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Expression of *bcl-2* and *nr-13* in Hen Ovarian Follicles during Development[1]

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ABSTRACT

Follicle atresia is initiated within the granulosa cell layer of ovarian follicles and is mediated via the process of apoptosis. In the hen, at least two populations of granulosa cells can be distinguished during follicle development on the basis of their inherent susceptibility or resistance to apoptosis, in vitro. Given the previously established correlation between expression of *bcl-x$_{Long}$* and hen granulosa cell resistance to apoptosis, the present studies were conducted to characterize expression of *bcl-2* and an avian *bcl-2* homologue, *nr-13*, in follicles at various stages of development. Levels of *nr-13* mRNA were significantly higher only in granulosa cells from the largest (F1) preovulatory follicle compared to 3- to 5-mm prehierarchal follicles. By comparison, *bcl-2* mRNA levels were 5- to 9-fold higher in granulosa cells from the three largest preovulatory follicles compared to those from follicles 9 to 12 mm in diameter and prehierarchal follicles. The increase in neither *nr-13* nor *bcl-2* was correlated with the stage of follicle development associated with the acquisition of resistance to apoptosis in granulosa cells (e.g., at the 9- to 12-mm stage). Results from the present studies do not support a close correlation between constitutive expression of *nr-13* or *bcl-2* mRNA and the transition from a state of apoptosis susceptibility to apoptosis resistance in hen granulosa cells. Thus, it is proposed that *nr-13* and *bcl-2* play more of a supportive role in regulating additional aspects of ovarian cell function such as cell proliferation and/or differentiation.

INTRODUCTION

Much recent research has centered on the role of Bcl-2-related proteins in regulating the initiation of apoptotic cell death. Since the initial characterization of the *bcl-2* gene [1], numerous studies have reported that induced *bcl-2* expression promotes survival in a variety of cell types [2–4], while a reduction in Bcl-2 protein (using antisense oligodeoxynucleotides) can induce apoptosis [5]. With specific regard to the ovary, ablation of *bcl-2* gene expression via a loss-of-function transgenic model decreases the number of oocytes and primordial follicles in the postnatal mouse [6], and over-expression of *bcl-2* in the ovary of transgenic mice results in decreased follicle apoptosis, enhanced folliculogenesis, and increased germ cell tumorigenesis [7]. The *bcl-2* gene is also expressed in avian species [8, 9] and is proposed to have functions similar to those described in mammalian cells. The high degree of evolutionary conservation of Bcl-2 actions on cellular survival across species is further exemplified by the ability of transfected mammalian Bcl-2 protein to block apoptotic cell death in the nematode *Caenorhabditis elegans* [10]. Nevertheless, Bcl-2 is not a universal protector against cell death (see, for example, [11]), and thus it has been proposed that other *bcl-2*-related "death-suppressor" genes may be more physiologically relevant in certain cell systems [12].

For instance, similar death-repressing functions have been proposed for a related protein, Bcl-x$_{Long}$ [13]. However, unlike *bcl-2*, the *bcl-x* gene is alternatively spliced to form a second protein, Bcl-x$_{Short}$, which when over-expressed in cultured cells counters the death-repressor effects of Bcl-2 and Bcl-x$_{Long}$ [14]. Nevertheless, a physiological role for Bcl-x$_{Short}$ has yet to be firmly established in any cell system, and the alternatively spliced form of the transcript is not detected in hen granulosa cells [15]. By comparison, *bcl-x$_{Long}$* mRNA is abundantly expressed within hen preovulatory follicle granulosa cells, and it has been speculated that such elevated expression serves to protect against spontaneous or agonist-induced cell death [15]. This proposal is supported by similar results that follow in vitro transfection of *bcl-x* in several nongonadal cell systems (e.g., T cells, [16]; B cells, [17]; breast carcinoma cells, [18]).

In addition, *nr-13*, a *bcl-2*-related gene recently characterized from the quail, is markedly up-regulated after *Rous sarcoma* virus (RSV)-induced tumor formation and has been indirectly associated with the suppression of apoptosis during brain development [19]. More recently, transfection of the *nr-13* gene into the murine bone marrow-derived cell line, Baf-3, has been demonstrated to have a potent anti-apoptotic effect [20]. While the *nr-13* gene currently has no closely related mammalian homologue, it shares considerable homology to the Bcl-2 family of proteins within the highly conserved BH1 and BH2 domains [19, 21].

The *bcl-2*-related family of genes also includes negative regulators of cell survival such as *bax*, *bad*, *bcl-x$_{Short}$*, and *bak*. It is widely believed that the cellular ratio of "death-suppressing" proteins to "death-inducing" proteins encoded by *bcl-2* gene family members ultimately dictates whether a cell will respond to a proximal apoptosis-inducing signal [22]. Alternatively, it has been proposed that changes in absolute levels of Bcl-2-related proteins within cells may not be directly associated with cellular resistance or susceptibility to apoptotic cell death in certain cell systems, but rather that the effects of negative regulators of cell viability are mediated via posttranslational mechanisms [12, 23]. In fact, the ultimate regulation of biological activity may be dependent upon cell type and/or stage of cell differentiation.

Ovarian follicle atresia is a process mediated via apoptosis, and this event in the hen, as in mammalian species [24, 25], is proposed to be initiated within the granulosa cell layer [15, 26]. The hen follicle model system used for the present studies is one in which two populations of gran-

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ulosa cells can be readily identified: those characterized as highly susceptible to apoptosis and collected from atresia-prone, prehierarchal follicles, and those that are comparatively resistant to apoptosis and collected from preovulatory follicles (which under normal physiological conditions rarely undergo atresia) [15, 27]. Selection of a single follicle per day into the preovulatory hierarchy occurs from a relatively small group (6–10 in number) of 6- to 8-mm diameter follicles. Although the fate of unselected follicles in this group has not been unequivocally established, it is thought that the majority succumb to atresia and are replaced in an ongoing process. Given that mRNA levels for *bcl-x*$_{Long}$ have been demonstrated to be markedly elevated within hen granulosa cells immediately after follicle selection into the preovulatory hierarchy (at the 9- to 12-mm stage of development) and to be positively associated with granulosa cell survival in vitro [15, 28], it was of interest to investigate expression profiles of both *nr-13* and *bcl-2* mRNA in follicles collected at the same stages of development, in vivo, and in granulosa cells cultured, in vitro. In addition, experiments were conducted to evaluate the ability of agonists that attenuate the progression of apoptotic cell death in granulosa cells to alter levels of mRNAs encoded by *bcl-2*-related genes.

MATERIALS AND METHODS

Animals and Reagents

Single-comb white Leghorn hens (H&H Poultry, Portland, IN), 25–40 wk of age and laying regular sequences of at least 8–10 eggs, were used in all studies described. Birds were housed individually in laying batteries, provided with free access to feed (Purina Layena Mash; Purina Mills, St. Louis, MO) and water, and exposed to a photoperiod of 15L:9D, with lights-on at 0000 h. Individual laying cycles were monitored by the daily timing of oviposition. Hens were killed approximately 16–18 h before a midsequence ovulation by cervical dislocation. All procedures described herein 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.

Oligonucleotide primers (for PCR amplification and sequencing) were synthesized by the University of Notre Dame Biotechnology Core Facility. Recombinant human transforming growth factor α (TGFα) was purchased from Bachem (Torrance, CA), and additional reagents were acquired from Sigma Chemical Co. (St. Louis, MO), unless otherwise stated.

Characterization of a Partial Chicken nr-13 *cDNA*

Total cellular RNA was isolated from granulosa tissue and reverse-transcribed into first-strand cDNA using oligo(deoxythymidine) primer and recombinant Moloney Murine Leukemia Virus reverse transcriptase in the presence of deoxynucleotides (Perkin-Elmer, Norwalk, CT). Sequences for oligonucleotide primer pairs were based on the published sequence for the quail *nr-13* cDNA [19]: forward primer, AAA ACT TGC TCA CCA AAC CCT TGG (base pairs [bp] 695–718); reverse primer, CAG AGA AAT TCC AGC CTC CAC TGG (bp 1272–1249).

First-strand cDNA was subjected to 35 cycles of polymerase chain reaction (PCR) amplification using GeneAmp core reagents (Perkin-Elmer; 2 min denaturation at 94°C first cycle and 1 min per cycle thereafter, 1 min annealing at 55 C, and 1 min extension at 72°C for the first 34 cycles, 7 min extension on the final cycle). Amplified 570-bp PCR products (522-bp chicken Nr-13 plus primer sequences) were resolved through a 1.2% agarose gel, isolated, and subcloned into the pCRII vector (Invitrogen, San Diego, CA) for large-scale plasmid preparation. Nucleic acid sequence analysis was conducted in both the forward and reverse directions from two different clones according to the dideoxychain termination method using [^{35}S]dATP (Amersham Corp., Arlington Heights, IL) and the Sequenase version 2.0 sequencing kit (United States Biochemical Corp., Cleveland, OH). Sequence data were assembled and analyzed using the MacVector and AssemblyLIGN programs (IBI, New Haven, CT).

Tissue Collection and Preparation

Tissues collected for analysis of *nr-13* mRNA expression included cerebrum, cerebellum, adrenal gland, kidney, heart, and liver. In addition, we collected and prepared, as previously described [29, 30], granulosa and theca tissue from the largest (F1), second largest (F2), and third largest (F3) preovulatory follicles, from follicles recently selected into the follicle hierarchy (9- to 12-mm follicles), and from prehierarchal (6- to 8-mm and 3- to 5-mm) follicles; and stromal tissue. Morphologically normal and early atretic follicles (3- to 5-mm diameter) were processed without separating granulosa and theca layers. Early atretic follicles were identified on the basis of the presence of follicle haemorrhagia, collapsed morphology, and opaque appearance [15].

Granulosa Cell Cultures

Granulosa cells were collected from preovulatory (F1 and F2) or prehierarchal (6- to 8-mm) follicles, pooled within their respective group, and dispersed in 0.3% collagenase (type 2; Worthington, Freehold, NJ) as previously described [29]. Granulosa cells were incubated as suspension cultures (2.5×10^6 per 12×75-mm polypropylene culture tube) in the absence or presence of treatments in 2 ml medium 199 supplemented with Hanks' salts (Gibco-BRL, Gaithersburg, MD; M199-HEPES) for 18 h at 37°C in a shaking water bath. Treatments included medium alone (control), 1 mM 8-bromo-cyclic AMP (8-br-cAMP), 3.3 nM TGFα, or the phorbol ester 167 nM phorbol 12-myristate 13-acetate (PMA). These agents and doses were selected for use on the basis of their previously established actions to affect cell viability and/or cell differentiation in granulosa cells [15, 27]. After suspension culture, cells were gently pelleted by centrifugation ($200 \times g$; 20°C; 5 min), media were discarded, and cells were frozen (−70°C) until further processed.

Alternatively, granulosa cells from 6- to 8-mm follicles were frozen at −70°C immediately after dispersion (T0 controls) or were plated on 60-mm polystyrene culture plates (Falcon 3004; Fisher Scientific Company, Pittsburgh, PA) at a density of approximately 1.5×10^6/ml in 2 ml M199-HEPES supplemented with 2.5% fetal bovine serum (Gibco-BRL). We have previously reported that the plating efficiency of such prehierarchal follicle granulosa cells is approximately 30% with the remaining cells determined to undergo apoptosis [27]. The following day, adherent cells were washed once with fresh medium and removed via trypsinization. Cells were pelleted at $200 \times g$ for 5 min, then stored at −70°C until processed further.

Northern Blot Analysis

Total cellular RNA from each tissue and stage of follicle development was isolated using Trizol Reagent (Gibco-BRL), and purity and quantity were assessed by measuring the optical density of each sample at 260 nm vs. 280 nm. Ten to 15 μg of total cellular RNA was electrophoresed on 1% agarose gels in the presence of formaldehyde, then transferred to nitrocellulose Nitro ME membranes (MSI, Westboro, MA) by capillary action overnight. Analysis of *nr-13*, *bcl-2*, and *bcl-x* mRNA was conducted using cDNA probes labeled by the random-prime labeling method using the Megaprime DNA labeling System (Amersham) and [α^{32}P]deoxycytidine triphosphate (dCTP; 3000 Ci/mmol; Amersham). The *nr-13* probe consisted of the 570-bp cloned product derived from PCR amplification, while the *bcl-2* probe was a 545-bp PCR product amplified from the open reading frame (beginning 17 bp to the 5′ end of the ATG start codon) of chicken *bcl-2* [9]. Messenger RNA for *bcl-*x_{Long} was analyzed using a 485-bp cDNA probe as previously described, and it is noted that these studies failed to identify a *bcl-*x_{Short} transcript expressed in the hen ovary [15]. Blots were prehybridized for 30 min at 60°C and subsequently hybridized overnight at 60°C, according to previously described methods [31–34]. Membranes were exposed to autoradiographic film at −70°C for 1–3 days (*nr-13* and *bcl-*x_{Long}) and subsequently analyzed by densitometric scanning (UltraScan XL laser densitometer; Pharmacia LKB, Piscataway, NJ), or exposed to phosphorimaging plates (1–2 days for *nr-13*; 7–10 days for *bcl-2*) and analyzed using a Storm 840 PhosphorImager system equipped with the ImageQuant data reduction system (Molecular Dynamics, Sunnyvale, CA). When appropriate (see data analysis), blots were rehybridized to a random-primed chicken 18S ribosomal RNA cDNA probe to standardize for equal loading of RNA samples. The 225-bp 18S probe was amplified by PCR based upon GenBank sequence data (Accession number D38360; unpublished data). Hybridization conditions for Northern blot analysis were identical to those described above.

Western Blot Analysis

Granulosa and theca tissues were collected from follicles at different stages of development as described above, and homogenized in protein lysis buffer (1.7 mM sodium monophosphate, 17 mM sodium diphosphate, 1% Triton X-100, 0.1% SDS, 0.1% sodium azide, 0.1 M NaCl) containing aprotinin (10 μg/ml), pepstatin (10 μg/ml), PMSF (1 mM), leupeptin (10 μg/ml), and sodium orthovanadate (0.1 M). Samples were vortexed and then centrifuged at 12 000 × *g* at room temperature for 10 min. The supernatant was collected, and protein was quantified using the Bio-Rad DC Protein Assay kit (Hercules, CA). Protein samples were subsequently diluted with sample buffer (1-strength): 125 mM Tris [pH 6.8], 2% SDS, 5% glycerol, 1% β-mercaptoethanol, 0.003% bromophenol blue dye), heated to 65°C for 10 min, then centrifuged at 12 000 × *g* for 10–20 min. Samples were loaded (with appropriate size markers) onto a 4% polyacrylamide stacking gel, 12% resolving gel and electrophoresed for 1 h at 150 V. Gels were washed (3 × 10 min) with transfer buffer (25 mM Tris, 190 mM glycine, 20% MeOH), then transferred to a nitrocellulose membrane (Hybond, Amersham) at 110 V, 180 mA for 1 h.

Membranes were blocked for 2 h at 20°C in Western blocking solution (5% nonfat dry milk, TBST: 10 mM Tris [pH 8.0], 100 mM sodium chloride, and 0.1% Tween 20). The mouse anti-chicken Bcl-2 (from Dr. Y. Euguchi, Osaka University Medical School, Osaka, Japan) and β-tubulin (cat. N-357, Amersham) monoclonal antisera were diluted 1:10 and 1:1000, respectively, in blocking solution and incubated with membranes at 4°C overnight; then membranes were washed (3 × 10 min) in blocking solution. Goat anti-mouse serum:horseradish peroxidase (Pierce, Rockford, IL) was diluted 1:10 000 in blocking solution and incubated with membranes for 1 h at 20°C. Membranes were washed in TBST (2 × 5 sec followed by 3 × 10 min). Finally, blots were incubated with ECL Western blotting detection reagent (Amersham) for 1 min and exposed to x-ray film for 5–60 min.

Data Analysis

Northern blot analyses were repeated on a minimum of three independent occasions with different pools of cells or tissues. Equal loading of RNA from granulosa and theca tissues during follicle development was verified after the evaluation of ethidium bromide-stained 28S and 18S bands of ribosomal RNA. This method of analysis has proven reliable for the measurement of a variety of genes expressed during hen follicle development [15, 31–34]. Because of the potential for treatment-related differences in cell viability after granulosa cell suspension culture, mRNA data from such studies were standardized to 18S ribosomal RNA. Messenger RNA levels or protein levels (fold difference as related to the appropriate control) are presented as the mean ± SEM of data from replicate experiments. Data were analyzed by the Student's *t*-test or by using one-way analysis of variance with significant interactions determined by the Fisher PLSD multiple range test.

RESULTS

Characterization of a Chicken nr-13 *cDNA and Tissue Distribution of* nr-13 *mRNA*

The nucleic acid sequence for a chicken *nr-13* cDNA encoding a portion of the 3′-untranslated region is presented in Figure 1. The amplified 522-bp chicken *nr-13* sequence was 91.6% homologous to the corresponding quail *nr-13* nucleic acid sequence. Whereas *nr-13* mRNA was expressed in all tissues examined, steady-state expression of the 1.7-kilobase (kb) transcript was consistently highest in ovarian stromal and adrenal tissues and lowest in the liver (Fig. 2).

bcl-2 *and* nr-13 *mRNA Levels during Follicle Development and Atresia*

Changes in *nr-13* mRNA levels within the granulosa layer during follicle development were relatively minor (less than 33%), and levels of the transcript were elevated only in F1 compared to 3- to 5-mm follicles ($p < 0.05$; Fig. 3, left panels). While there were no differences in *nr-13* mRNA in the theca layer relative to stage of follicle development, ovarian stromal tissue contained 2.3-fold higher levels compared to the theca ($p < 0.05$; Fig. 3, right panels).

A predominant transcript of approximately 6.5 kb in size was found for *bcl-2* mRNA at all stages of development. Highest levels of *bcl-2* mRNA were found in granulosa cells from F1 through F3 follicles whereas levels were significantly lower in granulosa from 9- to 12-mm follicles, and 3- to 5-mm and 6- to 8-mm prehierarchal follicles ($p < 0.05$; Fig. 4, left panels). No significant changes in *bcl-*

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cNR-13   CTATAGACAG CTTCACTCGC TACCCAGTAA CAGCACAAAC GTGTACCAAC CCACCATTTG   60
qNR-13         a  c                  g      g                  a   cca

cNR-13   TGCGTGTTCA CAATGTGTCT TCTCCAAACA TCCTCTGGAC TTTCTTTTGA ACGTTTGACC  120
qNR-13                 c      g          tg c  c                        c

cNR-13   AAAACATTAC CATGGTTCGT GTACTGTAAC TTCTGAGTGC TCAATGTCAT GCTAGGAGAA  180
qNR-13   t    g             t                                   c        a

cNR-13   AGCTATGGTG GACTCGTAAC TAAACCCTGT GTTCAAGGCA TTTACATGGA GGCAGTCCCA  240
qNR-13

cNR-13   GACCTCGCCT GCCATTCATA CTGTAACGTG GCCTCGCACC GACCGATAGA ACACGGTCTG  300
qNR-13                                          a        t

cNR-13   TGGCTTCACA CGCAGGTCCC AGCACTTGTT ACTGAAGGGC AACTCGTTGG TTGGTTTAGA  360
qNR-13              t  - tg                t   ga        ctcaa     a g

cNR-13   GCAGTCAGCA CCTGTGATGA GAGGCATTAT GGCTTGGTGT AATGTAAGGC CGATTTCATG  420
qNR-13         t  t           t      g           a               t

cNR-13   ACACTACAAG AGTAGAAGTG TTGATACAGG ACAGTAATGG CTGCAGTTCC AGTTCTTACT  480
qNR-13                  gt

cNR-13   GAAAGGAATT AAATATTAAA CCTTAATTGG TATAGTGAAA TC          522
qNR-13             t
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FIG. 1. Nucleic acid sequence of the PCR-amplified chicken (c) cDNA compared to quail (q) [19] *nr-13* cDNA. Blank spaces within the aligned quail sequence represent homologous bases; -, corresponding residue deleted from the c*nr-13* sequence; bold print, bases not found in the c*nr-13* sequence inserted at the site marked by ^.

2 mRNA levels were observed in theca tissue during development, and, as observed for *nr-13*, *bcl-2* mRNA was highest in stromal tissue ($p < 0.05$; Fig. 4, right panels). Western blot analysis confirmed that levels of the translated Bcl-2 protein were higher in preovulatory (F1) follicle granulosa cells compared to almost nondetectable levels in prehierarchal (6- to 8-mm diameter) follicle granulosa cells (Fig. 5). Curiously, levels of *nr-13* mRNA were decreased by 57% in atretic compared to normal 3- to 5-mm follicles, while *bcl-2* mRNA was increased by 1.6-fold in atretic follicles (Fig. 6; $p < 0.05$).

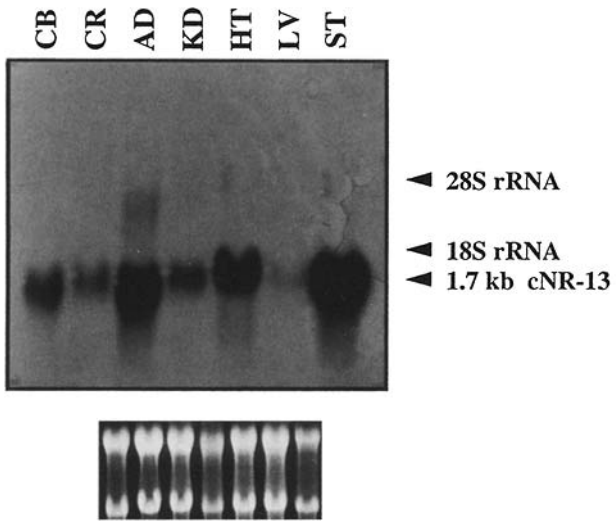

FIG. 2. **Top**) Distribution of *nr-13* mRNA in chicken tissues. **Bottom**) Ethidium bromide-stained gel for a relative comparison of total cellular RNA loading. CB, cerebellum; CR, cerebrum; AD, adrenal; KD, kidney; HT, heart; LV, liver; ST, ovarian stroma. Each tissue was independently analyzed a minimum of three times with similar results.

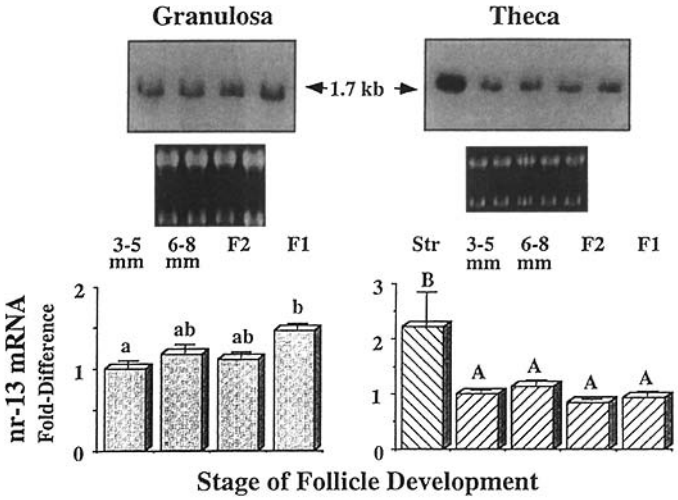

FIG. 3. Representative Northern blots of *nr-13* mRNA from granulosa and theca tissue during follicle development (upper panels), ethidium bromide-stained gels for 28 and 18S ribosomal (r) RNA (middle panels), and summary of densitometric scanning data (lower panels; mean densitometric units expressed as fold difference compared to 3- to 5-mm follicles; data based on 6–8 replicate experiments). a, b; A, B: mean ± SEM, $p < 0.05$.

bcl-2, nr-13 *and* bcl-x_{Long} *mRNA Levels in Cultured Granulosa Cells*

Levels of *nr-13* mRNA were 2.5-fold higher in the viable granulosa cells that form a primary culture after 24 h than in freshly collected (T0) cells ($p = 0.028$; Fig. 7, left panel). By comparison, *bcl-2* mRNA levels were lower in cells plated for 24 h ($p = 0.04$), but this decrease was only to an extent of 15% (Fig. 7, right panel).

After an 18-h suspension culture of granulosa cells from 6- to 8-mm follicles, levels of *bcl-2* mRNA were not altered by TGFα, cAMP, or PMA (Fig. 8, left panel). By comparison, mRNA levels for *bcl-x_{Long}* were increased (by 4.2-fold) in 6- to 8-mm follicle granulosa cells by suspension

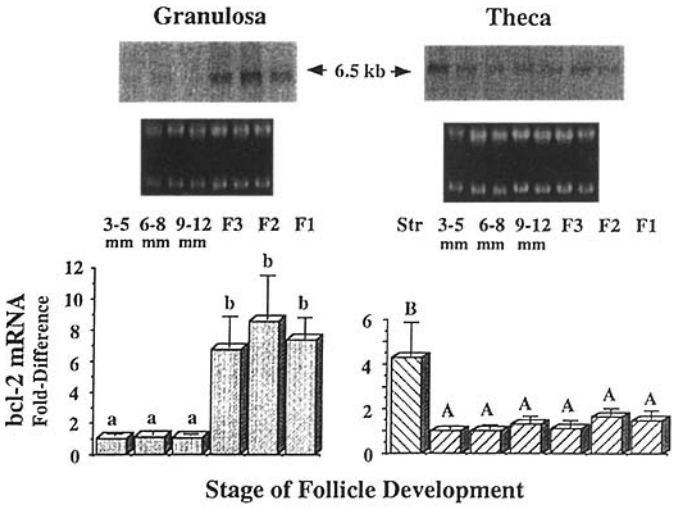

FIG. 4. Representative Northern blots of *bcl-2* mRNA from granulosa and theca tissue during follicle development (upper panels), ethidium bromide-stained gels for 28S and 18S rRNA (middle panels), and summary of densitometric scanning data (lower panels; mean densitometric units expressed as fold difference compared to 3- to 5-mm follicles; data based on 3–5 replicate experiments). Phosphorimages of granulosa and theca tissue were collected after an equal length of exposure. Str, stromal tissue. a, b; A, B: mean ± SEM, $p < 0.05$.

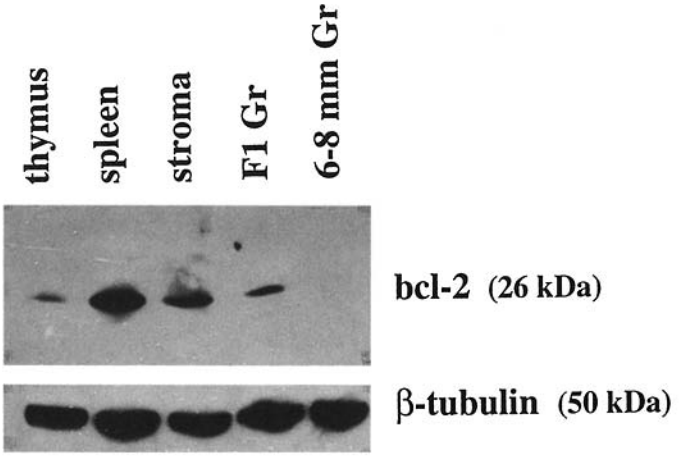

FIG. 5. Western blot analysis of a 26-kDa Bcl-2 protein in the hen thymus and spleen (positive control tissues) and in ovarian stromal, preovulatory follicle granulosa (F1 Gr) and prehierarchal follicle granulosa (6–8 mm Gr) tissues. Approximate equal loading of proteins determined by comparing to β-tubulin (bottom panel). Note that levels of Bcl-2 are readily detectable in F1 Gr but not readily detected in 6–8 mm Gr. This profile is consistent with that for mRNA expression (see Fig. 4). This blot was repeated two additional times with similar results.

culture in the presence of 8-br-cAMP compared to 18-h control cultured cells ($p < 0.001$; Fig. 8, right panel). Similarly, only 8-br-cAMP induced a significant (2-fold) increase in levels of *bcl-x*$_{Long}$ in preovulatory F1 follicle granulosa cells. Finally, levels of *nr-13* and *bcl-x*$_{Long}$ mRNA levels were unaltered after suspension culture with the remaining treatments, and *bcl-2* mRNA levels were significantly decreased by TGFα and cAMP treatments (Fig. 9).

DISCUSSION

The chicken *nr-13* partial cDNA showed considerable homology to the quail *nr-13* gene [19], considering that the characterized sequence lies within the 3′-untranslated region of the gene. Therefore, it was not surprising that the 1.7-kb mRNA transcript, determined by Northern blot analysis, was of similar size to that of the quail. Patterns of *nr-13* mRNA expression were also comparable to those from the quail in that lowest levels were found in the chicken liver and cerebellum, while higher levels were expressed in the heart (Fig. 2). Of additional interest was the novel finding that highest levels of the *nr-13* mRNA transcript were

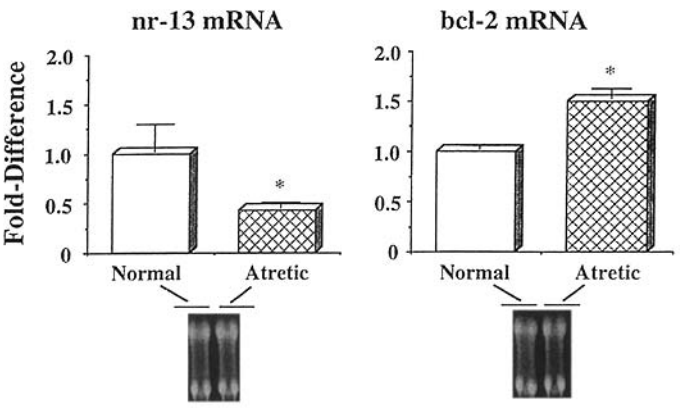

FIG. 6. *nr-13* and *bcl-2* mRNA levels (mean ± SEM; data based on 4 or 5 replicate observations) in whole atretic compared to normal (healthy) 3- to 5-mm diameter follicles. Bottom panels represent ethidium bromide-stained gels for 28S and 18S rRNA. *$p < 0.01$.

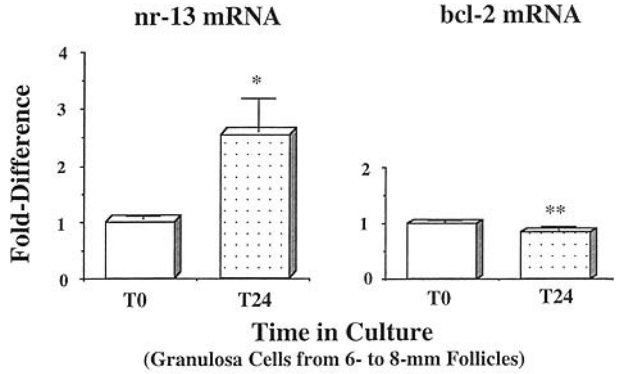

FIG. 7. *nr-13* and *bcl-2* mRNA levels in 6- to 8-mm follicle granulosa cells frozen immediately after enzymatic dispersion (T0) or after 24 h of culture (T24). Values represent the mean ± SEM (data based on 5 or 6 replicate observations) standardized to 18S rRNA. *$p = 0.028$; **$p = 0.04$.

consistently detected in endocrine tissues such as ovarian stroma and the adrenal. Thus, given the positive relationship previously reported between elevated levels of another related death-suppressing gene (e.g., *bcl-x*$_{Long}$) and granulosa cell resistance to apoptotic cell death [15], studies were subsequently pursued to evaluate expression of *nr-13* and *bcl-2* in granulosa and theca tissue during follicle development.

Whereas highest levels of *nr-13* mRNA in granulosa cells were found in F1 follicles, the relative increase in mRNA compared to the increase in prehierarchal follicles was markedly lower than that found for *bcl-x*$_{Long}$ (a 5.0- to 9.0-fold increase) [15]. Moreover, this stage-related increase in *nr-13* mRNA was not related to follicle selection and the acquisition of resistance to apoptotic cell death in granulosa cells, as levels of *nr-13* mRNA were similar in F2 follicle granulosa and 6- to 8-mm follicle granulosa (Fig. 3). By comparison, the 6.7- to 8.5-fold increase in *bcl-2* mRNA transcript in F3 to F1, over prehierarchal, follicle granulosa was similar to that found for *bcl-x*$_{Long}$ [15]. A second, and less prominent, *bcl-2* transcript (4.5 kb) has previously been detected in several chicken tissues (e.g., spleen, thymus, cerebellum) [9], but levels of this transcript in ovarian granulosa and theca tissues were generally low to nondetectable and were not measurably different with regard to follicle development. Consistent with a previous

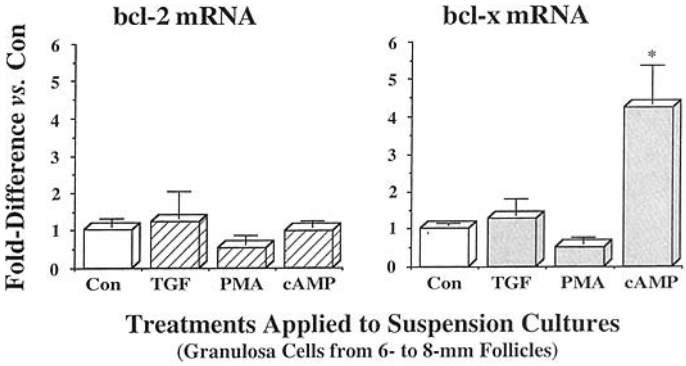

FIG. 8. Regulation of *bcl-2* and *bcl-x*$_{Long}$ mRNA levels in 6- to 8-mm follicle granulosa cells after an 18-h suspension culture in the absence (Con) or presence of TGFα (3.3 nM), PMA (167 nM), or 8-bromo-cAMP (cAMP; 1 mM). Values represent the mean ± SEM (data based on 3 or 4 replicate observations) standardized to 18S rRNA. *$p < 0.01$ compared to Con.

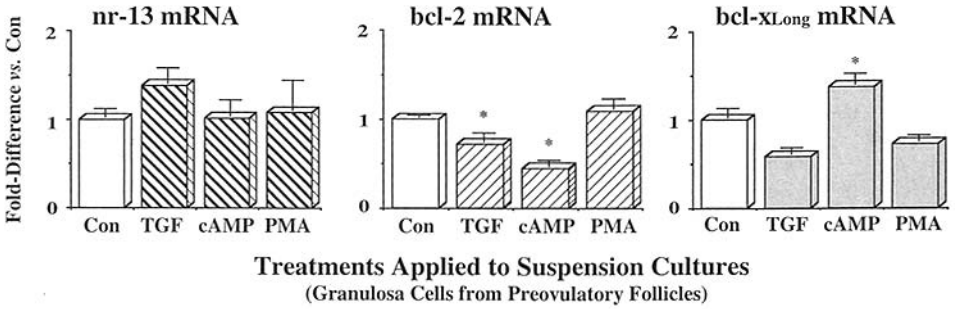

FIG. 9. Regulation of *nr-13*, *bcl-2*, and *bcl-x_{Long}* mRNA levels in preovulatory (F1 and F2) follicle granulosa cells following an 18 h suspension culture in the absence (Con) or presence of TGFα (3.3 nM), PMA (167 nM), or 8-bromo-cAMP (cAMP; 1 mM). Values represent the mean ± SEM (data based on 3 or 4 replicate observations) standardized to 18S rRNA. $*p <$ 0.05 compared to Con.

report [8] was the finding of a single 26-kDa Bcl-2 protein. Bcl-2 was detected in the thymus, spleen, ovarian stromal tissue, and granulosa from the largest preovulatory (F1) follicle but was not readily detected in granulosa cells from prehierarchal (6–8-mm diameter) follicles (Fig. 5). This is interpreted to indicate that the amount of protein present follows the pattern of mRNA expression.

Notable differences were found when patterns of *bcl-2* expression were compared to those for *bcl-x_{Long}*. Specifically, overall levels of the 6.5-kb *bcl-2* transcript were markedly lower than those for *bcl-x_{Long}*, as the exposure time required for detecting *bcl-2* mRNA using the PhosphorImaging system (7–10 days) was considerably longer than the time required for the detection of *bcl-x_{Long}* (1–2 days, by autoradiogram) [15]. Moreover, similar to *nr-13*, the increase in *bcl-2* mRNA expression was not closely associated with the stage of follicle selection or the acquisition of resistance to cell death, as *bcl-2* mRNA levels were not different within granulosa cells from recently recruited 9- to 12-mm compared to prehierarchal 3- to 5-mm or 6- to 8-mm follicles, but were increased in F3, F2, and F1 follicles.

It has previously been speculated that one requirement for maintaining avian follicle viability during the initial growth phase through the time of follicle recruitment into the preovulatory hierarchy is the constitutively elevated expression of one or more death-suppressing genes within the granulosa layer [35]. This proposal is supported by several observations related to *bcl-x_{Long}* gene expression. First, *bcl-x_{Long}* mRNA was elevated in 9- to 12-mm follicle granulosa cells, in vivo, compared to granulosa cells collected from 6- to 8-mm follicles [15]. This finding suggests a direct relationship between an increase in *bcl-x_{Long}* gene expression and follicle recruitment into the preovulatory hierarchy as well as the acquisition of granulosa cell resistance to cell death. Second, when granulosa cells from prehierarchal follicles were cultured for 24 h, only a select population of these cells (about 30% of the initial cells plated) forms a primary monolayer culture, while the remainder remain unattached and undergo apoptosis. Plated cells expressed 2.5-fold higher levels of *bcl-x_{Long}* mRNA compared to the initial population. By comparison, approximately 80% of preovulatory (F1) follicle granulosa cells cultured in a similar fashion remain viable after a 24-h culture, and *bcl-x_{Long}* mRNA levels from the adherent population of cells are not different compared to the original population [35]. Such findings suggest that the survival of granulosa cells, in vitro, is associated with elevated levels of *bcl-x_{Long}* mRNA. It is further speculated that the surviving granulosa cells from prehierarchal follicles may have originated primarily from follicles that were destined for selection into the preovulatory hierarchy, in vivo [35].

In the present studies, *nr-13* mRNA levels were increased (by 3-fold) in the 6–8-mm follicle granulosa cells remaining after a 24-h culture to an extent similar to those previously reported for *bcl-x_{Long}*, while *bcl-2* mRNA levels were slightly decreased (Fig. 7). Although the decrease in *bcl-2* mRNA is statistically significant, there is some question as to whether such a marginal decrease is of physiological relevance. Similarly, because constitutive expression of *nr-13* was not increased until the F1 stage of follicle development (even then only to a level 1.4-fold that in prehierarchal follicle granulosa; Fig. 3), it is suggested that the increase in *nr-13* mRNA following culture is not necessarily related to selection of apoptosis-resistant granulosa cells. Taken collectively, patterns of *nr-13* and *bcl-2* mRNA expression in granulosa cells are not consistent with the idea that elevated expression of either gene is prerequisite for prehierarchal follicle resistance to apoptosis, in vitro, and follicle recruitment, in vivo. On the other hand, it remains possible that expression of these death-suppressing genes provides a supportive role to regulate cell division or differentiation [36].

It is noted that, similar to the pattern of *bcl-x_{Long}* gene expression within theca tissue [15], *nr-13* and *bcl-2* mRNA levels did not change during follicle development. On the other hand, levels of both *bcl-2* and *nr-13* mRNA were markedly higher in stromal than in theca tissue, and this may be related to the proportionally greater presence of immune-related cells, which are known to express relatively high levels of *bcl-2* [9], within stromal tissue. Previous data indicate that apoptotic cell death during follicle atresia is initiated predominantly within, and subsequently occurs throughout, the granulosa but not the theca layer [15, 26]. If *bcl-2* and/or *nr-13* gene expression plays a role in conferring resistance to cell death within the theca layer, it is possible that the constitutive level of *nr-13* and *bcl-2* expression, combined with *bcl-x_{Long}*, is sufficient for this purpose at all times during follicle development. However, it is also recognized that, unlike the granulosa layer, which undergoes functional differentiation only subsequent to follicle selection, steroidogenic cells within the theca layer exist in a differentiated state at all times throughout development (for review, see [28]).

With regard to the latter, results from a recent study using a human neural-crest-derived tumor cell line indicate that the *bcl-2* gene product promotes cell differentiation but does not significantly affect cell survival in culture [37]. Thus, it can be alternatively speculated that a more important role for the Bcl-2 protein may be to modulate ovarian granulosa and theca cell differentiation. In addition, overexpression of *bcl-2* in rat-6 embryo fibroblasts has recently been reported to decrease the rate of cell proliferation by specifically extending the duration of the cell cycle G_1 phase [36]. Of relevance is the previous report that hen granulosa cells from F3 and F1 follicles are less mitotically active (indicated by [^{3}H]thymidine incorporation studies) than granulosa from prehierarchal and 9- to 12-mm follicles

[38]. Given the positive association between levels of *bcl-2* gene expression (present results) and loss of granulosa cell proliferation [38], it is possible that increased levels of *bcl-2* expression may play an additional role in decreasing the mitotic division rate of granulosa cells during the final stages of follicle maturation.

Previous studies have demonstrated that *bcl-2* gene expression can be induced in several cell types by extracellular factors (e.g., prolactin in Nb2 lymphoma cells [39]; progesterone in human uterine smooth muscle tumor cells [40]) or by intracellular signaling mechanisms (e.g., by activation of the cyclic AMP-responsive element binding protein in B lymphocytes [41]). On the other hand, in vivo priming of 25-day-old immature rats with eCG fails to alter *bcl-2* or *bcl-x* mRNA levels in RNA preparations from the whole ovary [42]. Nevertheless, it has not yet been determined whether the expression of *bcl-2*-related genes in a purified population of granulosa cells can be regulated by extracellular factors or cell signaling mechanisms that have previously been determined to attenuate apoptotic cell death. Specifically, TGFα and the cAMP analog, 8-br-cAMP, attenuated oligonucleosome formation in 6- to 8-mm follicle granulosa induced by suspension culture in serum-free medium for 6 h [15].

In the present studies, the response to these survival factors was compared to that elicited by the pharmacologic protein kinase C activator PMA, which neither attenuates nor promotes the progression of cell death in prehierarchal or preovulatory follicle granulosa cells [15, 27]. Results of these studies provided no evidence for the stimulation of *bcl-2* or *nr-13* gene regulation by TGFα or 8-br-cAMP in hen granulosa cells (Figs. 8 and 9). The inability of such factors to up-regulate *bcl-2* or *nr-13* mRNA levels is consistent with the recent proposal that most cell survival factors mediate their effects on *bcl-2* family members via post-translational mechanisms. For example, in FL5.12 cells, interleukin (IL)-3 promotes cell survival via phosphorylation of the death-promoting protein Bad, which prevents heterodimerization with Bcl-x_{Long} [23]. By comparison, gene regulation of *bcl-*x_{Long} in prehierarchal and preovulatory follicle granulosa cells is indicated by the finding that 8-br-cAMP induced a 1.4- to 4-fold increase in levels of *bcl-*x_{Long} mRNA (Figs. 8 and 9). This stimulatory effect cannot be explained by a decrease in the number of prehierarchal follicle granulosa cells actively undergoing apoptosis in 8-br-cAMP-treated cells compared to control cells, as 1) TGFα fails to increase *bcl-*x_{Long} mRNA, but is known to suppress oligonucleosome formation in prehierarchal follicle granulosa cells to an extent similar to 8-br-cAMP [15]; 2) preovulatory follicle granulosa cells are essentially resistant to apoptosis in suspension culture [15]; and 3) neither *bcl-2* nor *nr-13* mRNA levels are altered at either stage of development under similar suspension culture conditions. Recently, it has been reported that the ability of insulin-like growth factor-1 to attenuate apoptosis in rat pheochromocytoma PC12 cells is associated with its ability to increase *bcl-*x_{Long} gene expression [43]. Accordingly, it is speculated that FSH and/or vasoactive intestinal peptide may serve as physiological agonist(s) that regulate *bcl-*x_{Long} mRNA levels in 6- to 8-mm follicle granulosa [31, 44], while LH is the primary agonist in preovulatory follicle granulosa [28, 31, 33].

Finally, levels of a variety of gene transcripts, including *nr-13*, are significantly decreased during the early stages of hen follicle atresia (e.g., [15, 31–33]). These findings are perhaps not unexpected and suggest that a selective decrease in the expression of certain genes is associated with, and may actually precede, the onset of atresia. On the other hand, urokinase plasminogen activator (uPA) [34] and *bcl-2* (present data) mRNA levels are significantly higher in early atretic than in morphologically normal follicles. While the increase in uPA transcript is suggested to indicate an active role for this cell-remodeling enzyme during follicle death, a physiological role for increased *bcl-2* gene expression during this process is less clear. Nevertheless, this result is not entirely unlike that reported in rat follicles that undergo atresia, in vitro, since after incubation of whole antral follicles for 24 h in the absence of tropic hormone support, *bcl-2* mRNA levels are not altered compared to unincubated control follicles [42]. Moreover, a decrease in Bcl-x_{Long} protein precedes the decline in Bcl-2 that follows IL-2 withdrawal-induced apoptosis in CTLL-2 T cells [45]. Such results indicate that the down-regulation of bcl-2 may not be tightly coupled to the initiation of apoptosis in some cell systems, and further support the proposal that sustained *bcl-2* gene expression in ovarian follicles functions in a role other than solely as a death-suppressor.

In summary, results from the present studies provide novel information regarding the expression and regulation of *bcl-2*-related genes in ovarian granulosa cells. Differences in the levels of *nr-13*, *bcl-2*, and *bcl-*x_{Long} gene expression between granulosa and theca tissues and during follicle development were not unexpected, as there are a number of published reports that cell types vary in the relative expression of *bcl-2* and *bcl-*x_{Long}, and that the profiles of their relative expression can change with stage of cell differentiation (for review, see [25]). The pattern and level of *bcl-*x_{Long} mRNA expression during hen follicle development suggest that the *bcl-*x_{Long} has a more important role in modulating cell survival within granulosa cells, while *bcl-2* and *nr-13* may play roles in alternative processes such as regulation of cell proliferation and/or differentiation. Ongoing studies are now directed towards directly evaluating the physiological actions of the *bcl-2*-related family of proteins as regulators of granulosa cell viability, as well as their potential role in controlling cell proliferation and differentiation in this model system.

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