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Genetics

The whn transcription factor encoded by the *nude* locus contains an evolutionarily conserved and functionally indispensable activation domain

(hairlessness/congenital athymia/T-cell development/rat/*Fugu rubripes*)

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ABSTRACT **Mutations in the *whn* gene are associated with the phenotype of congenital athymia and hairlessness in mouse and rat. The *whn* gene encodes a presumptive transcription factor with a DNA binding domain of the forkhead/winged-helix class. Two previously described null alleles encode truncated whn proteins lacking the characteristic DNA binding domain. In the rat *rnu* allele described here, a nonsense mutation in exon 8 of the *whn* gene was identified. The truncated whn^{rnu} protein contains the DNA binding domain but lacks the 175 C-terminal amino acids of the wild-type protein. To facilitate the identification of functionally important regions in this region, a *whn* homolog from the pufferfish *Fugu rubripes* was isolated. Comparison of derived protein sequences with the mouse *whn* gene revealed the presence of a conserved acidic protein domain in the C terminus, in addition to the highly conserved DNA binding domain. Using fusions with a heterologous DNA binding domain, a strong transcriptional activation domain was localized to the C-terminal cluster of acidic amino acids. As the whn^{rnu} mutant protein lacks this domain, our results indicate that a transactivation function is essential for the activity of the whn transcription factor.**

The forkhead/winged-helix class of transcription factors (1, 2) is characterized by a conserved DNA binding domain, first recognized in the homeotic *Drosophila* gene *forkhead* (3, 4). The three-dimensional structure of this DNA binding domain complexed with DNA revealed a bundle of three α-helices attached to a three-stranded anti-parallel β-sheet (5) that is structurally related to other DNA-binding proteins, such as linker histones (6). Members of this class of transcriptional regulators have been implicated in the regulation of gene expression and in developmental decision making in various metazoan organisms (7–18). In two instances, forkhead/winged-helix proteins have been linked to tumorigenesis (19, 20). Whereas little overall protein homology has been found among the many members of this class of proteins, the primary sequences of the characteristic ≈100 amino acid DNA binding domains are highly conserved. Transcriptional activation domains (21) that have been identified in forkhead/winged-helix proteins vary considerably in amino acid composition, but include motifs known from other transcriptional activators (2).

Loss-of-function mutations in the mouse *whn* (for *w*inged-*h*elix *n*ude) gene, which also encodes a forkhead/winged-helix transcription factor, are associated with the *nude* phenotype of hairlessness and congenital athymia (22, 23). The *whn* gene was isolated by positional cloning and verified as the *nude* gene by the identification of deleterious mutations on the original *nude* alleles in mouse and rat (16), and by use of a targeted disruption of *whn* (24). Here, we describe the molecular analysis of a third *nude* allele, *rnu* (25), that encodes a truncated whn protein containing the forkhead/winged-helix DNA binding domain but lacking the C-terminal quarter of the wild-type protein. The characterization of this mutant led to the identification of a functionally indispensable and evolutionarily conserved transcriptional activation domain in the whn protein.

MATERIALS AND METHODS

***rnu* Rats.** Rats carrying the *rnu* mutation originating from the Institut für Versuchstierkunde (Hannover, Germany) were provided by Dr. H. Hedrich (Medizinische Hochschule, Hannover). The coding exons of the rat *whn* gene were amplified from genomic DNAs using primers derived from rat cDNA sequences (data not shown) and sequenced directly on both strands with the Prism Dye Deoxy Terminator Cycle Sequencing kit (Applied Biosystems) using an automatic DNA sequencer (Applied Biosystems 373A). To amplify all of exon 8, the following primers were used: 5′-GGAAAGACCCCATTGCTGTG (derived from exon 7 sequences) and 5′-GCTGAGGTGCATGTCTCCCA (derived from exon 9 sequences).

Isolation of *whn* Homologs. DNA was extracted from brain tissue of *Fugu rubripes* (kindly provided by T. Kagawa, National Institute for Physiological Sciences, Mdaiji, Okazaki, Japan), partially digested with *Sau*3AI, and cloned into the *Bam*HI site of λPS (26). The resulting library (M.S., Michael Nehls, and T.B., unpublished work) was screened at low stringency (hybridization and washes in 6× SSC and 0.1% SDS at 65°C) with a radiolabeled mouse *whn* cDNA (16) probe. The resulting clones belonged to two different groups, as judged by restriction enzyme analysis. One representative recombinant from each group was sequenced.

***GAL4* Fusion Genes.** Various parts of the whn protein were fused to the DNA binding domain of *GAL4* present in pAS2-1 (27) (for transfection into yeast) and pM (for transfection into mammalian cells) (28); all constructs were verified by DNA sequencing. Wild-type fragments were generated by using appropriate restriction fragments or by using PCR amplification of desired regions; mutant versions were generated by cloning of annealed synthetic oligonucleotides. pM-derived constructs were cotransfected by calcium phosphate precipitation into BHK cells with a control plasmid directing the expression of β-galactosidase (β-gal) (to correct for transfection efficiencies) and a reporter plasmid, pG5EC, in which the *CAT* gene is under the control of multimerized *GAL4* UAS

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Abbreviation: β-gal, β-galactosidase.

Data deposition: The sequences reported in this paper have been deposited in the GenBank data base (accession nos. X97020 and X97021).

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sequences (28). Forty hours after transfection, the cells were harvested, resuspended in 250 mM Tris·HCl (pH 7.5), and cytoplasmic extracts were prepared by three freeze-thaw cycles. Aliquots of these extracts were used for determination of β-gal activity (29) and the presence of CAT protein using an ELISA assay (Boehringer Mannheim). The results of the latter were normalized for β-gal activity and expressed relative to the activity of a *GAL4–VP16* fusion gene (28). Stability of GAL4 fusion proteins was determined by use of a polyclonal antibody (Upstate Biotechnology) directed against the GAL4 DNA binding domain. No significant differences in fusion protein levels were detected (data not shown). pAS2-1-derived constructs were transfected into strain Y190 (27) and assayed for β-gal activity as described (30).

RESULTS

A Nonsense Mutation in Exon 8 of the Rat *whn* Gene on the *rnu* Allele. Molecular analyses of the mouse *nu* and the rat *rnuN* alleles have shown that the transcripts emanating from these null alleles do not contain the characteristic DNA binding domain of the whn transcription factor (16). To determine whether a similar type of mutation could be found in a second rat nude allele, *rnu*, the structure of this *whn* allele was investigated. Filter hybridization analysis of *whn* transcripts in RNA isolated from rat skin homozygous for the *rnu* allele indicated normal levels of transcription and no apparent alteration in size (data not shown). However, sequence analysis of individual exons of the rat *whn* gene in *rnu* rats revealed a nonsense mutation in exon 8, resulting in a truncated protein of 473 amino acids, instead of 648 amino acids of the wild-type protein (Fig. 1). Similar results have been described by Segré *et al.* (31) by sequence analysis of *whn* cDNAs from *rnu* rats. This result suggested that a functionally important domain is encoded in the 175 C-terminal amino acids of the whn protein.

Isolation of a Pufferfish Homolog of *whn*. Since about 98% of amino acids are identical between mouse and rat *whn* (16, 31), no functionally important residues can be deduced by sequence comparison between these two closely related species. To facilitate the identification of functionally relevant domains, we sought to isolate a fish homolog of *whn*. To this end, an *F. rubripes* (pufferfish) genomic library was screened by low-stringency hybridization using a mouse *whn* cDNA probe. Several genomic clones were isolated and compared by sequence analysis. These experiments revealed readily detectable polymorphisms in presumptive introns, but complete sequence identity in putative exons (Fig. 2). This result strongly suggested that the fugu genome only contains a single homolog of *whn*, albeit in different allelic variants. The genomic sequence depicted in Fig. 2 spans exons 5 to 9 of the fugu *whn*

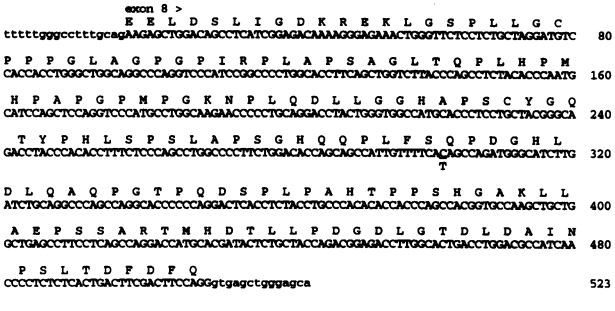

FIG. 1. Nucleotide sequence of exon 8 of the rat *whn* gene. Intron sequences are shown in lowercase type, the conceptual translation of exon sequences is given in single letter code. The location and phase of rat exon 8 is similar to that of mouse exon 8 (16). The C → T mutation on the *rnu* allele is indicated; it introduces a stop codon. Our sequence differs from that of Segré *et al.* (31) in one position (nucleotide 82), where we find an adenine instead of a cytosine residue.

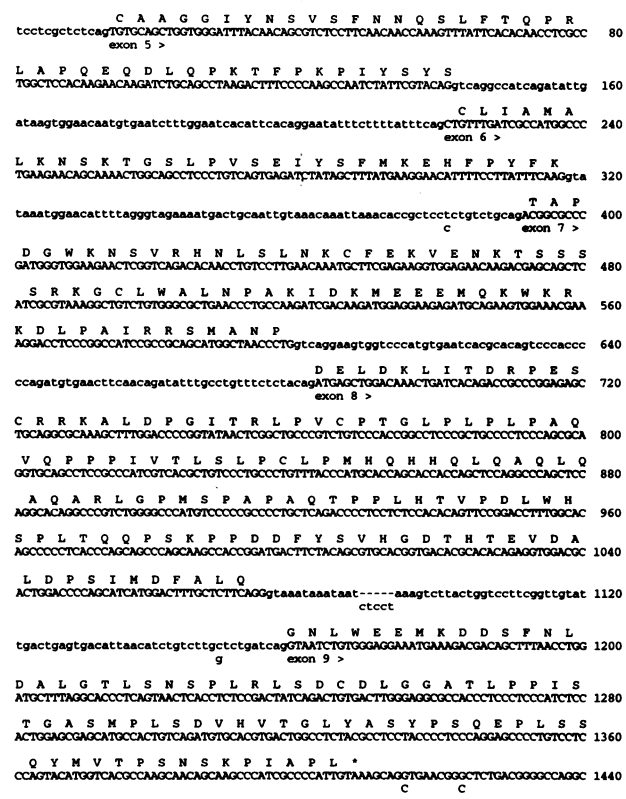

FIG. 2. Nucleotide sequence of part of the *whn* homolog from *F. rubripes*. The locations of intron/exon boundaries have been deduced from splice site consensus sequences and the mouse *whn* gene structure (16). Conceptual translation of exon sequences is given in single letter code. Nucleotide polymorphisms detected in introns (lowercase letters) or 3′ untranslated regions (uppercase letters) are indicated.

gene. As expected (32), the fugu gene has very small introns, ranging from 74 to only 83 bp. A comparison of derived protein sequences (Fig. 3) reveals that overall 49% of amino acids are identical between mouse and fugu. About 90% of all residues are identical in the presumptive DNA binding domain. In addition, a second stretch of high homology (49%) is observed at the end of exon 8 and the beginning of exon 9; this region is located C terminal to the site of the nonsense mutation in the whn^{rnu} allele and characterized by a high contents of acidic amino acids residues, often found in transcriptional activation domains (21).

Identification of a Transcriptional Activation Domain in the whn Protein. To explore the possibility that the loss of activity of the whn^{rnu} protein is due to the absence of a transcriptional activation domain, fusion genes appending various parts of the *whn* coding sequence to the GAL4-DNA binding domain were constructed. Initially, the entire N- and C-terminal domains of the whn protein were individually tested for such activity in yeast and primate cells. The results summarized in Fig. 4 indicate that a protein encompassing aa 1–285 is devoid of such activity, whereas the region located C terminal of the whn DNA binding domain (aa 347–648) contains a strong activation domain. A full-length *whn* cDNA appended to the GAL4 DNA binding domain also activated transcription of the reporter gene, albeit at lower levels than the C-terminal fragment alone (data not shown).

The analysis of deletion mutants of the C terminus indicated that activity in the transactivation assay is eliminated in constructs that do not contain the region C terminal of aa 511. However, full activity is retained in constructs devoid of the very C terminus of the whn protein (aa 563 to aa 648). This suggests that the region between aa 511 and aa 563 is necessary for transactivation (highlighted in Fig. 3). This conclusion is

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Exon 5 >                                                    Exon 6 >
m  234 YSPGGGSY-PVPYLGSPHYPYQRIAPQANAEGHQPLFPKPIYSYS   278 ILIFMALKNSKTGSLPVSEIYNFMTEHFPYKK
f       CAA..-I.NSVSFNNQSLFTQP.L...-EQDLQPKT.........      C..A.................S..K.....F.

Exon 7 >
m  310 TAPDGWKNSVRHNLSLNKCFEKVENKSGSSSRKGCLWALNPSKIDKMQEELQKWKRKDPIAVRKSMAKP
f       ..........................TS.............A.....E..M.......LP.I.R...N.

Exon 8 >
m  358 EELDSLIGDKREKLGSPLLGCPPPGLAGPGPIRPMAPSAGLSQPLHPMHPAPGPMPGKNPLQDLLGGHAPSCYGQTYPHLSP
f       D...K..T.RP.-------S.RRKA.-D..IT.LPVCPT..PL..-.AQVQ.P.I----------------------VT..L

                  *
m  SLAPSGHQQPLFPQPDGHLFLQAQPGTPQDSPLPAHTPPSHGAKLMAEPSSARTMHDTLLPDGDLGTDLDAINPSLTDFDFQ
f  PCL.MHQHHQ.QA.LQAQAR.GPMSPA.AQT.-.L..V.DLWHSPLTQQP.-KPPD.FYSVH..TH.EV..LD..IM..AL.


Exon 9 >
m  543 GNLWEQLKDDSLALDPLVLVTSSPTSSSMLPPPPAAHCFPPGPCLAETGNEAGELAPPGSGGSGALGDMHLSTLY
f       .....EM....FN..A.GTLSN..------------------LR.SDCDLGGAT.P.IST.A.MP.S.V.VTG..

        SAFVELESTPSSAAAGPAVYLSPGSKPLALA
        ASYPSQ.PLS.QYMVT.S-----N...I.PL
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FIG. 3. Comparison of derived whn protein sequences between mouse (m) and fugu (f). The DNA binding (contained within exons 5–7) and activation (contained within exon 8 and 9) domains are highlighted in boldface letters. Dashes have been introduced in alignments of exons 8 and 9 to maximize homology. Dots indicate identical residues. *, The site of the nonsense mutations in the *whn*^{rnu} allele.

directly supported by a fusion protein appending a peptide corresponding to aa 509 and 562 to the GAL4-DNA binding domain. Since the activity of this small domain is identical to that of the entire C terminus, it likely represents an independent protein module.

Conserved Activation Domain. To determine whether the fugu *whn* homolog also contains a transcriptional activation domain, fusion genes between the GAL4 DNA binding domain and various genomic fragments of the fugu gene were constructed. As shown in Fig. 4*B*, constructs containing exons 7 to 9, 8 and 9, and 9 were all active in this assay. The construct containing only exon 9, however, displayed slightly lower activity than the two larger ones. This finding suggests that an activation domain is localized within the C-terminal domain of the fugu whn protein, consisting of sequences from exons 8 and 9. This activity coincides with a stretch of significant sequence homology between mouse and fugu; within a stretch of about 42 amino acids, more than a quarter of all residues are acidic in both species (Fig. 3).

Site-directed mutagenesis was used to exchange aspartic acid residues in exon 8 of the mouse *whn* gene with alanine (Fig. 5). Whereas single amino acid exchanges in exon 8 have a moderate effect, three replacements cause a more drastic reduction. Therefore, as with other acidic domains, the number of acidic residues appears to correlate with the strength of the transactivation function (33).

DISCUSSION

A Molecular Explanation for the whn$^{rnu/rnu}$ Phenotype. Our results show that the whn protein, like other members of the forkhead/winged-helix class of transcription factors (34), contains an acidic transcriptional activation domain, located in the C terminus of the whn protein. This observation is particularly instructive in light of the known loss-of-function mutations in the *whn* gene, which are associated with the phenotype of congenital athymia and hairlessness (16, 31). A frame-shift mutation occurs in exon 3 on the mouse *nude* allele (16), whereas aberrant splicing creates *whn* mRNAs lacking exons 5 to 9 on the rat *rnuN* allele (16). The original molecular interpretation for these null alleles was the absence of the DNA binding domain (encoded in exons 5 to 7) in the derived protein sequences. The data presented here clearly indicate that besides the presumptive DNA binding domain, an acidic activation domain (overlapping the junction between exons 8 and 9) is also missing from these mutant proteins.

A second *nude* allele from the rat, *rnu*, carries a nonsense mutation in the middle of exon 8 (Fig. 1). The truncated whn^{rnu} protein therefore lacks the activation domain, although it contains the characteristic DNA binding domain. Nevertheless, the phenotypic consequences of this mutation are indistinguishable from those seen in the two other mutants (25),

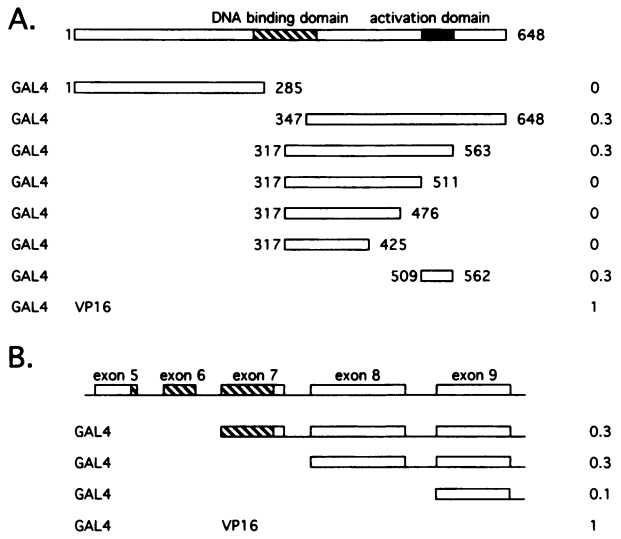

FIG. 4. Identification of a transcriptional activation domain in mouse (*A*) and fugu (*B*) *whn* genes. (*A*) Schematic of the mouse whn protein is shown in the top line. Fragments fused to the GAL4 DNA binding domain are indicated schematically below and are characterized by the relevant first and last amino acid residues of the whn protein sequence. The activity in a transcriptional activation assay after transfection into BHK cells relative to a GAL4-VP16 fusion is shown in the right column. These values are the means from four transfection experiments; the standard deviations were less than 20% of the mean values. Constructs GAL4 (whn 1–285) and GAL4 (347–648) were also tested in yeast strain Y190; only the latter construct activated the β-gal reporter gene (data not shown). (*B*) Schematic of the *F. rubripes whn* gene structure is shown in the top line. Fusion constructs with GAL4 are indicated below. Note that constructs containing exons 7–9 and exons 8 and 9 incorporated intronic sequences. Activity relative to a GAL4-VP16 construct is shown in the right column.

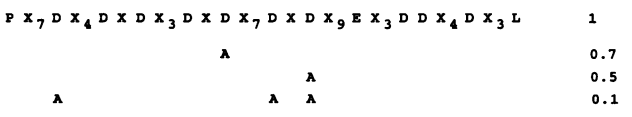

FIG. 5. Acidic amino acid residues are critical for the mouse whn transcriptional activation domain. *GAL4* fusion genes were prepared with wild-type and mutated versions of the mouse whn transcriptional activation domain (aa 509–562); the relevant replacements are indicated. The numbers in the right-hand column represent the relative strength in the transcriptional activation assay calculated from four transfection experiments; standard deviations are less than 20% of the mean values.

demonstrating that the transactivation domain is indispensable for whn function.

The identification of the C-terminal activation domain therefore provides a plausible molecular explanation for all three known loss-of-function alleles of *whn* genes. In light of these findings and a previous observation that a homeodomain-deleted *Drosophila* fushi tarazu protein still functions *in vivo* (35), it will be of interest to determine the *in vivo* properties of a whn protein without the DNA binding domain.

An Ortholog of whn in a Lower Vertebrate. The phenotype of the loss-of-function mutations in rodent *whn* genes suggests that the *whn* gene is involved in the differentiation of thymic epithelium (24, 36) and the keratinization of hair shafts (37). Because of *whn* involvement in two seemingly unrelated differentiation programs, we sought to determine whether a *whn* homolog existed in a lower vertebrate that develops a thymus but lacks hair. Since the thymus gland is already well developed in bony fish (38), a genomic library of pufferfish was screened for genes homologous to mouse *whn*. Pufferfish has a very compact genome that should greatly facilitate genomic sequencing efforts (32), an expectation that was borne out by the results presented here.

Although the sequence of the *whn* homolog isolated from the pufferfish genome is not complete, several features suggest that it represents an ortholog of the mouse *whn* gene. First, there is a remarkably high degree of identity in the derived protein sequences (overall identity of 49%), which is particularly striking in the DNA binding domain (90% identity) and extends to critical residues in the transcriptional activation domain. Second, the residues implicated in determining the sequence specificity of DNA binding in forkhead/winged-helix domains in two independent studies (39, 40) are identical between mouse and fugu genes, although a possible similarity of binding sites requires experimental verification. Third, the locations of all five introns covered in the sequenced fragment are identical to the mouse *whn* gene with respect both to phase and location within protein domains, although exons 8 and 9 are significantly shorter in fugu. Fourth, there is no evidence for a second gene similar to mouse *whn* in the fugu genome.

The loss of whn activity cannot be compensated for in mice and invariably affects both thymus and hair development. The identification of a *whn* homolog in pufferfish therefore suggests that the involvement in the process of hair keratinization represents a new function for the *whn* gene in mammals.

It will be of interest to investigate the possible presence of *whn* homologs in agnathan fish, such as lamprey, which do not possess a recognizable thymus gland (38). Such studies could help to trace the phylogenetic origin of the thymus in molecular terms.

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