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Conservation of Death Receptor-6 in Avian and Piscine Vertebrates

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One of the most recently identified members of the tumor necrosis factor receptor family, death receptor-6 (DR6), has been shown to mediate apoptosis following overexpression in HeLa cells. The avian and piscine orthologs of DR6 have now been identified, and the deduced amino acid sequence for each demonstrates a high level of conservation compared to the mammalian sequence. Expression of dr6 mRNA occurs widely across tissues of both the mature chicken and brook trout. It is now well-established that ovarian follicular atresia occurs via apoptosis originating within the granulosa cell layer. Accordingly, DR6 expression within the ovary was examined to assess the relationship between stage of follicle development and relative levels of this death receptor. Of particular interest was the finding that elevated levels of dr6 mRNA, as well as the translated protein, are expressed in atretic compared to healthy follicles of the hen ovary, thus providing the first association between DR6 expression and apoptosis, *in vivo.* We conclude that DR6 is a highly conserved and widely expressed death-domain-containing receptor and may be implicated in regulating follicle atresia within the vertebrate ovary. © 2001 Academic Press

Key Words: **death receptor; chicken; trout; ovary; TNFR family.**

Death receptor-6 (DR6) is a recently identified member of the tumor necrosis factor receptor (TNFR) family, and belongs to the subgroup of death domain-containing receptors (1). These receptors, including Fas (CD95/APO-1), TNFR1, DR3 (TRAMP/APO-3/WSL/LARD), DR4 (TRAIL-R1/APO-2), DR5 (TRAIL-R2), and DR6, are characterized by the presence of varying numbers of extracellular, cysteine-rich motifs that comprise the ligand binding domain, a transmembrane domain, and a cytoplasmic death domain. When activated by their respective ligands, each receptor appears to be capable of either initiating signaling pathways leading to activation of the caspase cascade and cell death, or activating the nuclear factor-kappaB (NF-κB) and/or Jun N-terminal kinase (JNK) pathways which have been linked to an immune or stress response.

Ovarian follicle atresia in a wide variety of vertebrate species is mediated via programmed cell death (apoptosis) initiated within the granulosa cell layer, and may eventually account for the loss of up to 99% of germ cells originally present in the ovary at or around the time of birth/hatch (2, 3). Moreover, apoptosis has recently been reported to occur during normal seasonal growth and postovulatory regression in the teleost ovary (4, 5). While growth factors (e.g., insulin-like growth factor-I, epidermal growth factor, transforming growth factor-α) and gonadotropins are known to play a role in the negative regulation of this pathway within the ovary (2, 6–9), receptor-mediated signaling events leading to induction of cell death are largely unknown. Tumor necrosis factor (TNF) family members, TNFα and Fas ligand (10) can initiate cell death in granulosa cells from mammalian ovarian follicles, yet often require the presence of protein synthesis inhibitors (e.g., cycloheximide) which reportedly block the production of potential death suppressing proteins (7, 11, 12). Both Fas and TNFR1 have been characterized from the chicken, and transcript levels have been determined to be regulated by different, but overlapping, signaling pathways in hen granulosa cells (13). In brook trout, the recent identification of a TNF family decoy receptor (TDcR) in the ovary (14) led to the search for related death-inducing receptors, and resulted in the identification of one death receptor [ovarian TNF receptor (OTR)] plus two TNF ligands [TNF-related apoptosis inducing ligand (TRAIL), and lyphotoxin-alpha] (15).

As a result of the continuing effort to identify endogenous mechanisms responsible for initiating cell death in the ovary, orthologs for DR6 were identified in both avian and piscine species. We have also described the

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distribution of DR6 expression in the hen and brook trout ovary, and provide evidence for a putative role in the regulation of ovarian follicle atresia.

MATERIALS AND METHODS

Animals and tissue collection. Single-comb white Leghorn hens (H & H Poultry, Portland, IN), 25–35 weeks of age and laying regular sequences of at least 5–6 eggs, were used in all studies described. Birds were housed individually in laying batteries, provided with free access to feed (Purina Layeena Mash, Purina Mills, St. Louis, MO) and water, and were exposed to a photoperiod of 15 h light, 9 h darkness, with lights on at midnight. Individual laying cycles were monitored by the daily timing of oviposition. Hens were killed approximately 16–18 h prior to a midsequence ovulation by cervical dislocation.

Tissues were collected from the chicken ovarian stroma, postovulatory follicle, oviduct, spleen, adrenal, bone marrow, and testes for isolation of RNA and protein. Ovarian follicle tissues included granulosa and theca from 3- to 5-mm-diameter and 6- to 8-mm-diameter (prehierarchal) follicles, and the largest (F1) and second largest (F2) preovulatory follicle. Morphologically normal and atretic follicles (3–8 mm) were processed without separating the granulosa and theca layers, as previously described (16). Samples were collected and processed immediately or frozen at −70°C.

Mature brook trout (*Salvelinus fontinalis;* 300–400 g) were purchased during the reproductive season from a commercial hatchery in Grand Haven, Michigan, and held under natural photoperiods in 1100-liter tanks supplied with flow-through well water at 12°C. Prior to collection of tissue, the reproductive stage of individual trout was determined by sampling follicles, *in vivo,* as previously described (17). Trout were overanesthetized in 2-phenoxyethanol and decapitated. Tissues were collected for mRNA and protein, and frozen immediately in liquid nitrogen.

All procedures described within were reviewed and approved by the University of Notre Dame Institutional Animal Care and Use Committee and were performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals.

Isolation and characterization of chicken and trout dr6 cDNAs. Screening of the GenBank EST database led to the initial recognition of chicken (Accession No. AI980074) and zebrafish (Accession No. AW153974) sequences with similarity to the human DR6 gene. A partial chicken dr6 cDNA was initially cloned using homologous primers DR6-F1 5′-GAT GGA GGA CAC CAC GCC-3′ and DR6-B1 5′-TCG GGG TTG AGG ATG TGC-3′ to obtain the expected 384-bp product from a poly(A)$^+$ mRNA template produced from prehierarchal and preovulatory follicle granulosa cells. Further cloning of the dr6 sequence encompassing the entire coding region required using rapid amplification of cDNA ends (RACE) PCR in the 5′ and 3′ directions (18). Brook trout dr6 cDNA was amplified using the degenerate primers, DR6-1 5′-TGY GAY AAR TGY CCI GCI GG-3′ and DR6-2 5′-CCR CAI ACR TTR TCI GTY TY-3′, to produce a 435-bp fragment.

PCR products were cloned into TA cloning vector pCR2.1 and transformed into INVαF′ cells (Invitrogen, Carlsbad, CA). Plasmids were sequenced using simultaneous bidirectional sequencing (SBS) reactions with the DYEnamic cycle sequencing kit (US79535) from Amersham Pharmacia Biotech (Baie d'Urde, Quebec, Canada) and IRD700- and IRD800-labeled vector or internal primers (LiCor, Lincoln, NB). The sequencing reactions were separated on a LiCor 4200L sequencer (LiCor) and analyzed with the Sequencher 4.0 (Gene Codes Corp., Ann Arbor, MI). Sequence analysis and alignments were performed using MacVector 6.5 software (Oxford Molecular Ltd., Genetics Computer Group, Madison, WI).

Northern blot analysis of dr6 in chicken and trout. Levels of dr6 mRNA were evaluated using Northern blot analysis of total RNA isolated from chicken tissues using Trizol Reagent (Gibco-BRL), and poly(A)$^+$ mRNA from trout (PolyAtract mRNA Isolation System, Promega, Madison, WI). Blots were probed with the 384- and 435-bp fragments for the chicken and trout dr6, respectively. Chicken specific probes were random-prime labeled using Megaprime DNA labeling system (Amersham Pharmacia Biotech, Piscataway, NJ) and [^{32}P]dCTP (3000 Ci/mmol; Amersham). Hybridizations were carried out at 60°C overnight followed by stringent washing conditions (13). Brook trout Northern blots were hybridized as previously described (19). Images were captured using the Storm 840 PhosphorImager system equipped with the ImageQuant data reduction system (Molecular Dynamics, Sunnyvale, CA). When indicated, blots were subsequently reprobed with a probe against 18S ribosomal RNA to standardize for loading of RNA.

Western blot analysis of DR6 Proteins were prepared and resolved by SDS–polyacrylamide gel electrophoresis, then transferred to nitrocellulose membrane (Micron Separations, Inc., Westborough, MA) as previously described (6). Membranes were blocked for 1 h at 20°C in Western blocking solution [5% nonfat dry milk TBST (10 mM Tris, pH 8.0; 100 mM sodium chloride; 0.1% Tween 20)], incubated at 4°C overnight in anti-DR6 antibody (Upstate Biotechnology, Lake Placid, NY) diluted 1:500 in blocking solution. Blots were subsequently washed for 5 min three times at 20°C in blocking solution, were incubated for 1 h at 20°C in goat anti-rabbit IgG conjugated to horseradish peroxidase (Pierce Chemical Co., Rockford, IL) diluted 1:10,000 in blocking solution, and were finally washed for 10 min three times in TBST. Antibody binding was visualized with enhanced chemiluminescence Western blotting detection reagent (Amersham Pharmacia Biotech, Piscataway, NJ). Prior to quantitation, the loading of protein was routinely analyzed by reblotting for α-tubulin (20). Data were quantitated by densitometry using an UltraScan XL laser densitometer (Pharmacia LKB, Piscataway, NJ). The use of the anti-human DR6 antibody for Western analysis of chicken protein was initially justified by the detection of comparable cross-reactive signals among chicken, human and mouse proteins.

Data analysis. For purposes of quantitation, replicate data within an experiment were expressed as fold-difference relative to a common reference sample. The data were subsequently compared by one-way ANOVA without including the reference sample (standardized to 1.0) (Statview; Abacus Concepts, Inc., Berkley, CA).

RESULTS AND DISCUSSION

Chicken and brook trout orthologs of the TNFR family member, DR6, have been identified, and each demonstrates a high degree of similarity to the mammalian counterpart. Compared to the human DR6, the deduced amino acid sequence from the full-length chicken DR6 is 69% similar and 76% identical, while the partial sequence from the trout is 60% similar and 77% identical (Fig. 1). Although the predicted initiation codon (surrounded by the consensus Kozak sequence, ACCATGG) of the chicken dr6 nucleotide sequence does not appear at a site identical to the human and mouse sequence, there exists a putative signal peptide ending at a comparable position [as verified using the program by Nielsen *et al.* (21)]. In addition, each of the deduced amino acid sequences from the four species shows conservation for all cysteine residues throughout the extracellular cysteine-rich, putative ligand binding, domain. Although the partial cDNA from the brook trout does not extend through the region containing the death domain, the deduced death domain from the chicken, human, and mouse species was

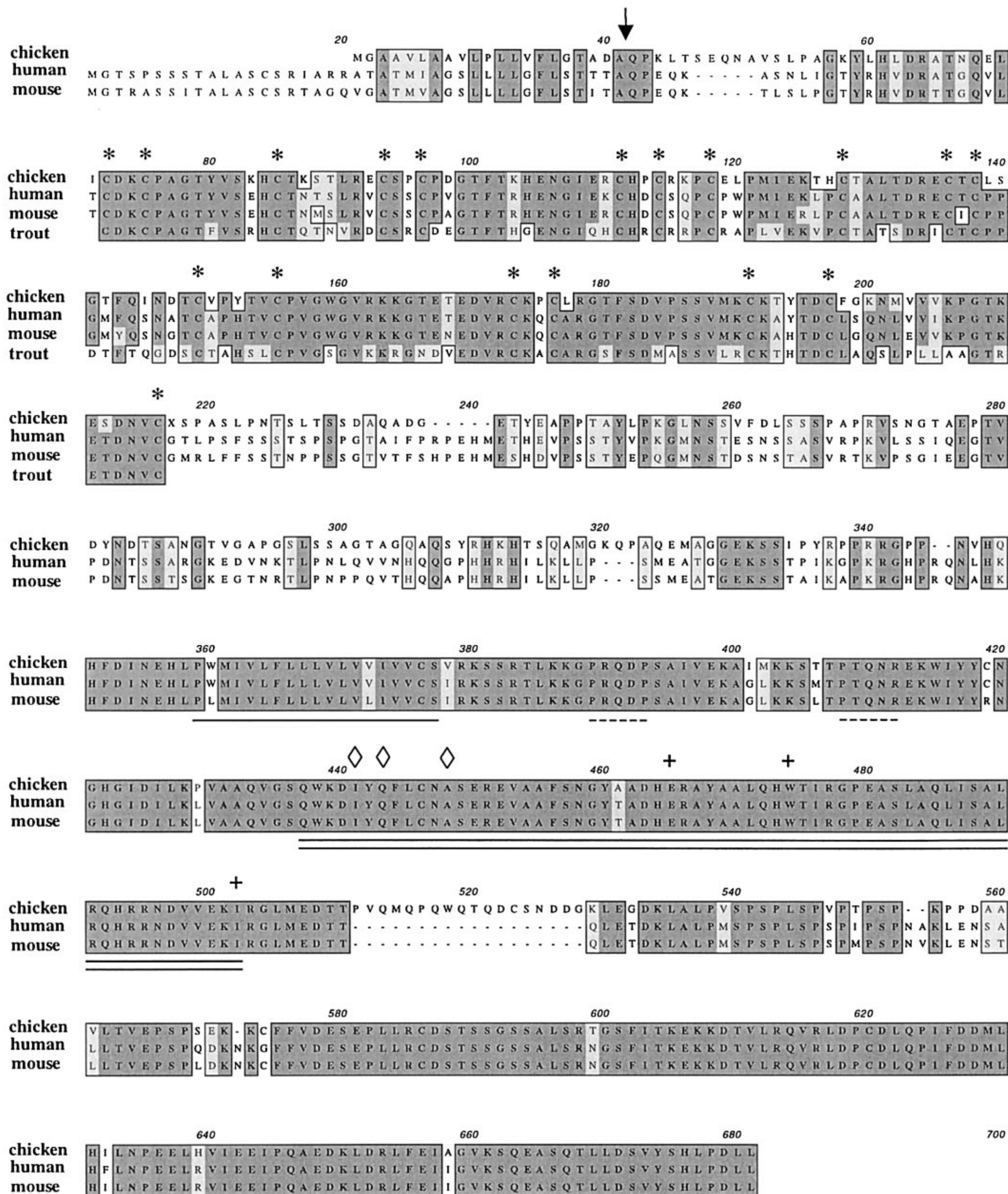

FIG. 1. Deduced amino acid sequence of DR6 from chicken (GenBank Accession No. AF349908) aligned to the human (AF068868), mouse (AF322069), and brook trout (partial cDNA; AF302499) sequences. Dark shading denotes identity while light shading represents conserved substitutions. Arrow indicates end of a putative signal peptide sequence, and asterisks (*) highlight cysteine residues within the cysteine-rich domains. Single underline indicates the putative transmembrane domain, double underline represents the death domain, and dashed lines represent potential TRAF binding sites. + denotes conserved, and diamonds indicate variant amino acids previously reported to be critical for TNFR1 receptor death-inducing function (1, 22).

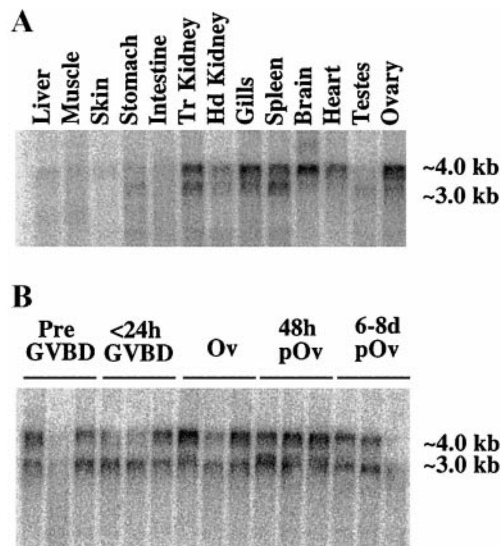

FIG. 2. (A) Northern blot showing tissue distribution of dr6 mRNA expression in the brook trout. Tr Kidney, trunk kidney; Hd Kidney, head kidney. (B) Northern blot of brook trout ovarian mRNA (0.5 μg/lane) collected from separate females prior to germinal vesicle breakdown (pre-GVBD), less than 24 h prior to GVBD, during ovulation (Ov), 48 h past ovulation (48h pOv) and 6–8 days postovulation (6–8d pOv).

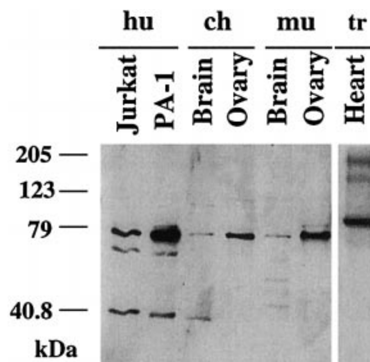

FIG. 3. Western blot of DR6 proteins from human (hu), chicken (ch), mouse (mu), and trout (tr) tissues. Human Jurkat cell lysate was from Upstate Biotechnologies, while PA-1 cells represent an ovarian epithelial tumor cell line.

determined to be virtually identical (98%). Generally, the death domain represents an amino acid sequence of varying length, which contains functionally critical sites for transmission of the death inducing signal as determined by point mutation analysis (22, 23). While the chicken, human and mouse DR6 sequences match the related TNFR1 receptor death domain at only three of the six critical positions (Glu^{185}, Trp^{194}, Ile^{502}; Fig. 1), the human DR6, when overexpressed, was found to induce cell death in the HeLa (but not MCF7) cell line (1, 24). Moreover, each of the three variant amino acids (Ile^{141}, Gln^{143}, Ala^{148}) is conserved among the chicken, human, and mouse DR6 predicted amino acid sequences, possibly indicating that DR6 utilizes some unique intracellular signaling pathways compared to other known death receptors. Cell death signaling generally occurs through adaptor proteins, such as TRADD or FADD, that dimerize via homophilic protein–protein interactions within the death domain of the transmembrane receptors. Adaptor proteins ultimately recruit initiator procaspases, which results in their autoactivation and subsequently the activation of effector caspases leading to the commitment to cell death. TRADD may also recruit TRAF1 or TRAF2 adaptor molecules that lead to activation of JNK and NF-κB (25), and significantly, DR6 contains two potential TRAF binding sites (Fig. 1). Similar to the TNFR1 and DR3 receptors, DR6 over-expression induced activation of both JNK (with or without the death domain) and NF-κB (only in the presence of the death domain) (1, 10). These alternative signaling pathways have the potential to lead to suppression rather than initiation of cell death (26). Moreover, DR6 contains an uncharacteristically long cytoplasmic region beyond the death domain, which is notably absent in other TNFR family members. Interestingly, this region is highly conserved between the chicken and human sequences with 96% identity within the final 100 amino acids, suggesting the possibility that this domain plays a functionally significant role.

At least two dr6 mRNA transcripts and one or two different size proteins are expressed in most tissues of

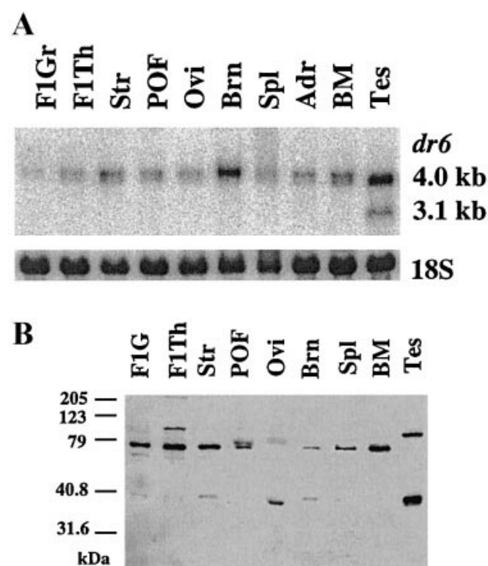

FIG. 4. (A) Representative Northern blot showing widespread expression of dr6 mRNA collected from hen tissues. 18S ribosomal RNA is provided as evidence of equal loading. F1G and F1Th, granulosa and theca from largest preovulatory follicle, respectively; Str, ovarian stromal tissue; POF, postovulatory follicle; Ovi, oviduct; Brn, brain; Spl, spleen; Adr, adrenal; BM, bone marrow; Tes, testes. (B) Western blot showing distribution of DR6 protein in various chicken tissues. Each blot was replicated with similar results.

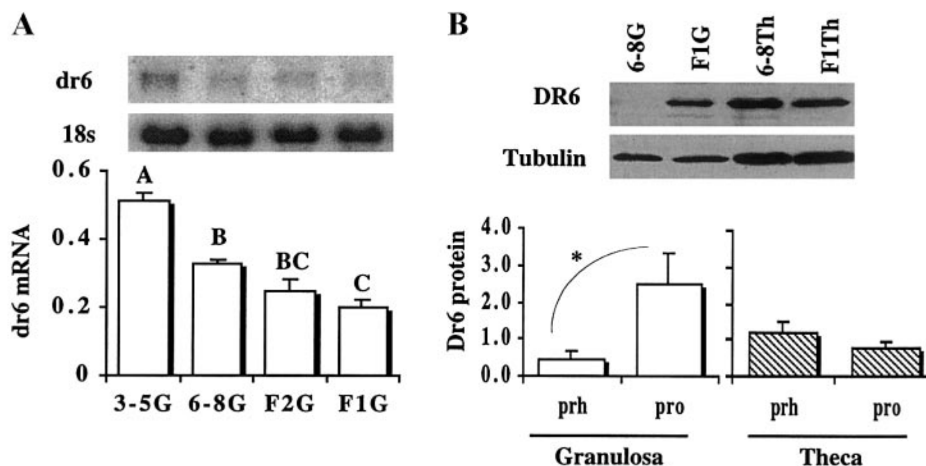

FIG. 5. (A) Expression of dr6 mRNA in hen granulosa during follicle development in the hen. F2 and F1, second largest and largest preovulatory follicle, respectively; 3–5 and 6–8, size of follicle (mm). Data are standardized to 18S ribosomal RNA, and are expressed as fold-difference versus a common reference tissue (stroma; not shown). (A, B, C) $P < 0.05$ by analysis of variance and the Fisher protected least-squares-difference multiple-range test ($n = 3$). (B) Representative Western blot showing DR6 protein in hen granulosa (G), theca (Th). Data represent levels of DR6 protein from prehierarchal (prh) versus preovulatory (pro) granulosa and theca tissue, standardized to α-tubulin and expressed as fold-difference relative to a common reference tissue (stroma; not shown). * $P < 0.05$ by unpaired t test.

both the trout and chicken (Figs. 2, 3, and 4). Brook trout express a predominant transcript of approximately 4.0 kb, with a frequently expressed second transcript of approximately 3.0 kb especially in the trunk kidney, gills, spleen, brain, and ovary. Northern analysis of chicken tissues shows a predominant transcript of 4.0 kb in all tissues (frequently appearing as a doublet), with an additional 3.1-kb transcript detected in the testes. The expression of two distinctly different transcripts is similar to that reported for human dr6 (1). Western analysis of protein from chicken testes also present two bands, one larger (~85 kDa) and one substantially smaller (~37 kDa) than the DR6 found in other tissues (~75 kDa) (Fig. 4). By comparison, trout DR6 protein reveals a predominant band of approximately 80 kDa which is also suggested to represent DR6 (Fig. 3), but given the lack of a complete cDNA from which to predict the amino acid sequence this size discrepancy compared to the chicken, human, and mouse cannot be explained at the present time. The combined results of Northern and Western blot analyses indicate both widespread expression of DR6 across various tissues and the possibility of alternatively processed proteins among the various vertebrate species; however, neither the identity nor function of such putative proteins has been addressed in any species.

The present results demonstrate that dr6 mRNA is expressed within the ovary at each stage of the ovulation cycle (trout) and follicle development (chicken) studied (Figs. 2 and 5). There are no differences in levels of dr6 mRNA (data not shown) or DR6 protein (Fig. 5B) in hen theca tissue relative to stage of follicle

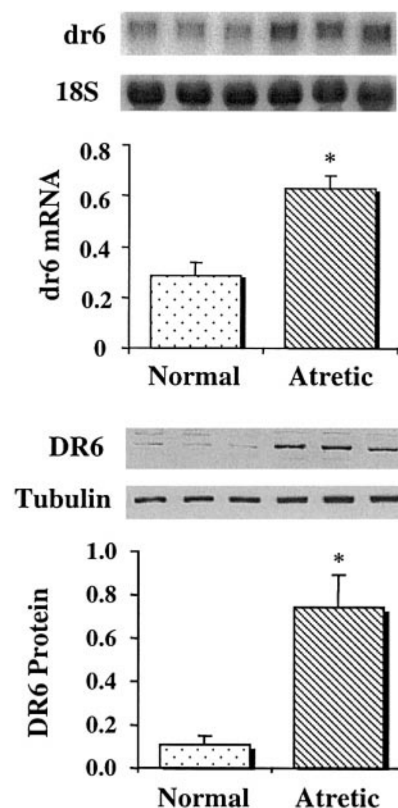

FIG. 6. Relative amounts of dr6 mRNA and protein detected in whole normal (viable) versus atretic 3- to 5-mm hen follicles. Data were standardized to 18S ribosomal RNA (dr6 mRNA) or α-tubulin (DR6 protein), prior to statistical analysis by unpaired t test. * $P < 0.05$ ($n = 3$).

development. Interestingly, there is an apparent discrepancy between the finding of higher levels of dr6 transcript within hen 3- to 5-mm (prehierarchal) follicle granulosa cells relative to preovulatory granulosa (Fig. 5A), compared to the relatively lower levels of protein present in the prehierarchal follicle granulosa (Fig. 5B). Such discrepancies between the relative amounts of a mRNA transcript and its protein expression have previously been noted (e.g., caspase-6; 27) and may suggest regulation at the translational rather than transcriptional level. In addition, functional DR6 activity may be regulated not only by the levels of receptor protein, but also by the availability of downstream adaptor proteins (discussed above) or presence of ligand(s). Unfortunately, the DR6 ligand has yet to be identified in any vertebrate. Alternatively, regulation of DR6 function may occur further downstream from receptor activation by cytoplasmic inhibitors such as X-linked Inhibitor of apoptosis protein (XIAP) which is known to prevent TNFα-induced cell death in rat granulosa cells (12), or c-FLIP, which inhibits the autoactivation of caspase-8 (28).

Finally, a comparison of normal, viable 3- to 5-mm hen ovarian follicles versus atretic follicles demonstrates a significant upregulation in DR6 mRNA and a single, approximately 75-kDa protein associated with atresia (Fig. 6). This finding represents the first evidence, *in vivo,* to correlate DR6 expression with ongoing apoptotic cell death. Given the potential for DR6 to activate a cell death and/or survival signaling cascade, the challenge for the immediate future will be to determine the functional signaling pathways and physiological role for DR6 in ovarian granulosa cells.

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