Bcl-w Forms Complexes with Bax and Bak, and Elevated Ratios of Bax/Bcl-w and Bak/Bcl-w Correspond to Spermatogonial and Spermatocyte Apoptosis in the Testis

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Bcl-w, a prosurvival member of the Bcl-2 family, is essential for spermatogenesis. However, the mechanisms by which Bcl-w participates in the regulation of apoptosis in the testis are largely unknown. To explore the potential role of Bcl-w in the regulation of apoptosis in the testis, the expression of Bcl-w mRNA and protein during testicular development and spermatogenesis, the dimerization with the proapoptosis members of the Bcl-2 family, and the responses to hormonal stimulation *in vitro* and apoptosis-inducing signals *in vivo* were investigated. Both Bcl-w mRNA and protein were detected in Sertoli cells, spermatogonia, and spermatocytes, as well as in Leydig cells. The steady-state levels of Bcl-w mRNA and protein were much higher in Sertoli cells than in spermatogonia and spermatocytes. In the adult rat testis, both Bcl-w mRNA and protein in Sertoli cells displayed a stage-specific expression pattern. Bcl-w could form complexes with Bax and Bak but not with Bad. Bax and Bak were immunohistochemically localized to the same cell types as Bcl-w, but with higher expression levels in spermatocytes and spermatogonia than in Sertoli cells. FSH could up-regulate Bcl-w mRNA levels in the seminiferous tubules cultured *in vitro*, whereas no effect was observed when testosterone was applied. Three animal models that display spermatogonial apoptosis induced by blockade of stem cell factor/c-kit interaction by a function-blocking anti-c-kit antibody, spermatocyte apoptosis induced by methoxyacetic acid, and apoptosis of spermatogonia, spermatocytes, and spermatids induced by testosterone withdrawal after ethylene dimethane sulfonate treatment were employed to check the changes of Bcl-w, Bax, and Bak protein levels during apoptosis of specific germ cells. In all three models, the ratios of Bax/Bcl-w and Bak/Bcl-w were significantly elevated. The present study suggests that Bcl-w is an important prosurvival factor of Sertoli cells, spermatogonia, and spermatocytes and participates in the regulation of apoptosis by binding proapoptotic factors Bax and Bak. The ratios of Bax/Bcl-w and Bak/Bcl-w may be decisive for the survival of Sertoli cells, spermatogonia, and spermatocytes. (Molecular Endocrinology 14: 682–699, 2000)

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INTRODUCTION

Apoptosis is a strictly regulated process involving sequential activation of specific signaling transduction pathway, disturbance of mitochondrial membrane function causing the release of intermembrane proteins into the cytosol, and finally stepwise degradation of the cell (1). Bcl-2 family members are key regulators of apoptosis. They may either promote cell survival (Bcl-2, Bcl-xL, Bcl-w, Mcl-1, A1/Bfl-1) or encourage cell demise (Bax, Bak, Bcl-Xs, Bad, Bid, Bik, Hrk, Bok) (1–3). It appears that members of the Bcl-2 family members interact among each other to form a dynamic equilibrium between homo- and heterodimers. Because members of those opposing factions can associate and seemingly titrate one another's function, their relative abundance in a particular cell type may determine its threshold for apoptosis (4). The competitive action of the pro- and antisurvival Bcl-2 family proteins regulates the activation of the pro-

teases (caspases) that dismantle the cell, but how they do so remains uncertain (1–3).

Bcl-w is a prosurvival member of Bcl-2 family proteins, and its physiological significance has been highlighted by two studies generating *bcl-w*-deficient mice (5, 6). Male *bcl-w*-deficient mice display normal testicular development before puberty, whereas after puberty Sertoli cells and germ cells of all types are severely reduced in number, and numerous apoptotic cells and no mature sperm are present in the seminiferous tubules, indicating an essential role of Bcl-w in normal spermatogenesis. However, the molecular mechanisms accounting for the testicular phenotypes in the *bcl-w*-deficient mice are still largely unknown.

In the present study, we analyzed the expression of Bcl-w mRNA and protein in the immature and mature rat testes, the potential dimerization of Bcl-w with the proapoptosis factors of Bcl-2 family proteins Bax, Bak, and Bad, and the regulation of Bcl-w mRNA levels by FSH and testosterone (T), as well as the responses of Bcl-w to specific germ cell-apoptosis-inducing signals by using three animal models.

RESULTS

Developmental and Stage-Specific Expression of Bcl-w mRNA in the Rat Testis

Bcl-w mRNA was detectable throughout all stages of testicular development. A peak was observed on day 20 after birth, and thereafter the expression levels slightly declined (Fig. 1, A and B).

Bcl-w mRNA was detected in the seminiferous tubules of all stages of the epithelial cycle. However, Bcl-w mRNA expression displays a stage-specific pattern (Fig. 1, C and D). Bcl-w mRNA levels were higher at stages II-VI, IX-XII, and XIII-I than at stages VII-VIII ($P < 0.05$, n = 3). Bcl-w mRNA was also detected from the purified Leydig cells from adult rat testis (Fig. 1C).

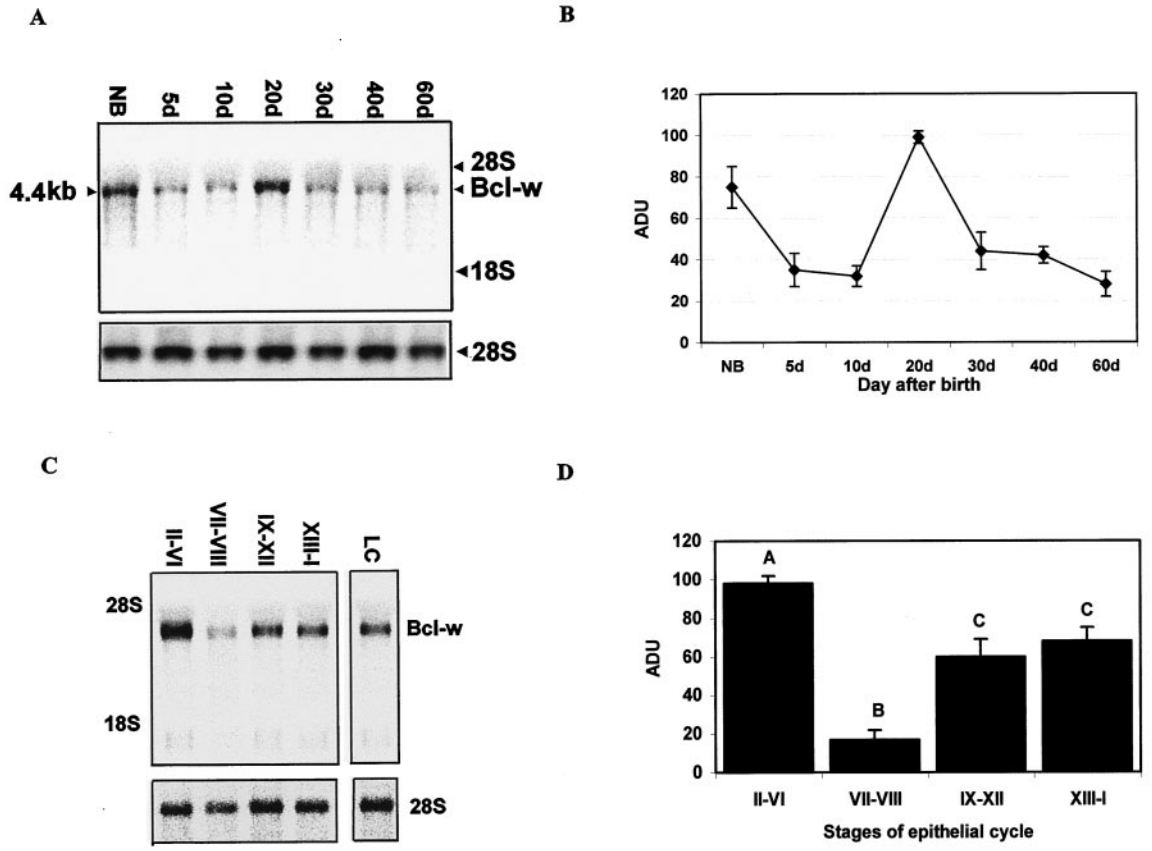

Fig. 1. Developmental and Stage-Specific Expression of Bcl-w mRNA in the Rat Testis

A, A Northern hybridization for Bcl-w mRNA expression during testicular development. Ages (NB, newborn; 5–60d, 5–60 days of age) are marked on the *top* and the positions of 28S and 18S rRNA are labeled on the *right*. B, Quantitative analysis of Bcl-w mRNA levels during testicular development. Data are presented as mean ± SEM of three independent experiments using RNA isolated from different animals. ADU, Arbitrary densitometric value. C, Stage-specific expression of Bcl-w mRNA in the rat seminiferous epithelium. A Northern hybridization for measuring the steady-state Bcl-w mRNA levels in four pooled stages of the rat seminiferous epithelium and purified Leydig cells (LC). *Roman numerals* refer to stages of the epithelial cycle. D, Quantification of Northern hybridization results of stage-specific expression from three independent experiments. Bars represent mean ± SEM. ADU, Arbitrary densitometric value. Data with *different letters* are significantly different ($P < 0.05$, n = 3).

Localization of Bcl-w mRNA and Protein

In the newborn rat testis, specific hybridization signals were detected in both Sertoli cell and gonocytes (Fig. 2). At 10 and 20 days of age, the signals were present in Sertoli cells, spermatogonia, and spermatocytes. In the adult testis, the most intense signals were confined to the cytoplasm of Sertoli cells and spermatogonia, whereas spermatocytes showed weaker signals (Fig. 3). Stage-specific pattern of signals were observed, with more intense signals at stages I–VI and IX–VIX, and less intense signals at stages VII–VIII (Fig. 3, A and A′).

The strong immunostaining was localized to the cytoplasm of Sertoli cells, while the weak staining was observed in the cytoplasm of spermatogonia and spermatocytes. Sertoli cell staining displayed a stage-specific pattern with strongest staining at stages II–VI, strong at stages IX—XII and XIII-I, and weak at stages VII–VIII. Leydig cells were also positively stained (Fig. 4).

The localization and stage-specific expression of Bcl-w mRNA and protein is summarized in Fig. 5.

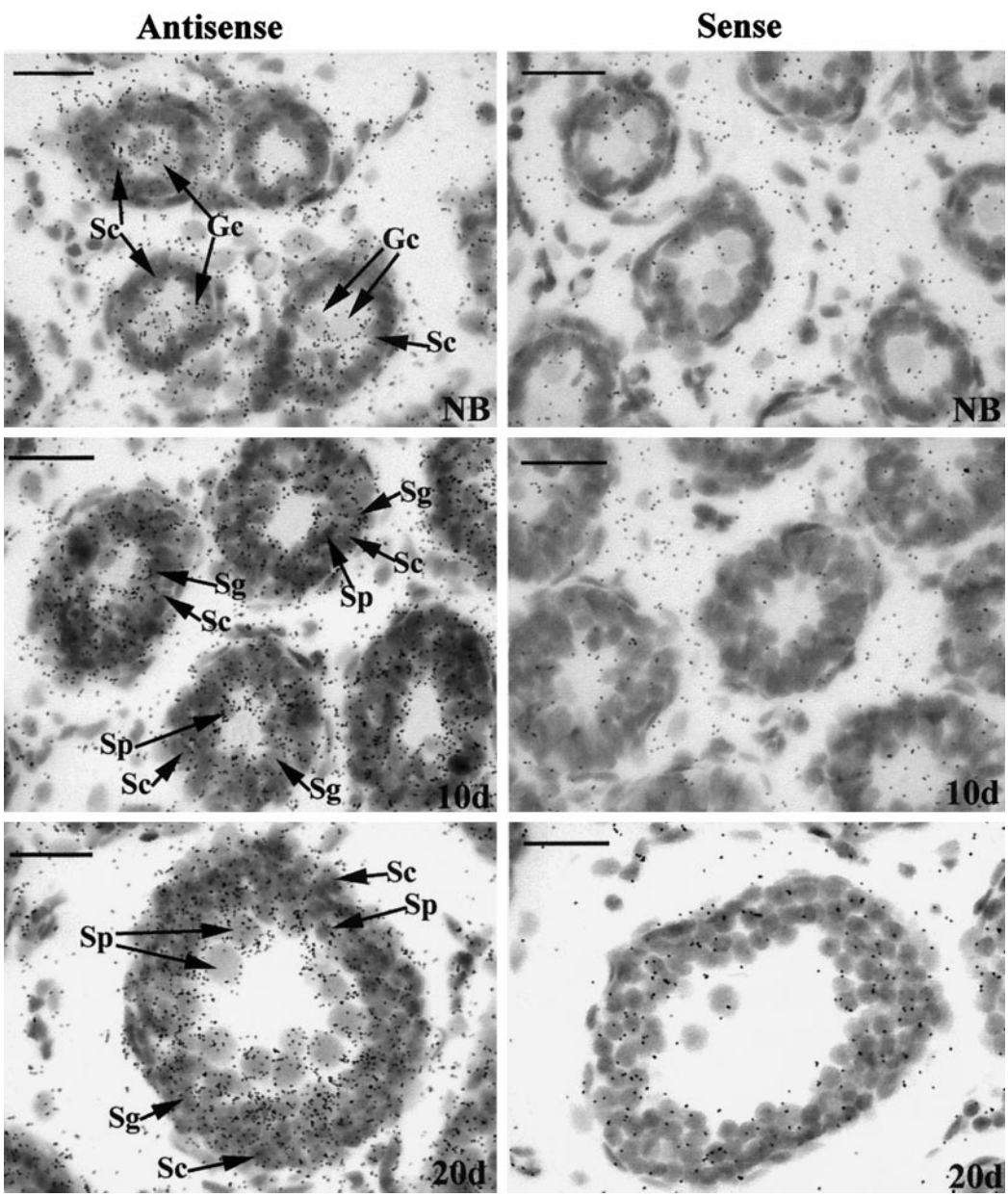

Fig. 2. Localization of Bcl-w mRNA in the Immature Rat Testis

In situ hybridization using antisense probe is shown in the *left panel*. The control slides using sense probe is on the *right*. *Black spots* represent hybridization signals (silver grains). The age of the rat used is marked in each panel. Signals can discerned in both Sertoli cells and germ cells. NB, Newborn; Sc, Sertoli cell; Gc, gonocyte; Sg, spermatogonia; Sp, spermatocyte. *Bars* = 0.25 μm.

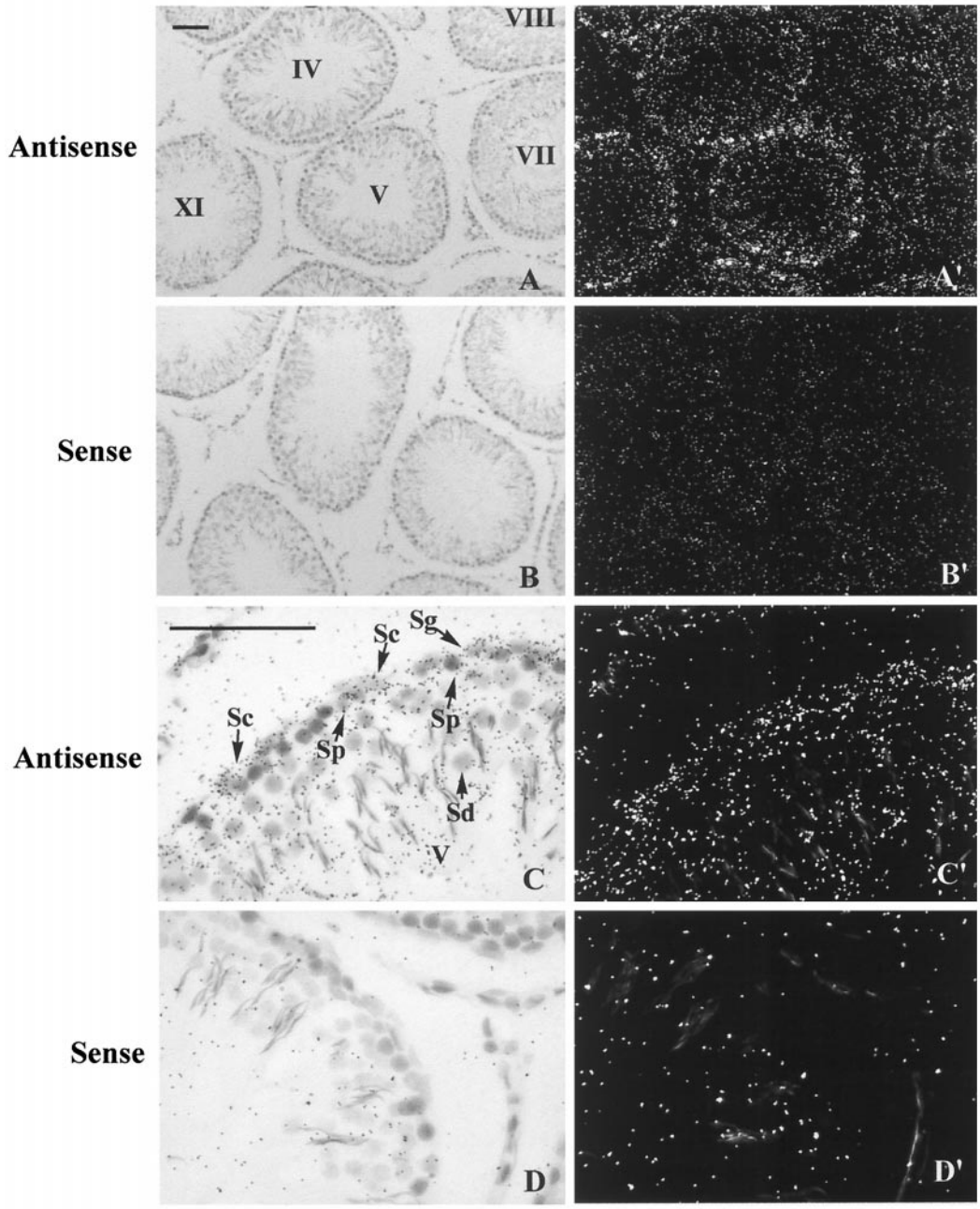

Fig. 3. Bcl-w mRNA Localization in the Adult Rat Testis

The bright field is shown in the *left panel,* and the corresponding dark field is shown on the *right*. The specific signals are mainly confined to the basal compartment of the seminiferous epithelium. The background signals were evaluated according to the sense probe (B,B′ D,D′). *Bars* = 0.5 μm. Note that the signals at stages VII–VIII are less intense than those at other stages (see A and A′). Sc, Sertoli cell; Sg, spermatogonia; Sp, spermatocyte; Sd, spermatid.

FSH Up-Regulates Bcl-w mRNA Levels *in Vitro*

No significant effect was observed after 8 h incubation in the presence of FSH (data not shown). After 30 h incubation in the presence of FSH, significant increases of the steady-state Bcl-w mRNA levels were found at stages II–VI, IX—XII, and XIII-I ($P < 0.05$, n = 3), whereas no significant effect was found at stages VII–VIII (Fig. 6). No significant effect was observed when the seminiferous tubules were incubated in the presence of T (data not shown).

Bcl-w Forms Complexes with Bax and Bak, but Not with Bad

By immunoblotting analysis, Bcl-w was detected from the immunoprecipitates of whole testis lysates prepared with anti-Bax, or anti-Bak antibody, while no

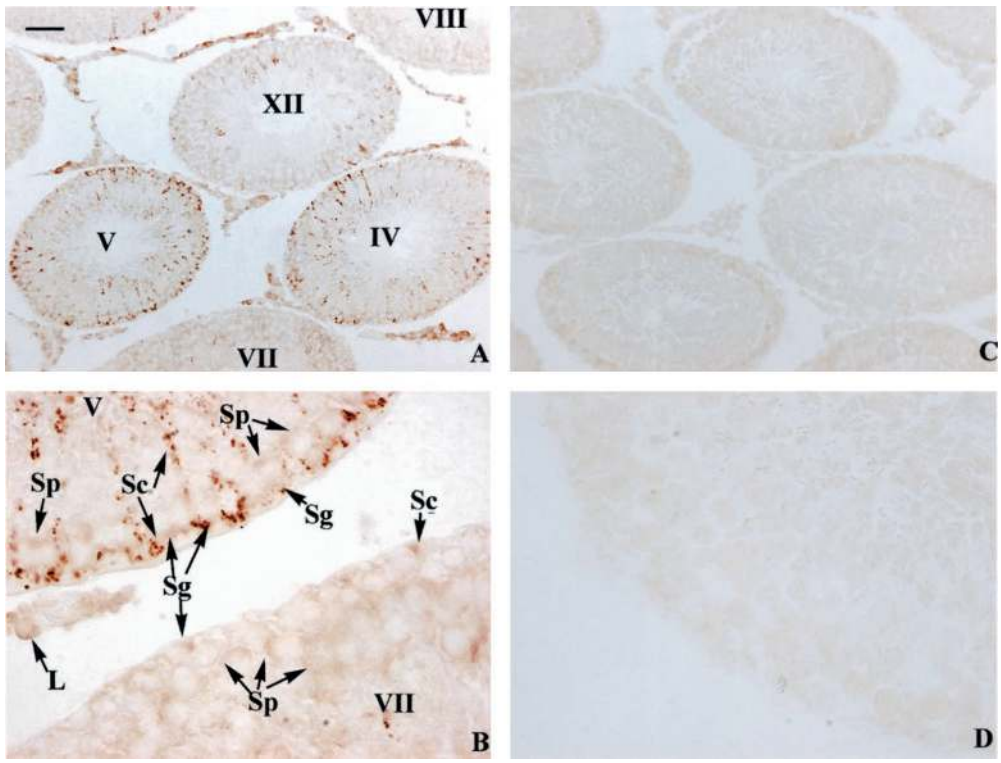

Fig. 4. Bcl-w Protein Localization in the Adult Rat Testis

Immunostaining using rabbit anti-Bcl-w polyclonal antibody is shown in the *left panel* at low (*top*) and high magnification (*bottom*). The control staining performed using preabsorbed antibody is shown in the *right panel. Bars* = 0.5 μm. Note that the immunostaining in the Sertoli cells at stage VII is much weaker than those at other stages. Sc, Sertoli cell; Sg, spermatogonia; Sp, spermatocyte; L, Leydig cell.

Bcl-w was detected from Bad immunoprecipitates (Fig. 7). The lysates of purified Sertoli cells, spermatogonia, and spermatocytes were immunoprecipitated using anti-Bax, anti-Bak, or anti-Bad antibody, and then the immunoprecipitates were used subsequently for detection of Bcl-w, Bax, Bak, and Bad. Similarly, Bcl-w was detected from Bax- and Bak-immunoprecipitates prepared from the lysates of purified Sertoli cells, spermatogonia, and spermatocytes (Fig. 7). The samples into which no antibody or rabbit IgG was added during immunoprecipitation were used as negative controls, and no signal was detected (Fig. 7). Bax and Bak were detected from Sertoli cells, spermatogonia, and spermatocytes, while Bad was present in Sertoli cells and spermatogonia, but not in spermatocytes (Fig. 7). None of these four Bcl-2 family proteins were detected from spermatids (data not shown).

Localization of Bax and Bak Proteins in the Rat Testis

Since Bcl-w could be coimmunoprecipitated with Bax and Bak, the localization of these two proteins was further studied. Immunostaining using anti-Bax antibody was localized to the cytoplasms of Sertoli cells, spermatogonia, and spermatocytes (Fig. 8, A–D). Leydig cells were stained sporadically. The immunostaining pattern of anti-Bak antibody was similar to that of anti-Bax antibody (Fig. 8, E–H). The cytoplasms of Sertoli cells, spermatogonia, and spermatocytes were all positively stained. No immunoreaction was found in spermatids. Few Leydig cells displayed positive staining.

In contrast to the immunostaining pattern of Bcl-w, the staining of both Bax and Bak was stronger in spermatogonia and spermatocytes than in Sertoli cells. The relative abundance of Bcl-w, Bax, and Bak in the adult rat testis is summarized in Table 1.

Subcellular Distribution of Bcl-w, Bax, and Bak

Soluble, crude membrane and nuclear fractions of Sertoli cells, spermatogonia, and spermatocytes were prepared by hypotonic lysis, Dounce homogenization, and differential centrifugation. The protein samples were analyzed by Western blotting (Fig. 9). Bcl-w was detected predominantly in the crude membrane fraction. In Sertoli cells, Bcl-w appeared to exist in both the soluble fraction and the high-speed membrane pellet (Fig. 9). Bax and Bak were found as soluble proteins in all three types of cells (Fig. 9). β-Actin was used as protein quality control with each experiment (Fig. 9). None of these three proteins was detected in the nuclear fraction (data not shown).

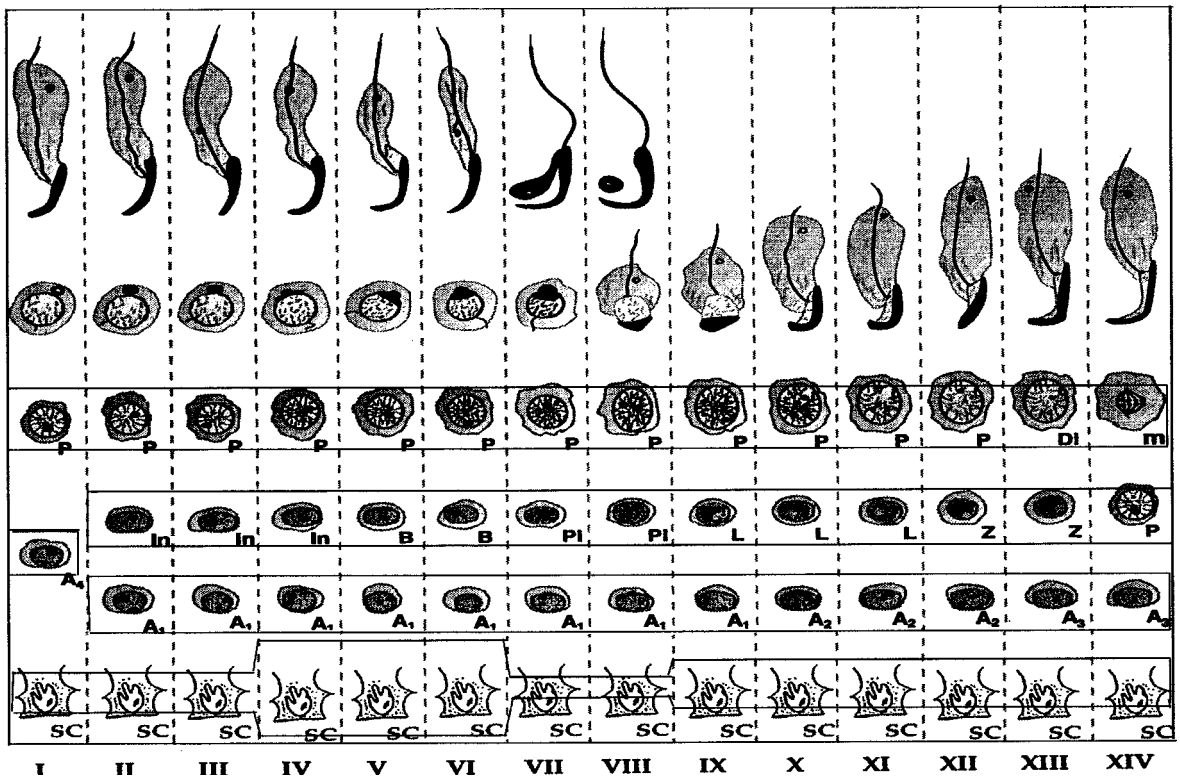

Fig. 5. Schematic Illustration of the Expression of Bcl-w mRNA and Protein in the Rat Seminiferous Epithelium

Cells in the frame express Bcl-w, and the width of the frame represents the relative abundance of Bcl-w. The specific cell associations in the *vertical columns* represent specific stages (*Roman numerals*) of the epithelial cycle. Sc, Sertoli cells; A1–4, type A spermatogonia; In, intermediate spermatogonia; B, type B spermatogonia; Pl, preleptotene spermatocytes; L, leptotene spermatocytes; Z, zygotene spermatocytes; P, pachytene spermatocytes; Di, diplotene spermatocytes; m, meiosis. Cells that are not marked are spermatids.

Expression of Bcl-w, Bax, and Bak in *bcl-w*-Deficient Mice

At 4 weeks of age, histology of the testis of bcl-w$^{-/-}$ mice still appears roughly normal with the exception of a significantly increased number of apoptotic germ cells (6). Using paraffin testis sections from 4-week-old bcl-w$^{-/-}$ and bcl-w$^{+/+}$ mice, we also checked the expression pattern of Bcl-w, Bax, and Bak proteins. Bcl-w was predominantly expressed in Sertoli cells and weakly in spermatogonia and spermatocytes. Bax and Bak were expressed mainly in spermatocytes and spermatogonia and moderately in Sertoli cells. The expression pattern of these three Bcl-2 family proteins in the murine testis (Fig. 10, A, D, and G) was quite similar to that in the rat testis (Figs. 4 and 8 and Table 1). In bcl-w$^{-/-}$ mice, Bcl-w was undetectable (Fig. 10B), while Bax was highly expressed in spermatocytes, spermatogonia, and Sertoli cells (Fig. 10E). Bak was also expressed in the bcl-w$^{-/-}$ mouse testis, but the immunoreactivity of Bak was higher in Sertoli cells than in spermatocytes and spermatogonia (Fig. 10H). No specific staining was found in the control sections using preabsorbed antibodies (Fig. 10, C, F, and I)

Responses of Bcl-w, Bax, and Bak to Spermatogonial Apoptosis Induced by Blockade of Stem Cell Factor (SCF)/c-kit Interaction

Blockade of SCF/c-kit interaction by a function-blocking anti-c-kit antibody, ACK-2, can deplete proliferating spermatogonia (7, 8), which are located at stages I, IV, VI, IX, XII, and XIV of the epithelial cycle. As shown in Fig. 11, the number of *in situ* 3′-end labeling (ISEL)-positive spermatogonia at stage I was increased in a time-dependent manner during 96 h of blockade (Fig. 11, A and B). DNA laddering analysis revealed that DNA fragmentation took place also in a time-dependent manner, which was consistent with the ISEL staining data (Fig. 11C).

Bcl-w protein levels declined significantly (Fig. 12, A and B; $P < 0.05$, n = 3) after 48 h of blockade by ACK-2. During 48–96 h of blockade, the Bcl-w protein levels were reduced to about half of the control levels. Both Bax and Bak levels were significantly increased after 48 h of blockade (Fig. 2, A and B; $P < 0.05$, n = 3). After 72 h of blockades, both levels were increased 2.5-fold higher than those in the controls (Fig. 12, A and B). The ratios of Bax/Bcl-w and Bak/Bcl-w were

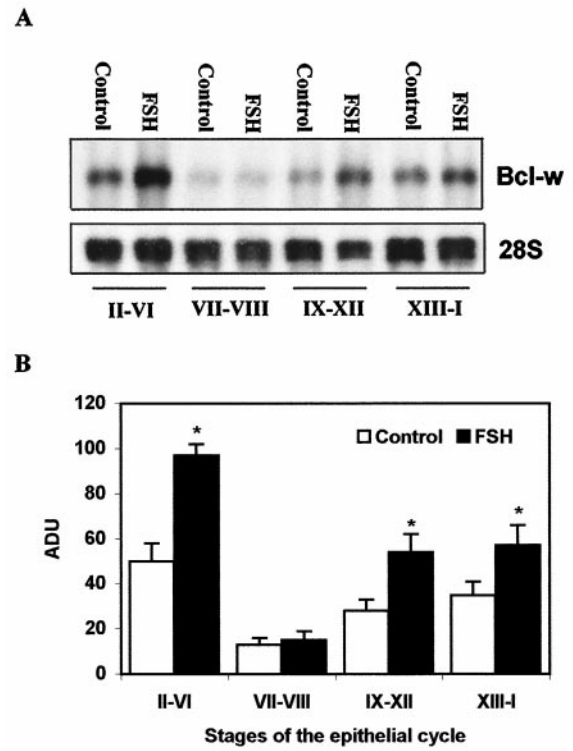

Fig. 6. Stage-Specific Up-Regulation of Bcl-w mRNA Levels by FSH *in Vitro*

A**,** A Northern hybridization result. B, Quantitative analysis of the response of Bcl-w mRNA levels to FSH *in vitro.* Data are presented as mean ± SEM of three independent experiments. *, $P < 0.05$, as compared with the controls. ADU, Arbitrary densitometric value.

significantly elevated (Fig. 12C; $P < 0.01$, n = 3) during 48 h- 96 h of blockade.

Responses of Bcl-w, Bax, and Bak to Spermatocyte Apoptosis Induced by Methoxyacetic Acid (MAA)

Spermatocytes at stages I–VI can be selectively depleted through apoptosis within 24 h after oral administration of MAA (9–11). Consequently, at day 4 after MAA treatment, the seminiferous tubules at certain stages are devoid of spermatocytes. At day 18, certain stages lack round spermatids, and at day 30, there are no elongating spermatids at defined stages.

ISEL staining revealed that most of the spermatocytes are still ISEL-negative at 6 h after MAA treatment. Spermatocytes start to undergo apoptosis at around 8 h, and apoptosis peaks at 12 h, and upon 24 h after MAA treatment, most of apoptotic spermatocytes at stages I–VI have been depleted (data not shown). Bcl-w protein levels were significantly reduced (Fig. 12, D and E; $P < 0.05$, n = 3), whereas Bax and Bak levels were increased significantly (Fig. 12, D and E; $P < 0.01$, n = 3) at 12 h after MAA treatment, when spermatocytes were undergoing intensive apoptosis. Bcl-w levels were lower than those of the controls at day 4, when seminiferous tubules at stages I–VI were devoid of spermatocytes. At days 18 and 30, Bcl-w levels were much higher than those in the controls (Fig. 12, D and E). Ratios of Bax/Bcl-w and Bak/Bcl-w were significantly elevated at 12 h and 24 h after MAA treatment (Fig. 12F; $P < 0.01$, n = 3).

Responses of Bcl-w, Bax, and Bak to the Germ Cell Apoptosis Induced by T Withdrawal after Ethylene Dimethane Sulfonate (EDS) Treatment

Germ cells, mainly spermatocytes and spermatids, undergo massive apoptosis between days 5 and 15 after EDS treatment due to lack of T caused by Leydig cell depletion (12–14). In the present study, controls (3 rats/time point) displayed similar levels at every time point (0 day, 1–4 days, 7 days, 10 days, 20 days, 40 days, data not shown) studied, and therefore only the controls (n = 3) at 1 day are shown in Fig. 12, G–I. Bcl-w protein levels were significantly reduced at days 4–15, as compared with the controls (Fig. 12, G and H; $P < 0.01$, n = 3). Two- to 5-fold increases of Bax protein levels were found during the same period of time (Fig. 12, G and H; $P < 0.01$, n = 3). Bak protein levels were significantly increased at day 10 after EDS treatment (Fig. 12, G and H; $P < 0.05$, n = 3). The ratios of Bax/Bcl-w and Bak/Bcl-w were significantly elevated (Fig. 11I; $P < 0.01$, n = 3) during days 4–15 after EDS treatment.

DISCUSSION

During testicular development, Bcl-w was expressed persistently in Sertoli cells and defined germ cells. The expression profile of Bcl-w mRNA during testicular development is consistent with that of Bcl-w protein (15), indicating the transcriptional regulation of *bcl-w* gene expression. In addition, Bcl-w, Bcl-xl, Bax, Bad, Bak, and p53 were all expressed in immature testis, and the dynamic changes in their expression correlate with the first wave of germ cell apoptosis, which takes place around 10–20 days after birth in mice (16, 17) and 10–30 days in the rat (15). In the adult rat testis, the mRNA localization is consistent with the protein localization, showing similar expression sites and stage-specific expression patterns. Both Bcl-w mRNA and protein were dominantly expressed in Sertoli cells. Thus, the stage-specific expression pattern of Bcl-w mRNA and protein in the seminiferous epithelium apparently results from the stage-specific expression in Sertoli cells. The mechanism of stage-specific expression of Bcl-w is currently unknown. Given that Bcl-w is a prosurvival factor, the stage-specific expression pattern in Sertoli cells might potentially represent stage-specific susceptibility of Sertoli cells to apoptosis. Interestingly, Bax and Bak were also detected in Sertoli cells, but at much lower levels than in spermatogonia

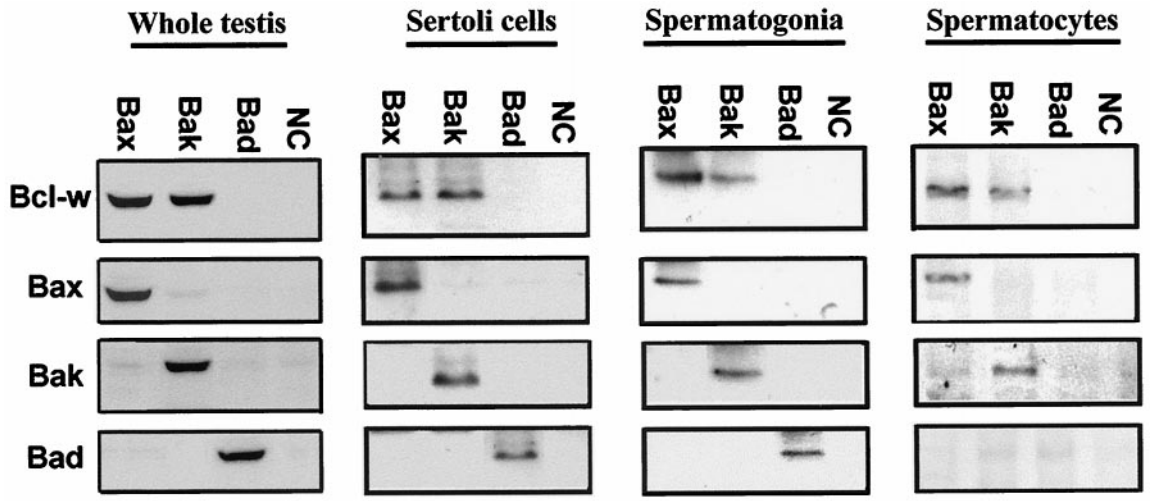

Fig. 7. Coimmunoprecipitation of Bcl-w with Bax and Bak

The lysates of whole testis, purified Sertoli cells, spermatogonia, spermatocytes, and spermatids were first immunoprecipitated using anti-Bax, anti-Bak, or anti-Bad antibody (marked on the *top* of each lane), and then the immunoprecipitates were subjected to immunoblotting by anti-Bcl-w antibody (the *first panel from the top*). The membrane was then stripped and subsequently detected using anti-Bax, anti-Bak, or anti-Bad antibody (the *second through fourth panels from the top*). NC stands for negative control sample, in which no antibody or rabbit IgG was added during immunoprecipitation.

and spermatocytes (Table 1). Bcl-2 is not expressed in the seminiferous tubule (18). Bcl-xL appears to be restricted to spermatids (19) and Mcl-1 is present in Leydig cells in the adult testis (20). So far, Bcl-w appears to be the only one of the prosurvival members of the Bcl-2 family that has been localized to Sertoli cells in the adult testis. The dominant expression of prosurvival factor Bcl-w over the proapoptosis regulators, Bax and Bak, in Sertoli cells might represent a mechanism by which Sertoli cells are less prone to apoptosis than germ cells. *In situ* hybridization signals in Leydig cells were very difficult to judge due to the resolution limit of the method used. However, immunoreaction of Leydig cells to Bcl-w antibody and detection of Bcl-w mRNA from the purified Leydig cells proved that Leydig cells do express Bcl-w. Another recent study also demonstrates that Leydig cells express Bcl-w (21).

By using the whole testis lysate, Bcl-w was found to be coimmunoprecipitated with Bax and Bak. However, it has been documented that in the presence of detergent, Bcl-2 family proteins can associate (22). Since the distribution of Bcl-w and Bax/Bak are mainly Sertoli cells and spermatocytes, respectively, it is reasonable to assume that interaction of these molecules can occur during processing of the tissue. Therefore, we prepared highly purified Sertoli cells, spermatogonia, spermatocytes, and spermatids and performed immunoprecipitation and cellular fractionation. Consistently, Bcl-w was found to be associated with Bax and Bak, but not with Bad in all three types of cells, indicating that the heterodimers of Bcl-w/Bax and Bcl-w/Bak do exist in each type of cell that expresses them *in vivo*. Bcl-w, Bax, and Bak were all detected in Sertoli cells, spermatogonia, and spermatocytes, but not in spermatids. The results further confirm that Sertoli cells, spermatogonia, and spermatocytes are the sites of the production of these three proteins. Bad was detected in Sertoli cells and spermatogonia by immunoprecipitation, which is consistent with the previous immunohistochemical data showing that Bad was localized to these cells (23).

Bcl-2 family proteins are believed to be a group of membrane proteins that regulate apoptosis through *in vivo* dimerizations. With few exceptions (Bad, Bid, A1, E1B19K), the Bcl-2 family proteins possess a C-terminal hydrophobic transmembrane domain of approximately 20 residues, which determines their insertion into different intracellular membranes including those of the endoplasmic reticulum, the nuclear envelope, and mitochondrial membranes (1). The intracellular distribution of the Bcl-2 family proteins can depend on the cell type and also on the family member (1). In murine thymocytes, Bax was found predominantly as a soluble protein, while Bcl-2 was not detected in the soluble fraction, but was present in both high-speed membrane and nuclear fractions (24). In human leukemia cells, Bcl-2 was mainly located to mitochondria and was not present in the cytosol and Bcl-X_L distributed in both mitochondria as a membrane-bound protein and the cytosol as a soluble protein. Bcl-X_S, Bax, and Bad, on the other hand, were mainly located to the cytosol (25). In the present study, we demonstrate that Bcl-w, a homolog of Bcl-2, is predominantly an integral membrane protein, whereas Bax and Bak are mainly cytosolic in the healthy Sertoli cells, spermatogonia, and spermatocytes. The observation that Bax, Bak, and a significant portion of Bcl-w in Sertoli cells are cytosolic suggests that their C-terminal hydrophobic domains may be hidden either

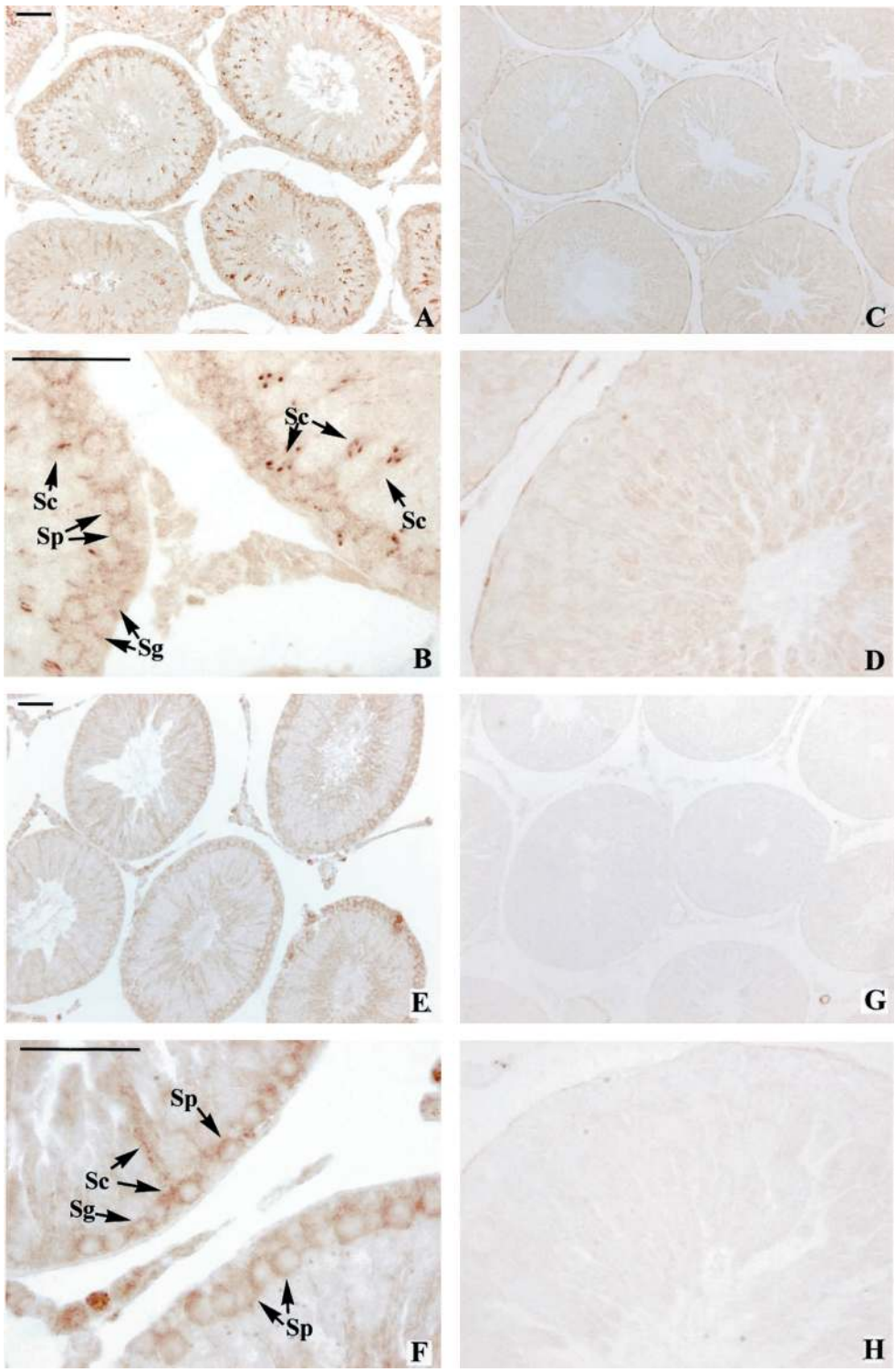

Fig. 8. Localization of Bax and Bak Proteins in the Adult Rat Testis

Immunostaining using specific rabbit anti-Bax polyclonal antibody (A and B) and rabbit anti-Bak antibody (E and F) is shown in the *left panel*. The control staining using preabsorbed antibodies is shown in the *right panel* (C and D for Bax, G and H for Bak). *Bars* = 0.5 μm. Sc, Sertoli cell; Sg, spermatogonia; Sp, spermatocyte.

within the interior of the proteins or may be involved in binding other cytosolic factors. Interestingly, recent evidence suggests that the subcellular localization of at least certain members of the Bcl-2 family changes during apoptosis (24, 25). For example, Bax and Bcl-xL, but not Bcl-2, have been found to redistribute from

Table 1. Relative Abundance of Bcl-w, Bax, and Bak Proteins in the Rat Testis

Cell Type	Bcl-w	Bax	Bak
Sertoli cells	+++	+	+
Spermatogonia	+	+	+
Spermatocytes	+	++	++

Criteria for semiquantification: +, weak; ++, strong; +++, very strong immunoreactivity.

the cytosol to intracellular membranes upon induction of thymocyte apoptosis, and Bax, Bad, and Bcl-Xs can redistribute from cytosol to mitochondria in two human leukemia cell lines. Therefore, it would be very interesting to determine, in a future study, how these Bcl-2 family proteins potentially translocate in response to the induction of germ cell apoptosis.

FSH has a prosurvival effect on germ cells *in vitro* (26). Since in the *in vitro* seminiferous tubule culture system most of the apoptotic cells are germ cells and Sertoli cells are more resistant to apoptosis than germ cells, it is plausible to ascribe the changes of mRNA levels in response to FSH stimulation to germ cells. Thus, up-regulation of Bcl-w mRNA levels by FSH might account for its prosurvival effect on germ cells *in vitro*. Since FSH receptor is mainly located on Sertoli cells, the prosurvival effect of FSH must be mediated through Sertoli cells (27). In fact, FSH prosurvival effect has been found to be partially mediated through SCF/c-kit interaction (28), and SCF from Sertoli cells could up-regulate Bcl-w protein levels in germ cells during 48 h culture *in vitro* (15).

Prosurvival members of Bcl-2 family exert prosurvival function by forming complexes with antisurvival members of Bcl-2 family and therefore suppressing their death-inducing effects (4). In the adult rat testis, Bcl-w forms complexes with Bax and Bak, but not with Bad. Consistently, Bax and Bak have similar expression sites to Bcl-w. Higher levels of Bax and Bak and lower levels of Bcl-w in spermatogonia and spermatocytes might imply that these types of cells are more susceptible to apoptosis than Sertoli cells. Neither Bcl-w, Bax, nor Bak is expressed in spermatids, suggesting that spermatid apoptosis might be regulated by other members of the Bcl-2 family. Indeed, Bcl-xL (19) and Diva (29) appear to be expressed only in spermatids, but little is known about these two factors in spermatid apoptosis. A recent study shows that Bcl-w forms a complex with Bax, but not with Bak, in the mammary gland (30). This might reflect the tissue-specific action of Bcl-2 family members since Bcl-2 family members, both the proapoptotic and antiapoptotic, are differentially expressed in different tissues and cells, and therefore the dimerization status could be different as well (1–3).

The changes of Bcl-w levels during apoptosis of specific germ cell types induced by different methods strongly support that Bcl-w is a prosurvival factor of spermatogonia and spermatocytes *in vivo*. It has been well characterized that the proliferating spermatogonia can be depleted when SCF/c-kit interaction is blocked by a function-blocking antibody, ACK-2, in the testis (7, 8). In the present study, spermatogonial apoptosis was induced. After 4 days of treatment with ACK-2, the number of apoptotic spermatogonia increased dramatically. DNA laddering analysis indicated that the ISEL-positive cells were apoptotic rather than necrotic. The time-dependent reduction of Bcl-w and elevation of Bax and Bak correlate with the time-dependent spermatogonial apoptosis. Three specific findings from this study are of interest: 1) depletion of proliferating spermatogonia by blockade of SCF/c-kit interaction is mediated via apoptosis, implicating the involvement of SCF/c-kit system in the regulation of spermatogonial apoptosis; 2) increased ratios of Bax/Bcl-w and Bak/Bcl-w correlate with spermatogonial death; 3) blockade of SCF/c-kit interaction differentially affects Bcl-w, Bax, and Bak expression, implying the involvement of other downstream factors or transcription factors in the regulation of gene expression of these three regulators of apoptosis.

MAA has been found to be able to selectively deplete spermatocytes through apoptosis within 24 h after oral administration (9–11). In the present study, we used this model to specifically induce spermatocyte apoptosis and monitored the changes of Bcl-w, Bax, and Bak levels every 6 h during the first 24 h. Morphological observation and ISEL staining indicate that MAA depletes spermatocytes at stages I-VI within 24 h after administration, and spermatocyte apoptosis peaked at 12 h after treatment (data not shown). There are three findings from this model that deserve further comment: 1) selective induction of apoptosis of spermatocytes at stages I–VI by MAA correlates with increased ratios of Bax/Bcl-w and Bak/Bcl-w; 2) reduction of Bcl-w levels at day 4 after MAA treatment is apparently due to the depletion of spermatocytes, which express Bcl-w. Elevations of Bcl-w levels at day 18 and day 30 result from the enrichment of Bcl-w-expressing cells in the testis after depletion of round spermatids and elongating spermatids. These results further validate our localization data, showing that Sertoli cells, spermatogonia, and spermatocytes are the expression sites of Bcl-w; 3) since Bcl-w, Bax, and Bak are commonly expressed in spermatocytes at all stages in the epithelial cycle, the selective apoptosis of spermatocytes suggests that there might be some switching points that are responsible for turning on the apoptosis machinery only at stages I-VI.

It is well known that EDS can selectively deplete Leydig cells within 48 h after administration, and T declines abruptly to an undetectable level at day 3 and remains there until day 15 (12, 13, 31, 32). From day 5 to day 15, germ cells, mainly spermatocytes and spermatids, undergo a massive wave of apoptosis as a consequence of T withdrawal. In the present study, the reduced levels of Bcl-w and elevated levels of Bax and Bak correlate with the wave of apoptosis. The reversed ratios at day 20 correlate with the reduced

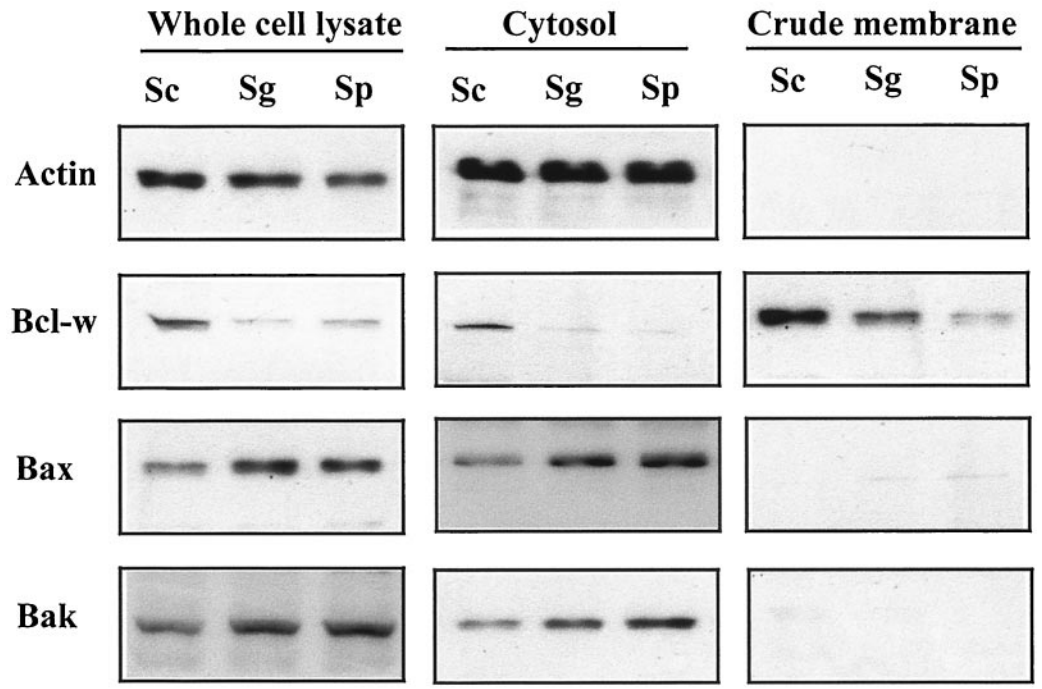

Fig. 9. The Expression of Bcl-w, Bax, and Bak at the Whole Cell Level and Subcellular Distribution in Sertoli Cells (Sc), Spermatogonia (Sg), and Spermatocytes (Sp)

Purified Sertoli cells (from 20-day-old rats), spermatogonia (from 9-day-old rats), and spermatocytes (from adult rats) were fractionated into soluble and crude membrane fractions. The whole cells and the crude membrane fraction were dissolved with 0.5% NP-40 containing lysis buffer. The protein extracts were separated on 12.5% SDS-PAGE and transferred onto nitrocellulose membrane followed by detection using anti-Bcl-w, anti-Bax, and anti-Bak antibodies. β-Actin served as a control in each experiment.

number of apoptotic germ cells since at this time point the testosterone level has already increased and the number of apoptotic germ cells has decreased dramatically (Refs. 12 and 32 and W. Yan, J. Kero, I. Huhtaniemi, and J. Toppari, paper submitted). Since Bcl-w and its partners, Bax and Bak, are expressed in spermatogonia and spermatocytes, the changes should result from the apoptotic events taking place in these cells. Thus, the increased ratios of Bax/Bcl-w and Bak/Bcl-w might produce more homodimers, such as Bax/Bax, Bax/Bak, or Bak/Bak, which promote apoptosis. Several studies (Refs. 12 and 32 and W. Yan, J. Kero, I. Huhtaniemi, and J. Toppari, paper submitted) have shown that after EDS treatment, not only does the testosterone level decrease, and the LH level goes up as a feedback, but the FSH level increases as well. Therefore, the changes of these three Bcl-2 family members could not be simply ascribed to the effect of T withdrawal. However, as FSH could up-regulate Bcl-w levels and in the EDS-treated rats Bcl-w levels were down-regulated when FSH levels were severalfold higher than the controls and testosterone levels were undetectable from day 2 to day 10 (Ref. 32 and W. Yan, J. Kero, I. Huhtaniemi, and J. Toppari, paper submitted), it is very likely that down-regulation of Bcl-w and up-regulation of Bax and Bak were due to lack of testosterone.

Both Bcl-2 and Bcl-xL levels were measured by Western blotting for all three models used. We could not detect Bcl-2 in the adult rat testis, while the positive control (prostate protein) gave a clear Bcl-2 band in Western analysis (data not shown). This is consistent with several previous reports showing that in the mature testis Bcl-2 is absent (16, 18). Bcl-xL levels are relatively low in the adult rat testis. We could not detect significant changes of Bcl-xL levels in the ACK-2-treated rats and MAA-treated rats (data not shown). However, in the EDS model, we did find changes of Bcl-xL levels (data not shown). Since it is localized to round spermatids (19), which express neither Bcl-w nor Bax nor Bak, it is likely that Bcl-xL might be involved in regulation of spermatid apoptosis.

Immunohistochemical detection of Bcl-w, Bax, and Bak in the Bcl-w deficient mice enabled us, first, to test the specificity of the antibodies used in the present study, and second, to explain possibly the reason why both Sertoli cells and germ cells start to undergo apoptosis after puberty in the absence of bcl-w. There is no discernable abnormality during testicular development in the *bcl-w*-deficient mice before puberty (6). However, this does not mean that Bcl-w has no role or the proapoptotic members are not present yet by this stage. In fact, in the immature testis, the antiapoptotic members, including Bcl-xL, Bcl-w, and Mcl-1, are all

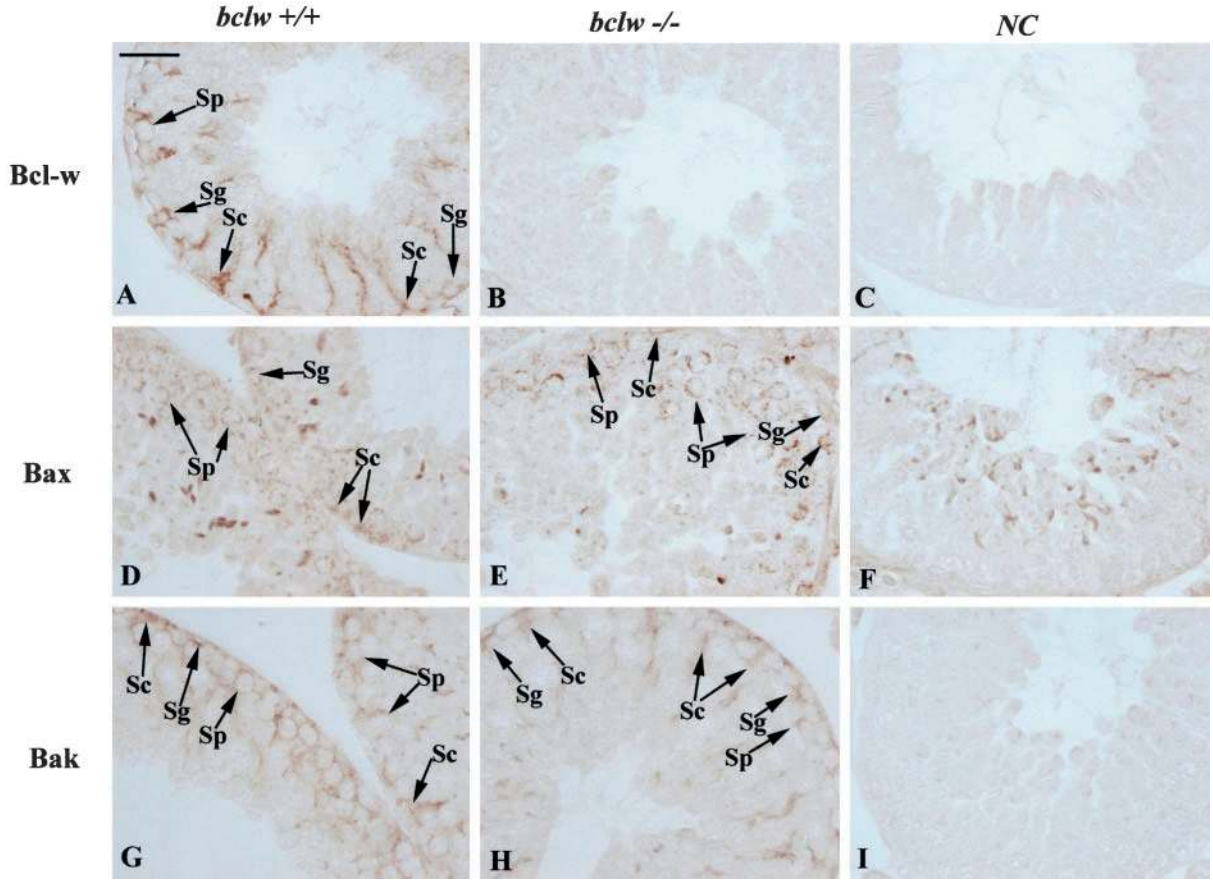

Fig. 10. Expression of Bcl-w, Bax, and Bak Proteins in bcl-w$^{-/-}$ and bcl-w$^{+/+}$ Mice

Bouin-fixed, paraffin-embedded testis sections were subjected to immunohistochemical staining using anti-Bcl-w (A and B), anti-Bax (D and E), or anti-Bak antibody (G and H). Preabsorbed antibodies were used for control staining (C for Bcl-w, F for Bax, and I for Bak). Sc, Sertoli cell; Sg, spermatogonia; Sp, spermatocyte. *Bar* = 0.5 μm.

highly expressed by almost all cell types including Sertoli cells and germ cells in the seminiferous epithelium (15). Similarly, the proapoptotic members of Bcl-2 family, including Bax, Bak, and Bad, are also expressed in the immature testis, and the expression sites are quite similar to those of the antiapoptotic members (15). It appears that the differential localization of Bcl-2 family proteins to different cell types in the seminiferous epithelium takes place around puberty. Thus, it is very likely that the absence of Bcl-w could be compensated by other antiapoptotic members of Bcl-w family, *e.g.* Bcl-xL, during prepubertal development. After puberty the failed spermatogenesis in *bcl-w*-deficient mice might result mainly from cell-extrinsic effects rather than cell-intrinsic effects (6), given the fact that the Bcl-w is much more abundant in Sertoli cells than in spermatocytes and spermatogonia. It is very likely that Sertoli cells are first implicated due to the absence of Bcl-w and undergo apoptosis via Bax- and/or Bak-mediated cell death. Death of Sertoli cells is devastating for germ cells, particularly for elongating spermatids and round spermatids, which is consistent with the findings that these two types of germ cells suffer from the most severe reduction in number in both lines of *bcl-w*-deficient mice (5, 6). However, depletion of spermatogonia and spermatocytes may be either mediated via Bax and Bak, due to lack of Bcl-w in a cell-intrinsic way, or due to Sertoli cell death in a cell-extrinsic manner.

Taken together, the present study suggests that Bcl-w is an important prosurvival factor of Sertoli cells, spermatogonia, and spermatocytes and participates in the regulation of apoptosis by binding proapoptotic factors Bax and Bak. The ratios of Bax/Bcl-w and Bak/Bcl-w might be decisive for the fates of Sertoli cells, spermatogonia, and spermatocytes.

MATERIALS AND METHODS

Experimental Animals and Treatments

Sprague Dawley male rats at the ages of 1 day, 5 days, 10 days, 20 days, 30 days, 40 days, and 2–3 months were used as experimental animals. They were housed two per cage in a controlled environment at 21 C with a 14-h light, 10-h dark cycle with free access to water and food.

All animal experiments were approved by the Turku University Committee on Ethics of Animal Experimentation.

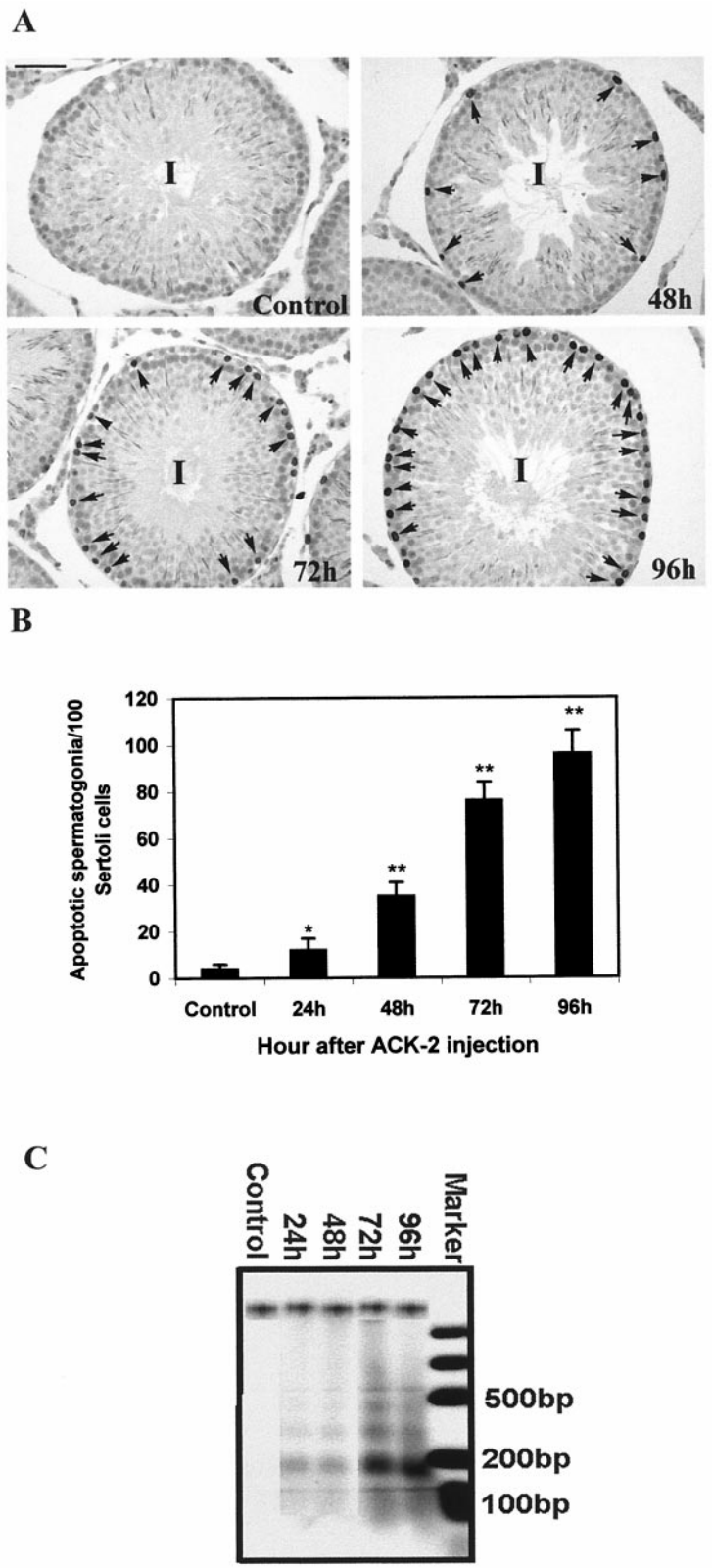

Fig. 11. Spermatogonial Apoptosis Induced by Blocking SCF/c-kit Interaction by ACK-2

A, Microphotographs show ISEL staining result, time-dependent apoptosis of spermatogonia at stage I at 48 h, 72 h, and 96 h after ACK-2 injection. *Bar* = 0.25 μm. *Arrows* point to ISEL-positive spermatogonia. B, Quantification of time-dependent apoptosis of spermatogonia induced by ACK-2. Data are presented as mean ± SEM of 12 cross-sections from three rats. *, $P < 0.05$; **, $P < 0.01$, as compared with the controls. C, Testicular DNA fragmentation pattern after injection of ACK-2.

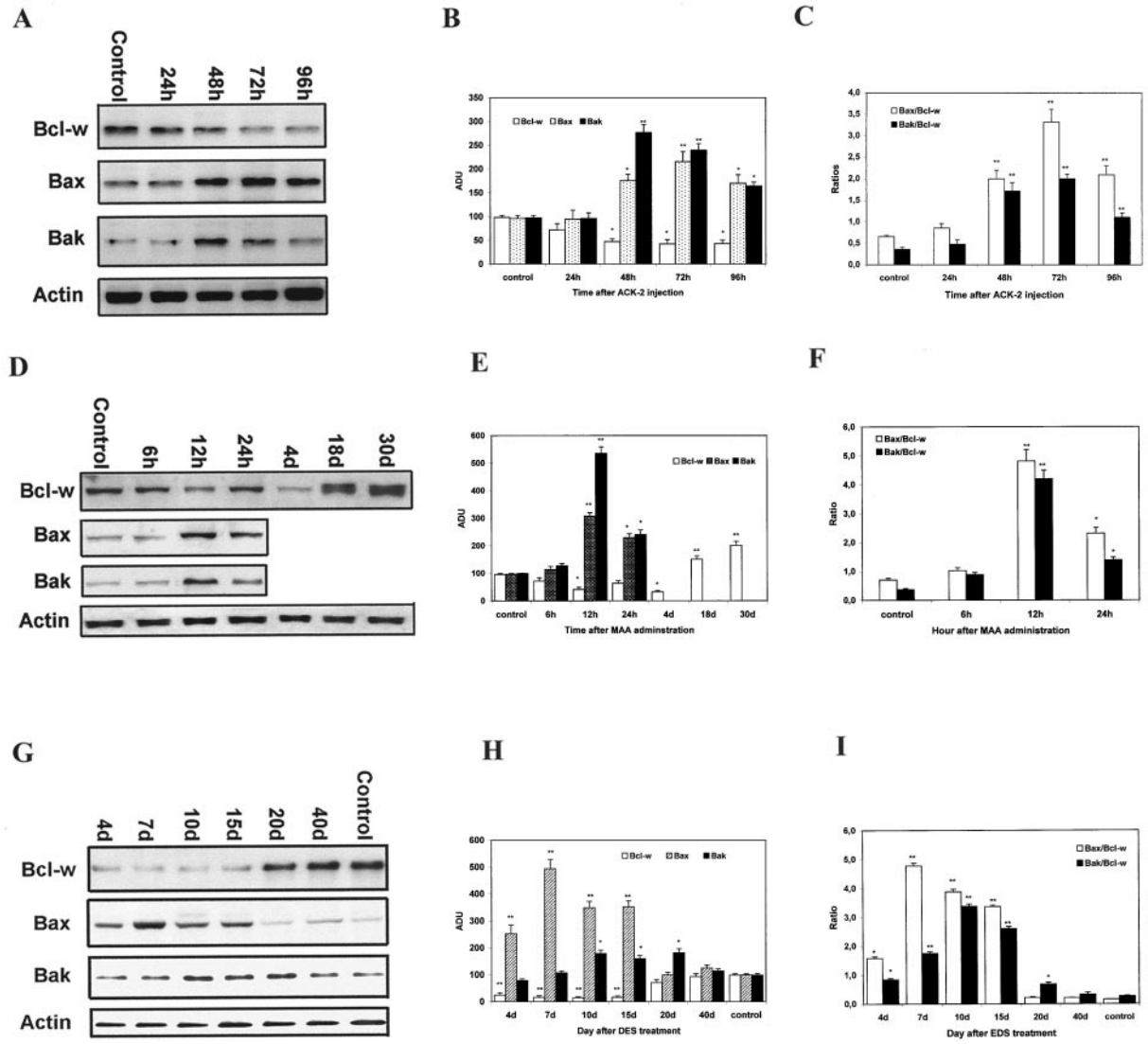

Fig. 12. Changes of Bcl-w, Bax, and Bak Protein Levels during Germ Cell Apoptosis Induced by Blocking SCF/c-kit Interaction by Injection of ACK-2 Antibody (Model 1, A–C), MAA Treatment (Model 2, D–F), and Testosterone Withdrawal after EDS Treatment (Model 3, G–I)

The representative Western blotting results were shown in the *left panel* (A for model 1, D for model 2, and G for model 3). The quantitative analysis of changes in the levels of Bcl-w, Bax, and Bak proteins during germ cell apoptosis are shown in the *middle panel* (B for model 1, E for model 2, and H for model 3). Changes of the ratios of Bax/Bcl-w and Bak/Bcl-w during germ cell apoptosis are displayed in the *right panel* (C for model 1, F for model 2, and I for model 3). Data are presented as mean ± SEM of three independent experiments. *, $P < 0.05$; **, $P < 0.01$, as compared with the controls.

To induce apoptosis of proliferating spermatogonia by blocking SCF/c-kit interaction (7, 8), a function-blocking anti-c-kit antibody, ACK-2 (kindly provided by Dr. T. Kunisada, Department of Immunology, Faculty of Medicine, Tottori University, Japan) was injected i.v. at 3.5 mg/kg body weight in physiological saline. The injection was given twice, once every 48 h. Rats that received ACK-2 injection were killed at 24 h, 48 h, 72 h, and 96 h, respectively. One testis was snap frozen in the liquid nitrogen and then stored at −70 C for isolation of RNA; the other was fixed overnight at 4 C in 4% paraformaldehyde followed by dehydration and embedding onto paraffin for detection of spermatogonial apoptosis by ISEL.

For induction of spermatocyte apoptosis, MAA (Aldrich Chemie, Steinheim, Germany) was diluted in physiological saline and administered orally with a single dose of 650 mg/kg BW. The control rats received physiological saline. The rats were killed at 6 h, 12 h, 24 h, 4 days, 18 days, and 30 days, respectively. One testis was snap frozen in liquid nitrogen, and the other was fixed in 4% paraformaldehyde for preparation of paraffin block for checking apoptosis by ISEL staining.

To study the response of Bcl-w to germ cell death induced by testosterone withdrawal after EDS treatment, the rats were injected i.p. with a single dose of EDS (75 mg/kg BW). EDS was synthesized as previously described (33) and dissolved in dimethylsulfoxide (DMSO)-water (1:3, vol/vol). Control animals (3 rats/time point) for every time point [1 day (1d), 2d, 3d, 4d, 7d, 10d, 20d, 40d] received injection of vehicle. Rats (n = 3/group) were killed by cervical dislocation under CO_2

anesthesia at day 1, 2, 3, 4, 7, 10, 15, 20, 30, and 40 after administration of EDS. The testes were snap frozen in the liquid nitrogen and then stored at −70 C for isolation of RNA.

Riboprobe Preparation

The *bcl-w* cDNA was kindly provided by Dr. Jerry M. Adams (The Walter and Eliza Hall Institute of Medical Research, Victoria, Australia). The fragment, corresponding to nucleotide (nt) 1131-nt 1943 of the *bcl-w* cDNA, was subcloned into pBluescript II KS$^-$ (Stratagene, La Jolla, CA). The plasmid was linearized with *Eco*RI or *Xho*I for preparation of antisense or sense probe using T_3 or T_7 RNA polymerase (Promega Corp., Madison, WI), respectively. For Northern hybridization, ^{32}P-UTP was used for labeling the antisense riboprobe: for *in situ* hybridization, ^{35}S-UTP was used for labeling both antisense and sense probes.

Northern Hybridization

RNA preparation, gel fractionation, and Northern blotting, as well as hybridization, were performed as described previously (34).

In Situ Hybridization

Five-micrometer thick sections were cut from paraffin-embedded testis samples and mounted onto SuperForst Plus glass slides (Menzel-Gläser, Steinheim, Germany). The slides were then incubated at 37 C overnight and then stored at 4 C before use. *In situ* hybridization was performed as described previously (35).

Preparation of Purified Sertoli Cells, Germ Cells, and Leydig Cells

Sertoli cells were isolated from 20-day-old Sprague Dawley rats as described previously (36, 37). Spermatogonia were prepared from 9-day-old Sprague Dawley rats using a protocol described by Bellvé (38) with minor modifications introduced by Dym *et al.* (39). This procedure involved the use of enzymatic dissociation followed by filtration through 80- and 40-μm nylon mesh. Cells from the dissociated seminiferous tubules were separated by sedimentation velocity at unit gravity at 4 C using a 2–4% BSA gradient in Ham's F-12/DMEM. The cell suspension were bottom loaded into an SP-120 chamber in 30 ml of Ham's F12/DMEM containing 0.5% BSA, and a gradient was simultaneously generated using 275 ml each of medium supplemented with 2 and 4% BSA, respectively. The cells were allowed to sediment for a standard period of 2.5 h, and 300 ml were then collected from the bottom of the gradient and centrifuged at 100 $\times$ *g* for 10 min. Pellet cells were then resuspended in Ham's F12/DMEM supplemented with gentamycin (50 μg/ml) and 10% FCS and incubated at a density of 2.5 $\times$ 10^6/ml in a humidified atmosphere of 5% CO_2-95% air. After 14 h of culture, contaminant cells were plated, and nonadherent spermatogonia were used. Postmitotic germ cells were obtained from 90-day-old rat testes by mechanical dissociation (38). These cells were separated by centrifugal elutriation into two populations: primary spermatocytes and early spermatids. Flow rate and/or rotor speed were changed progressively, as described by Pineau *et al.* (40). Cell viability was evaluated by the trypan blue exclusion test and was found to be at least 95%. Pachytene spermatocytes and early spermatid fractions were found to be about 90% pure. Leydig cells were isolated and purified from rat testis as described previously (41), and the purity was ≥85%.

Preparation of Subcellular Fractions

Soluble, crude membrane and nuclear fractions of the purified Sertoli cells, spermatogonia, and spermatocytes were prepared as described by Hsu *et al.* (24). Briefly, cells were suspended at a cell density of 5 $\times$ 10^7 cells per ml in 1 ml of buffer A containing 10 mM HEPES, pH 7.4, 38 mM NaCl, phenylmethylsulfonyl fluoride (25 μg/ml), aprotinin (1 μg/ml), and leupeptin (10 μg/ml). The cell suspension was homogenized in a glass Dounce homogenizer and then centrifuged at 900 $\times$ *g* to pellet the nuclei in a Sorvall SA-600 rotor. The postnuclear supernatant was further centrifuged at 130,000 $\times$ *g* in a Ti 80 rotor (Beckman Coulter, Inc., Palo Alto, CA) to pellet the membranes. The crude membranes were solublized in a volume of the lysis buffer (buffer A supplemented with 0.5% NP-40) equal to that of the supernatant. The lysates were subjected to Western blotting.

Immunoprecipitation

Whole testis, purified Sertoli cells, or germ cells were lysed in a buffer containing 25 mM Tris-Hcl, 120 mM NaCl, 0.5% NP-40, 4 mM NaF, 100 μM Na_3VO_4, 100 KIU/ml aprotinin, 1 mM phenylmethylsulfonylfluoride , and 10 μg/ml leupeptin at 4 C for 30 min with vigorous shaking. Cell lysates were centrifuged for 20 min at 13,000 rpm, and then the supernatants were transferred to new tubes for measuring concentration of protein as described previously (42).

An aliquot of 300 μg of protein was incubated with 1 μg of polyclonal rabbit antirat Bax (PharMingen, San Diego, CA), Bak (G-23, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or Bad (K-17, Santa Cruz Biotechnology, Inc.) for 2 h at 4 C with gentle mixing. An aliquot of 20 μl of Protein A-agarose (Pharmacia Biotech, Uppsala, Sweden) was added, and the incubation proceeded for another 2 h at 4 C with gentle shaking. The mixture was centrifuged at 2500 rpm for 5 min at 4 C, and the pellet was washed four times with the buffer containing 20 mM Tris-Hcl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40, 100 mM NaF, 200 μM Na_3VO_4, 100 IU/ml aprotinin, 1 mM phenylmethylsulfonylfluoride and 10 μg/ml leupeptin and once with TBS. The pellet was resuspended in the sample buffer (400 mM Tris-HCl, 40% glycerol, 8% SDS, 0.4 M dithiothreitol, and 0.1% bromophenol blue) and boiled for 2.5 min. The suspension was centrifuged at 4 C, and the supernatant was loaded onto a 12.5% SDS-polyacrylamide gel using Mini Protean II system (Bio-Rad Laboratories, Inc. Hercules, CA). After electrophoresis, the protein was electrophoretically transferred onto a nitrocellulose membrane (Hybond, Amersham Pharmacia Biotech, Aylesbury, UK). Immunoprecipitation experiments were repeated three times using cells from different rats.

Immunoblotting

The membrane was incubated in a blocking buffer (10 mM Tris-HCl, pH 8.0, 0.1% Tween 20, and 5% non-fat milk powder) at room temperature for at least 1 h, followed by incubation in the blocking buffer containing polyclonal rabbit anti-bcl-w antibody (StressGen Biotechnologies Corp., Victoria, British Columbia, Canada) at 1:100–1:200 dilution for 1 h. After three times of wash in a washing buffer (10 mM Tris-HCl, pH 8.0, 0.1% Tween 20), the membrane was incubated in the blocking buffer containing horseradish peroxidase-conjugated donkey antirabbit antibody (Amersham Pharmacia Biotech) at 1:200 dilution for 1 h. After three times of wash, the membrane was subjected to chemiluminescent detection using an ECL Western Blotting Detection Kit (Amersham Pharmacia Biotech) and, finally, the membrane was exposed for 1–10 min to x-ray film (Fuji Photo Film Co., Ltd., Tokyo, Japan). The membranes were stripped using the method provided by the manufacture of the membrane (Hybond, Amersham Pharmacia Biotech) and reprobed using a

mouse anti-Actin monoclonal antibody (ICN Biomedicals, Inc., Aurora, OH) for normalization of the loading.

Tissue Culture and Hormone Stimulation

Seminiferous tubule segments were isolated in DMEM/F12 (1:1) (Life Technologies, Inc., Paisley, Scotland, UK) supplemented with 15 mM HEPES, 1.25 g/liter sodium bicarbonate, 10 mg/liter gentamycin sulfate, 60 mg/liter G-penicillin, 1 g/liter BSA and 0.1 mM 3-isobutyl-1-methylxanthin (MIX) (Aldrich Chemie) under a stereomicroscope by transillumination-assisted microdissection technique as described previously (43).

Twenty pieces of 5-mm seminiferous tubule segments from stages II–VI, VII–VIII, IX–XII, or XIII–I were incubated in 1 ml of above mentioned culture medium in the presence and absence of FSH (10 ng/ml), testosterone (T) (10^{-6} M), FSH+T for 8 h and 30 h. After incubation, RNA was isolated as described previously (34), and Bcl-w mRNA levels were detected by Northern blot hybridization.

Immunohistochemistry

Two 5-μm-thick consecutive sections were cut from each sample and mounted onto polylysine-coated slides. One section was used for immunohistochemical staining of Bcl-w, Bax, and Bak, and the other was used for periodic acid-Schiff-hematoxylin staining for accurate determination of stages.

After rehydration, the slides were washed twice in TBS buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl,) for 5 min each followed by microwave antigen retrieval at 700 W for 15 min in 10 mM sodium citrate solution, pH 6.0. After two washes with TBS, an aliquot of 50 μl of blocking solution (TBS containing 1% BSA, 3% FCS, and 3% normal horse serum) was applied to each section and incubated for 1 h at room temperature. After blocking, an aliquot of 50 μl primary antibody (1:200 diluted in TBS containing 1% BSA) was applied to each section and incubated at 4 C overnight. Incubation with secondary antibody and visualization of positive cells were performed using Vectastain Elite-kit (Vector Laboratories, Inc., Burlingame CA) according to the manufacturer's instructions.

As a control experiment, the testis sections from *bcl-w* $^{-/-}$ (line 043 and line 044) and *bcl*-w$^{+/+}$ (line 042) mice, which were kindly provided by Dr. Suzanne Cory (The Walter and Eliza Hall Institute of Medical Research, Victoria, Australia), were employed to perform immunohistochemical staining using anti-Bax, Bak, and Bcl-w antibodies. Preabsorbed antibodies by corresponding peptides used for immunization and rabbit IgG were also used as control antibodies.

A semiquantitative analysis was conducted for estimating the relative abundance of Bcl-w, Bax, and Bak proteins in Sertoli cells, spermatogonia, and spermatocytes in the rat. Immunoreactivity in different types of cells and in different stages was semiquantified according to the following criteria: +, weak; ++, strong; +++, very strong immunoreaction.

ISEL Staining of Apoptotic Spermatogonia

Two consecutive sections (5-μm thick) were cut from each paraffin block; one for ISEL staining and the other for periodic acid-Schiff-hematoxylin staining. ISEL staining was performed as described (28). Briefly, after rehydration, the sections were incubated in 2 × SSC at 80 C for 20 min followed by washing twice with water and once with Proteinase K buffer (20 mM Tris-HCl, pH 7.4, 2 mM $CaCl_2$) for 5 min each. The slides were then treated with proteinase K (10 μg/ml, Roche Molecular Biochemicals, Indianapolis, IN) in proteinase K buffer at 37 C for 30 min. An aliquot of 20 μl of 3′-end labeling reaction mixture containing 4 μl 5×TdT buffer (Promega Corp.), 0.1 μl Dig-11-ddUTP (10 nmol/μl, Roche Molecular Biochemicals), 0.2 μl dd-ATP (5 mM, Promega Corp.), 1 μl terminal deoxynucleotidyl transferase (Roche Molecular Biochemicals) and 14.7 μl nuclease-free water (Promega Corp.) was applied to one cross-section. The slides were kept in a humidified box and incubated at 37 C for 1 h and then washed three times with TBST buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 0.1% Tween-20) for 10 min each. An anti-Dig-horseradish peroxidase monoclonal antibody (DAKO Corp., Glostrup, Denmark, 1:200 dilution in TBST containing 1% BSA,) was applied, and the slides were incubated in a humidified box at room temperature for 1 h and then washed three times with TBST for 5 min each time. Finally, the labeled cells were visualized by 3,3′-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO) for 0.5–2 min.

Nonradioactive DNA Laddering

DNA was isolated from five pieces of 2 mm-long tubule segments by phenol/chloroform extraction after digestion in TES buffer (10 mM Tris-HCl, pH 7.6, 10 mM EDTA, 100 mM NaCl) containing 1% SDS, proteinase K (100 μg/ml, Roche Molecular Biochemicals), and RNase A (10 μg/μl, Roche Molecular Biochemicals) at 55 C for 30 min. The 3′-end labeling was performed in a 20 μl reaction volume containing 100 ng DNA, 4 μl 1× Terminal transferase buffer (Promega Corp.), 0.5 μl terminal deoxynucleotidyl transferase (20 U/μl, Promega Corp.), 0.1 μl DIG-11-ddUTP (10 nmol/μl, Roche Molecular Biochemicals). The reaction mixture was incubated for 30 min at 37 C and then 1 μl 0.5 M EDTA, pH 8.0, was added to terminate the reaction. The reaction mixture was size fractionated in a 1.6% agarose gel followed by blotting onto a nylon membrane overnight with 10×SSC. The DNA was fixed by baking in an oven at 80 C for 1 h followed by UV cross-linking. The membrane was incubated in 20 ml 1× blocking buffer (Roche Molecular Biochemicals) containing 1 μl anti-DIG-AP (Roche Molecular Biochemicals) at room temperature for 1 h followed by three times of washing with TBST buffer for 15 min each. One milliliter chemiluminescent substrate CSPD (Roche Molecular Biochemicals) was applied onto the blot. The blot was incubated at room temperature for 10 min and then sealed in a plastic bag. The blot was exposed to x-ray film (Fuji Photo Film Co., Ltd., Tokyo, Japan) for 5–10 min.

Quantitative Analysis of Northern Hybridization and Western Blotting Results

The x-ray films of Northern hybridization and Western blotting results were first scanned by a UMAX scanner (UMAX Inc., Fremont, CA) and a Photoperfect software package (Binuscan Inc., New York, NY). The images were saved as TIFF-type files (*.tif, Microsoft Corp. Co. and Aldus Co., New York, NY) and then quantified by TINA 2.0 densitometric analytical system (Raytest Isotopenmeβ gerate GmbH, Straubenhardt, Germany) according to the manufacturer's instructions. For Northern blotting results, after normalization to 28S rRNA, the densitometric value of a control was designated as 100%, and values of other controls and treated samples were expressed as the percentages of the control. For Western blotting results, after normalization to actin, similarly, the densitometric value of a control was designated as 100% and values of other controls and treated samples were expressed as the percentages of the control. The values of the controls that were designated as 100% were excluded from statistical analysis.

Statistical Analysis

The values from three independent experiments were pooled for the calculation of the SEMs and for one-way ANOVAs and

Duncan's new multiple range test to determine the significant differences between different experimental groups by using StatView 4.51 statistic program (Abacus Concepts Inc., Berkeley, CA). $P < 0.05$ was considered statistically significant.

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