ and 3′ RACE. We identified RACE products encoding the full-length protein, as well as truncated forms that lack the 5′ and 3′ RS domains. Similar alternate splicing of the human tra-2α transcript has been reported previously (23). We also detected transcripts with a retained intron downstream from the translational start site, near the 5′ end of the mRNA. This intron encodes 47 amino acids and then has an in-frame stop codon, so that intron-containing transcripts are unlikely to yield functional protein. Retention of an intron in tra-2 mRNA also occurs in *Drosophila* where it regulates protein expression (24), and it may play a similar role in MN-1 cells. To confirm that these splice variants are expressed in MN-1 cells, poly(A) RNA was analyzed by Northern blot with domain specific probes (Fig. 1c). The principal 2.2 kb transcript detected by these probes is the major androgen-responsive species, and encodes the full-length protein. A minority of the 2.2-kb transcripts has the retained intron. Also detected were less abundant, truncated transcripts encoding only the 5′ end of the mRNA including the retained intron, or the RNA recognition motif (summarized in Fig. 1e). Androgen-dependent induction of tra-2α mRNA and transcript alternate splicing were confirmed in two independent, androgen receptor-expressing MN-1 clones (data not shown).

Expression of tra-2α mRNA in tissues of adult male mice was assessed by multi-tissue Northern blot using domain specific probes (Fig. 1d). The tra-2α transcript undergoes tissue-specific alternate splicing. The most abundant mRNA species in the brain, as in MN-1 cells,

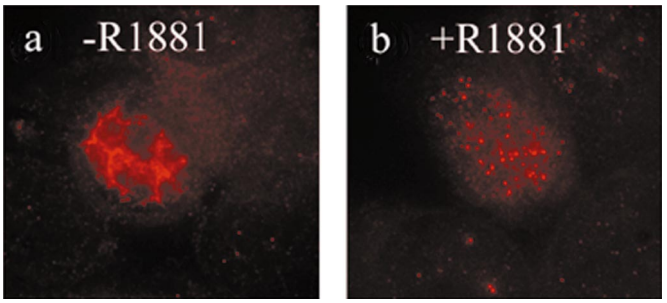

FIG. 2. Androgen-dependent redistribution of tra-2α protein. MN-1 clone AR5 was transfected with a tra-2α expression construct containing a C-terminal V5 epitope tag. Cells were grown in media containing charcoal and dextran-stripped serum for 24 h, then treated with 10 nM R1881 (b) or vehicle control (a) for 20 min. Protein distribution was visualized with an anti-V5 antibody by deconvolution microscopy.

encodes the full-length protein. Truncated species only recognized by the RRM domain probe were abundant in lung and testis (Fig. 1d, lanes 4 and 8), and a small transcript only detected by the 3′ RS domain probe was identified in skeletal muscle (Fig. 1d, lane 6). These alternatively spliced transcripts (summarized in Fig. 1e) may encode proteins of different function.

Androgen-Dependent Redistribution of tra-2α Protein

To determine whether androgens exert other effects on tra-2α protein in addition to influencing gene expression, the full-length protein was expressed as a fusion with a C-terminal V5 epitope tag. Androgen-responsive MN-1 cells were transfected with this construct, cultured in media containing serum stripped of steroids for 24 h, and then treated with 10 nM R1881 or vehicle control for 20 min. Localization of tra-2α protein was determined by immunofluorescence (Fig. 2) in transfected cells that co-expressed a GFP-antibiotic resistance fusion protein driven from a separate promoter (data not shown). We observed an androgen-dependent redistribution of tra-2α within the nucleus. Tra-2α was diffusely distributed in the nucleus (Fig. 2a) of the majority of cells treated with vehicle alone (69%). Androgen stimulation resulted in the intranuclear redistribution of protein to a speckled pattern (Fig. 2b) in the majority of cells (75%). This redistribution may reflect an overall change in the dynamic interaction of tra-2α other splicing factors, many of which are normally localized to nuclear speckles (25, 26).

fxh, the Mammalian Homologue of fox-1, Is Androgen-Responsive

Our subtractive screen led to the identification of a second androgen-responsive transcript that encodes a protein containing an RRM motif, the well-characterized RNA recognition domain that is also present in tra-2α. The predicted mouse and human proteins, the latter of which was identified by searching the database, are remarkably similar to the *C. elegans* protein fox-1 (Fig. 3), an RNA-binding protein involved in sex determination (28–30). The mouse and human genes encode predicted proteins of 377 amino acids. Over an 84 amino acid stretch that includes the RRM motif, the predicted mouse and *C. elegans* proteins show 75% identity and 86% similarity. Based on these sequence similarities, we have named this gene *fxh,* for fox-1 homologue. Mouse and human fxh are also strikingly similar to a protein that interacts with ataxin-2, the protein mutated in spinocerebellar ataxia type 2 (31). These mouse and human proteins may form a family of related RNA-binding proteins that are structurally similar to *C. elegans* fox-1.

Expression of fxh mRNA in MN-1 cells is induced by androgen stimulation (Fig. 4a). The fxh transcript is widely expressed in mouse tissues as three distinct forms of 7, 3, and 1.4 kb (Fig. 4b), which vary in relative abundance in a tissue-specific manner. The largest transcript predominates in MN-1 cells and in the brain of adult male mice. Although splice variants affecting the 5′ and 3′ untranslated regions were identified while cloning the full-length mouse cDNA, no changes within the coding region were detected. This is in contrast to human fxh, where ESTs in the database show alternate splicing near the 3′ end of the coding region, resulting in the deletion of 13 amino acids and a frameshift affecting the C-terminal residues (Fig. 3).

Mouse fxh was expressed as a fusion protein with a C-terminal V5 epitope tag. On Western blot, the protein is approximately 55 kDa, subtracting the mass of the epitope tag (Fig. 5a). When transfected into androgen-responsive MN-1 cells, the protein is found diffusely within the nucleus (Fig. 5b). No androgen-dependent effects on localization of fxh were observed (data not shown).

In Vivo Androgen Effects on Expression of tra-2α and fxh

The testicular feminized (*tfm*) rat provides an animal model in which to study the long-term, *in vivo* effects of androgens on development and gene expression. These animals contain a loss-of-function mutation in the androgen receptor gene (32), leading to feminized genetic males with undescended testes. The abundance of androgen-responsive cells in the testis prompted us to look there for androgen receptor-dependent effects on gene expression. Tra-2α and fxh mRNA expression was compared in testis from littermate wildtype and *tfm* mutant genetic males (Fig. 6). We observed a striking, androgen-dependent effect on mRNA expression, primarily manifested by a shift in the predominant fxh transcript and by the loss of the smaller tra-2α transcript. In contrast, no androgen-dependent effect on

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mfxh      MEKKKMVTQGNQEPTTTPDAMVQPFTTIPFPPPPPQNGIPTEYGVPHTQDYAGQTSEHNLT  60
hfxh      MEKKKMVTQGNQEPTTTPDAMVQPFTTIPFPPPPPQNGIPTEYGVPHTQDYAGQT EHNLT

mfxh      LYGSTQPHGEQSSNSPSNQNGSLTQTEGGAQTDGQQSQTQSSENSESKSTPKRLHVSNIP  120
hfxh      LYGSTQ HGEQSSNSPS QNGSLTQTEGGAQTDGQQSQTQSSENSESKSTPKRLHVSNIP
cefox-1                                                  PKRLHVSNIP

mfxh      FRFRDPDLRQMFGQFGKILDVEIIFNERGSKGFGFVTFENSADADRAREKLHGTVVEGRK  180
hfxh      FRFRDPDLRQMFGQFGKILDVEIIFNERGSKGFGFVTFENSADADRAREKLHGTVVEGRK
cefox-1   FRFRDPDL+ MF +FG + DVEIIFNERGSKGFGFVT E   DA+RAR++LHG+++EGRK

mfxh      IEVNNATARVMTNKKMVTPYANGWKLSPVVGAVYGPELYAASSFQADVSLGNEAAVPLSG  240
hfxh      IEVNNATARVMTNKKMVTPYANGWKLSPVVGAVYGPELYAASSFQADVSLGN+AAVPLSG
cefox-1   IEVN ATARV + K

mfxh      RGGINTYIPLIIPGFPYPTAATTAAAFRGAHLRGRGRTVYGAVRAVPPTAIPAYPGVVYQ  300
hfxh      RGGINTYIPLIIPGFPYPTAATTAAAFRGAHLRGRGRTVYGAVRAVPPTAIPAYPGVVYQ

mfxh      DGFYGADLYGGYAAYRYAQPATATAATAAAAAAAAYSDGYGRVYTADPYHALAPAASYGV  360
hfxh      DGFYGADLYGGYAAYRYAQPATATAATAAAAAAAATSDGYGRVVTADPYHALAPAASYGV

mfxh      GAVASLYRGGYSRFAPY 377
hfxh      GAVASLYRGGYSRFAPY
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FIG. 3. Comparison of mouse, human, and *C. elegans* fox-1 homologues. The predicted amino acid sequence of the mouse fxh (mfxh) protein, and comparison with human fxh (hfxh) and *C. elegans* fox-1. Identical amino acids are shown, and conservative changes are indicated by a + sign. Amino acids highlighted in bold indicate conserved residues that comprise the putative RNA recognition domain (RRM motif). Residues underlined in hfxh are deleted in some alternatively spliced clones, with a resulting frameshift affecting the C-terminal residues.

tra-2β mRNA expression was observed, similar to our results in cell culture. These effects of androgen on the fxh and tra-2α transcripts in the testis likely represent differences in mRNA processing, perhaps mediated by other androgen-responsive RNA-binding proteins and reflective of changes in cellular make-up.

DISCUSSION

We conducted a subtractive screen for androgen-responsive genes to identify pathways activated by androgens in motor neurons. We show androgen-inducible expression of two genes that encode proteins with RRM motifs, a well-characterized domain found in many RNA-binding proteins (20). Androgens upregulated the expression of *tra-2α*, an enhancer of RNA splicing, and promoted the redistribution of tra-2α protein within the nucleus. Androgens also upregulated the expression of a novel gene homologous to *C. elegans fox-1*. These data indicate that androgens exert their effects, in part, by modulating the expression and function of genes involved in RNA processing.

Androgen regulation of RNA-binding proteins may result in broad effects on protein expression. Steroid

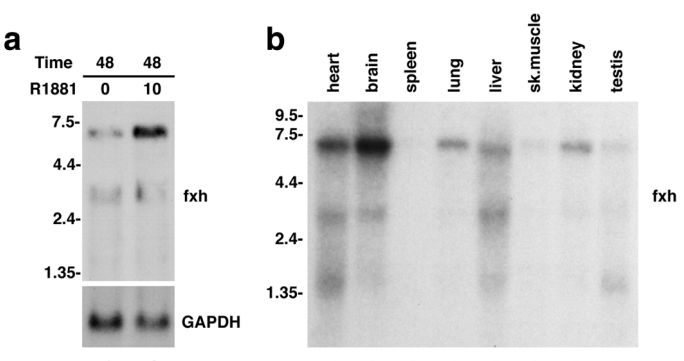

FIG. 4. Mouse fxh mRNA expression. (a) Induction of fxh mRNA by R1881. Northern blot of total RNA (10 μg/lane) from MN-1 clone AR5 (48 h unstimulated, lane 1; 48 h stimulation with 10 nM R1881, lane 2). (b) Fxh mRNA expression in tissues of adult male mice.

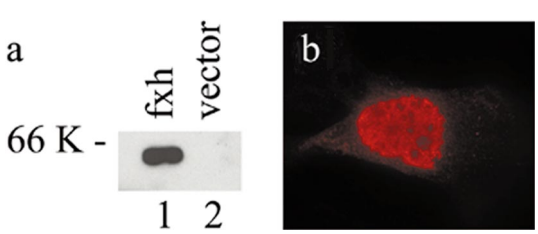

FIG. 5. Mouse fxh protein expression. (A) HEK 293T cells were transfected with a mouse fxh expression construct (lane 1) or empty vector (lane 2), and proteins were analyzed by Western blot using an antibody against a C-terminal V5 epitope tag. Molecular weight markers are indicated on the left. (B) MN-1 clone AR5 was transfected with a mouse fxh expression construct, and protein distribution was visualized with an anti-V5 antibody by deconvolution microscopy.

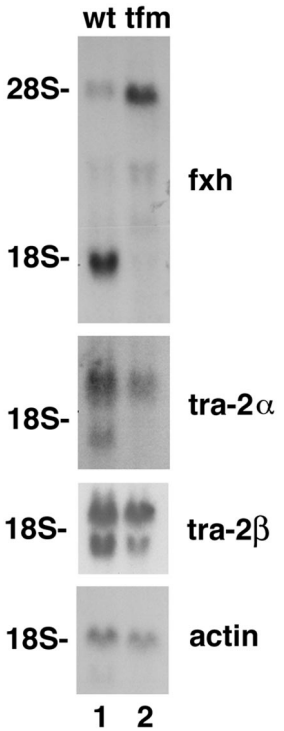

FIG. 6. Tra-2α, fxh, and tra-2β mRNA expression in testis of wildtype and *tfm* mutant male rats. Northern blot of total RNA (10 μg/lane) isolated from testis of littermate wildtype (lane 1) and tfm (lane 2) male rats.

hormones can effect alternate splicing of specific transcripts *in vivo* (32), though the mechanisms underlying this regulation are unknown. The extent to which alternate splicing contributes to genetic complexity in humans was highlighted by analysis of the working draft sequence of the genome. It is estimated that almost 50% of human genes encode transcripts that are regulated this way, many of which undergo multiple alternate splicing events (33). Tra-2α and β are enhancers of RNA splicing that function similarly by binding to exonic GAA repeats (19). These proteins, and their *Drosophila* homologue tra-2, are closely related to a family of RNA-binding proteins designated SR proteins. Members of this structurally defined group contain one or two N-terminal RRM motifs and a C-terminal RS domain (35). SR proteins are important constitutive components of the splicing machinery, and also influence alternate splicing in a concentration-dependent manner.

Many splicing factors are predominantly localized to subnuclear domains. SR proteins are highly mobile within the nucleus, and their distribution to regions of active transcription may be modified by phosphorylation of RS domains (25, 26). Phosphorylation of SR proteins can be mediated by the Clk family of kinases or by the SR protein kinases, which when overexpressed in cell culture release SR proteins from speckles (35–39). Phosphorylation status also affects the function of SR proteins as splicing enhancers (40–43). Similarly, both the intranuclear localization and function of *Drosophila* tra-2 are regulated by protein phosphorylation mediated by the LAMMER kinase *Darkener of apricot* (44). Here we have shown that androgens affect the intranuclear localization of tra-2α, and promote the redistribution of protein to a speckled pattern within the nucleus. Such intranuclear redistribution may have important functional consequences and may reflect alterations in phosphorylation status of the protein, although this remains to be proven experimentally.

That *tra-2*α and *fxh* are androgen-responsive genes in mammals is intriguing given the function of the invertebrate homologues. In *Drosophila*, functional knock-out of tra-2 causes genetic females to develop into sterile males (45), the opposite of the effect of loss of androgen receptor function in mammals. Replacement with human tra-2α results in a partial rescue of this phenotype (23), demonstrating striking functional conservation. In addition to its role during *Drosophila* development, tra-2 also influences sexual orientation and courtship behavior in adult animals (46–48). In *C. elegans*, fox-1 plays a similar critical role in sex determination by posttranscriptionally regulating the expression of *xol-1* (28). A downstream component of the *C. elegans* sex determination pathway, *tra-1*, encodes a protein that represses transcription of *egl-1*, part of the somatic cell death pathway (49), thereby regulating survival of sexually dimorphic neurons in this organism. In mammals, androgens influence the survival of certain neuronal populations during development, including sexually dimorphic spinal motor neurons, and exert important trophic and behavioral effects in adults. Our findings suggest that the differential regulation of target genes mediated by the RNA-binding proteins tra-2α and fxh may contribute to these androgen effects. As such, tra-2α and fxh may well play a conserved role in the expression of sexually dimorphic traits.

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