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Apoptosis during an Early Stage of Nephrogenesis Induces Renal Hypoplasia in *bcl-2*-Deficient Mice

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Renal development in* bcl-2*-deficient mice was monitored to examine the temporal and spatial function of this gene during nephrogenesis* in vivo. *Extensive apoptosis occurred during abnormal nephrogenesis in* bcl-2*-deficient mice. In embryos and newborn mice, the sequence of morphological events was monitored by morphology in conjunction with morphometry, and* bcl-2 −/−, bcl-2 +/−, *and* bcl-2 +/+ *mice were compared. In* bcl-2 −/− *mice, initial induction of nephrons was detected by embryonic day 13 (E-13) as normal. Then, apoptotic cells became five times more frequent at E-13 to E-16 with a significant reduction (1/5) in nephron number at E-17 to E-19 in* bcl-2 −/− *mice compared with* bcl-2 +/+ *mice. No morphological difference was evident between* bcl-2 +/− *mice and* bcl-2 +/+ *mice by morphometry. Apoptotic cells were found mainly among the mesenchyme and less frequently in tubuli. Little apoptosis among ureteric buds was noted. In* bcl-2 −/− *mice at E-17 to E-19, inactive branching and insufficient convolution of ureteric buds were accompanied by fulminant apoptosis in the mesenchyme. Neonatal* bcl-2 −/− *mice lacked the nephrogenic zone, exhibiting renal hypoplasia. Thus,* bcl-2 *seems to inhibit apoptosis in renal stem cells during the induction of nephrons* in vivo. *(Am J Pathol 1996, 148:1601–1611)

During normal development of the kidney, differentiation and proliferation of renal stem cells obviously play a crucial role in morphogenesis.[1–3] Several growth factors, namely, insulin-like growth factor-1,[4,5] insulin-like growth factor-2, hepatocyte growth factor, and transforming growth factor-α, have been postulated to promote metanephric development *in vitro*.[6,7] In addition, apoptosis[8] is known to be involved in many forms of organogenesis including that of kidneys.[9,10] During kidney development, apoptotic cells have been observed to account for several percent of the total number of cells involved.[9] By contrast, inhibition of apoptosis has been suggested to be an important phenomenon in nephrogenesis. Using an organ culture system, Koseki et al[11] demonstrated recently that spinal cord, a strong inducer of the differentiation of mesenchymal cells to epithelial cells, repressed programmed cell death (apoptosis) of mesenchymal cells in a manner that was essential for survival of cells before epithelial induction. Thus, regulation of apoptosis (maintenance of a balance between cell death and cell survival) seems to determine the morphology of the kidneys. However, the mechanisms by which apoptosis is regulated during nephrogenesis *in vivo* have not been well characterized.

The *bcl-2* gene was identified as a gene responsible for human follicular lymphomas,[12] and it exhibits considerable homology to an apoptosis-inhibitory gene, *ced-9*, which was identified in *Caenorhabditis elegans*.[13] The *bcl-2* protein functions as an apoptosis-inhibiting factor in several cell lines.[14] Moreover, previous reports have demonstrated *bcl-2* immunoreactivity in several organs and tissues, including kidneys in mouse and human embryos.[15–18] From these observations, it seems possible that *bcl-2*

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might function to inhibit apoptosis during the development of nephrons *in vivo*.

Recently, we and others have established *bcl-2*-deficient mice by homologous recombination techniques (knockout mice).[19–21] These mice have polycystic kidneys after birth. Sorenson and colleagues[22] demonstrated renal hypoplasia in neonatal *bcl-2* homozygous (*bcl-2* −/−) mice. In short-term organ cultures of metanephroi from *bcl-2* −/− mice, fulminant apoptosis and metanephric hypoplasia were observed.[22] However, the stage of kidney development at which *bcl-2* functions and the extent to which nephrogenic stem cells are protected by *bcl-2* from apoptosis remain unclear.

In the present study, we monitored the sequence of kidney development *in vivo* by morphometric analysis of *bcl-2* −/−, *bcl-2* heterozygous (*bcl-2* +/−), and wild-type (*bcl-2* +/+) mice to identify temporal and spatial aspects of the function of *bcl-2* during kidney development. Our results suggest the possibility that protection of renal stem cells from apoptosis during an early phase of nephrogenesis by *bcl-2* might play a crucial role in normal nephrogenesis *in vivo*.

Materials and Methods

Animals

Mice, heterozygous for the mutant *bcl-2* allele,[19] were housed in a barrier facility. After mating of female and male *bcl-2* +/− mice, vaginal plugs were checked to allow estimations of the gestational age of embryos. All animals were subjected to genotype screening to determine the phenotype, as described below.

Genotyping by the Polymerase Chain Reaction (PCR)

A small portion of the tail from each mouse was incubated overnight at 56°C in 300 μl of lysis buffer (100 mmol/L Tris-HCL, pH 8.5, 5 mmol/L EDTA, pH 8.0, 200 mmol/L NaCl, 0.2% sodium dodecyl sulfate, and 15 μl of a solution of 10 mg/ml proteinase K). The next day, 300 μl of TE buffer (10 mmol/L Tris-HCL, 1 mmol/L EDTA, pH 8.0) and phenol saturated by TE were added to the samples and they were centrifuged at 15,000 × *g* for 3 minutes at room temperature. This procedure was repeated three times. Then the supernatant was extracted once with phenol/chloroform/isoamyl alcohol (24:24:1) and once with chloroform. The DNA was precipitated with

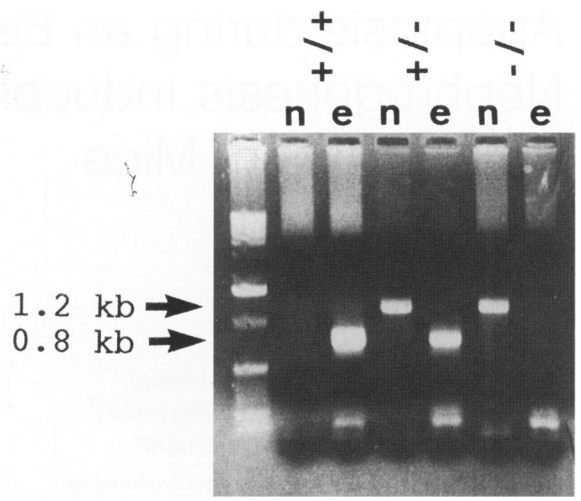

Figure 1. *Examples of genotype screening by PCR. The expected size of the PCR products of a gene for neomycin resistance (n) was 1.2 kb and that of endogenous* bcl-2 *(e) was 0.8 kb. In* bcl-2 *−/− mice, the neomycin resistance gene was present in the absence of an endogenous* bcl-2 *gene.*

2.5 volumes of ethanol and 0.1 volume of 3 mol/L sodium acetate (pH 5.2) and resuspended in 50 μl of TE plus RNAse (10 mg/ml). The concentration of the DNA was approximately 300 to 400 ng/ml. A total of 2 μl of the sample of DNA was added to 10X TE buffer, 0.5 μl of *Taq* DNA polymerase (Toyobo, Osaka, Japan), 3 μl of 10 mmol/L $MgCl_2$, 0.5 ml of 25 mmol/L nucleotide-3-phosphate. These solutions were mixed with either 2 μl of a solution of 3′ and 5′ noncoding region sequences of neomycin-resistant gene or *bcl-2* determined from a cDNA clone. A pair of sequences of the neomycin-resistant gene was 5′-TGCTAAAGCGCATGCTCCAGACTG and 3′-ATTCGTTCTCTTTATACTACCAAGG; and those of *bcl-2* was 5′-CGTCCCGCCTCTTCACCTTTCAGC and 3′-ATCCTCCCCCAGTTCACCC. The mixture was subjected to PCR for 35 cycles. The products of PCR were then subjected to gel electrophoresis. Patterns specific for the *bcl-2* −/−, *bcl-2* +/−, and *bcl-2* +/+ genotypes are shown in Figure 1.

Pathology

In total, 84 mice (E-13 to E-19 embryos and day 0 to day 2 neonates; *bcl-2* −/− = 23, *bcl-2* +/− = 42, *bcl-2* +/+ = 19) were fixed in Carnoy's fixative, in buffered formalin, or in glutaraldehyde. The number of mice in each phenotype by each developing stage is described in Table 1 and the legend of Figure 4.

Light microscopy was performed with formalin-fixed, paraffin-embedded sections that had been stained with hematoxylin and eosin and with periodic

Table 1. *Morphometry in E-17 to E-19 Embryo*

Phenotype	Nephrogenic zone per kidney area ($/\mu m^2 \times 10^5$)	Glomerular number per section	Glomerular number per kidney area ($/\mu m^2 \times 10^5$)
+/+	0.234 ± 0.01	70.6 ± 5.4	3.14 ± 0.25
+/−	0.227 ± 0.01	66.9 ± 5.4	2.90 ± 0.15
−/−	0.177 ± 0.02*	15.6 ± 3.5*	1.22 ± 0.18*

Data are expressed as mean ± SE; n = 8 (+/+), 17 (+/−), and 8 (−/−).
*P < 0.01 in +/+ *versus* −/− and P < 0.01 in +/− *versus* −/−.

acid-Schiff's reagent. Glutaraldehyde-fixed materials were used for electron microscopy and prepared by standard procedures. To identify ureteric buds, Carnoy's-fixed, paraffin-embedded sections were labeled with lectin from *Dolichos biflorus* (E-Y Labs, San Mateo, CA) as described by Avner and Sweeney[23] with modification by subsequent staining of streptavidin and biotin as previously described.[24]

Detection of Apoptosis

Apoptotic cells were detected by a previously described nick end-labeling method[25] with some modifications. Briefly, formalin-fixed, paraffin-embedded sections were deparaffinized with xylene passed through a graded alcohol series. Then they were washed three times with sterile distilled water and incubated with proteinase K (80 μg/ml) for 15 minutes. Endogenous peroxidase activity was blocked by immersing the slides in 2% H_2O_2 in distilled water for 5 minutes. After three washes in distilled water, slides were incubated for 5 minutes with TdT buffer (30 mmol/L Tris-HCl, 140 mmol/L sodium cacodylate, and 1 mmol/L cobalt chloride). The slides were then incubated at 37°C for 60 minutes with TdT reaction buffer, which consisted of 5X TdT buffer containing 1 μmol/L dATP (Takara, Otsu, Japan), 1.5 mmol of biotin 16 dUTP (Boehringer Mannheim, Mannheim, Germany), 35 U of TdT (GIBCO BRL, Grand Island, USA.). The reaction was stopped by distilled water for 15 minutes. After three washes in phosphate-buffered saline (PBS), the avidin-biotin complex was applied to the sections for 60 minutes at room temperature. After three additional washes in PBS, color was developed by incubation with 3,3′-diaminobenzidine in PBS with 1% H_2O_2. Finally, sections were counterstained with methyl green.

Morphometry

Using a computer image analysis system (SPICA system, Avionix, Tokyo, Japan), we estimated 1) the density of nick end-labeled positive cells per unit area of each section, 2) the ratio of the nephrogenic zone to the kidney area, and 3) the density of glomeruli in the kidney area. As nephrogenesis was active in the entire kidney from E-13 to E-16 and limited in the nephrogenic zone from E-17 to E-19, the density of nick end-labeled cells was estimated separately as follows. The density of apoptotic cells for E-13 to E-16 stages is equal to the nick end-labeled positive cells divided by the whole kidney area; for E-17 to E-19 stages, it is equal to nick end-labeled positive cells in the nephrogenic zone divided by the area of the nephrogenic zone. The density of the glomeruli in E-17 to E-19 stages is equal to the number of glomeruli in the nephrogenic zone divided by the area of the nephrogenic zone (glomeruli beyond the S-shaped body stage were defined as glomeruli). The ratio of the nephrogenic zone to the kidney area for E-17 to E-19 stages is equal to the area of the nephrogenic zone divided by the whole kidney area.

The nephrogenic zone was defined as the area composed of undifferentiated mesenchymal cells, metanephric condensates, ureteric buds, and glomeruli that were less mature than those at the S-shaped body stage. The border of the nephrogenic zone and the area of completion of nephrogenesis was defined as the outermost layer of mature tubuli. Mature glomeruli were not included in the nephrogenic zone.

Statistical Analysis

Data were expressed as means +/− standard errors (SE). Statistical analysis was performed by one-way analysis of variance or Bonnferroni or Dunn's procedures. Statistical significance was recognized at $P < 0.05$.

Results

E-13 to E-16 Stage

At E-13, the earliest stage of nephrogenesis in this study, no morphological differences among *bcl-2* −/−, *bcl-2* +/−, and *bcl-2* +/+ mice were evident. Initial tubulogenesis and branching of ureteric buds appeared normal in *bcl-2* −/− mice (Figure 2). Thus, metanephric development before the initial stage of nephron induction was normal despite *bcl-2* deficiency. During E-13 to E-16, gross difference of the kidneys was indistinguishable among three genotypes. Even by histology, *bcl-2* −/− mice displayed branching of ureteric buds that reached to the sub-

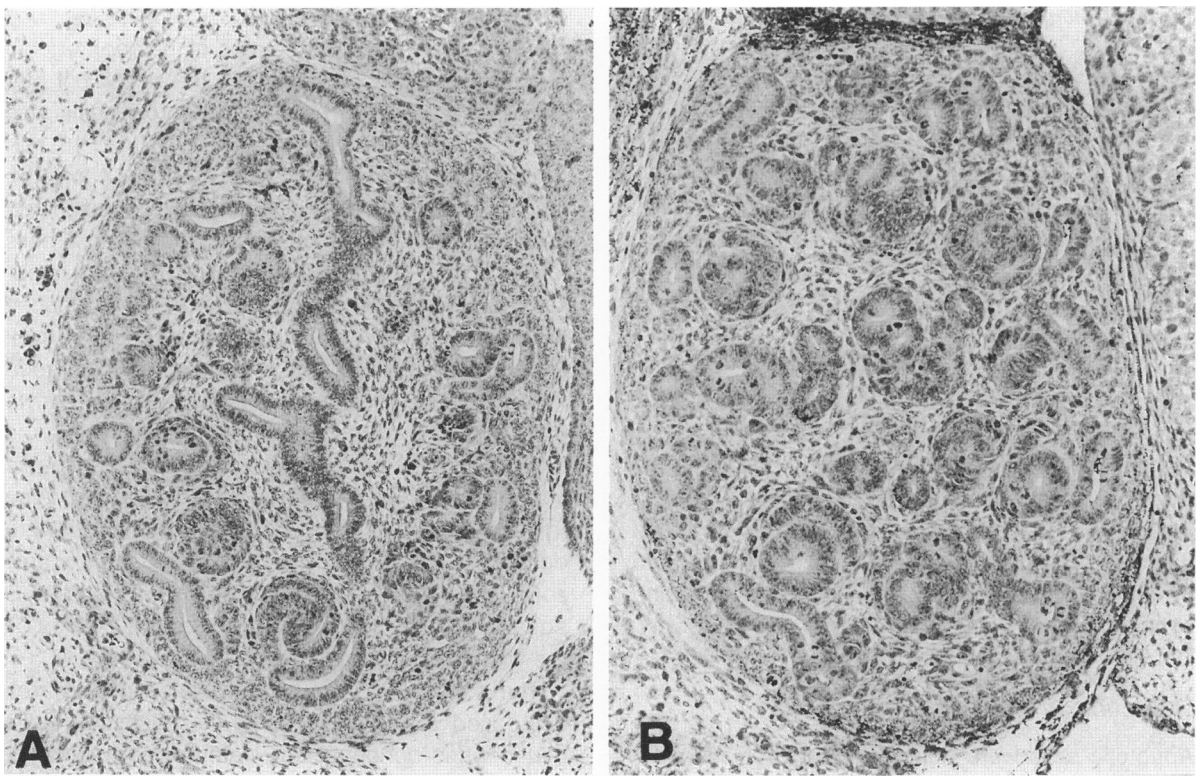

Figure 2. *Kidneys at E-13. No apparent morphological differences were detected between* bcl-2 +/− (**A**) *and* bcl-2 −/− (**B**) *mice. Note that ureteric buds locate in the subcapsular layer and initial tubulogenesis is visible to a similar extent in both samples. Magnification,* ×200.

capsular layer with nephrogenic condensate and tubulogenesis as observed in *bcl-2* +/− or *bcl-2* +/+ mice.

Nick end-labeled cells in *bcl-2* −/− mice were apparently much more numerous than in *bcl-2* +/+ mice (Figure 3). Numerically, five times more labeled cells were found in *bcl-2* −/− mice than in *bcl-2* +/+ mice (Figure 4). Thus, excessive apoptosis took place before any apparent morphological abnormalities. In *bcl-2* −/− mice, apoptotic cells and apoptotic bodies were often noted among uninduced mesenchymal cells and less frequently among induced tubular and peritubular cells (Figure 3B). In *bcl-2* +/+ and *bcl-2* +/− mice, few nick end-labeled cells were found among the subcapsular and peritubular mesenchymal cells (Figure 3A). Apoptotic cells in ureteric buds were scarcely ever observed in all genotypes. Although no statistically significant difference was evident, the *bcl-2* +/− mice tended to have approximately three times more nick end-labeled cells per kidney area than *bcl-2* +/+ mice (Figure 4A). Electron microscopy confirmed that nick end-labeled cells were apoptotic cells. Few apoptotic figures were ever found in *bcl-2* +/+ mice. By contrast, in *bcl-2* −/− mice, apoptotic cells with characteristic features were found among mesenchymal cells (Figure 5). Isolated apoptotic cells were usually surrounded by intact mesenchymal cells. Projections from intact mesenchymal cells were in contact with the cell membrane of apoptotic cells (Figure 5B). In some cases, two or three apoptotic cells were seen together. Apoptotic bodies were found in the cytoplasm of mesenchymal cells as well as in tubular epithelial cells. The ultrastructure of apoptotic cells in mice of different phenotypes was similar.

E-17 to E-19 Stage

Morphological abnormalities in kidneys in *bcl-2* −/− mice appeared first at E-17. At this stage, *bcl-2* +/+ and *bcl-2* +/− mice both showed normal nephrogenesis. In these mice, mature glomeruli lined up adjacent to the deep cortex that demarcated the medulla (Figure 6A). Tubulogenesis was active, with maturation of glomerular morphology and development of a centrifugal pattern. Ureteric buds were clearly recognizable at the subcapsular layer by their convoluted shape. Mesenchymal condensates were evident beside the convoluted ureteric buds. In *bcl-2* −/− mice, by contrast, the nephrogenic zone was thin and was characterized by immature tubuli that were scattered among the remaining uninduced mesenchyme (Figure 6B). Centrifugal maturation of

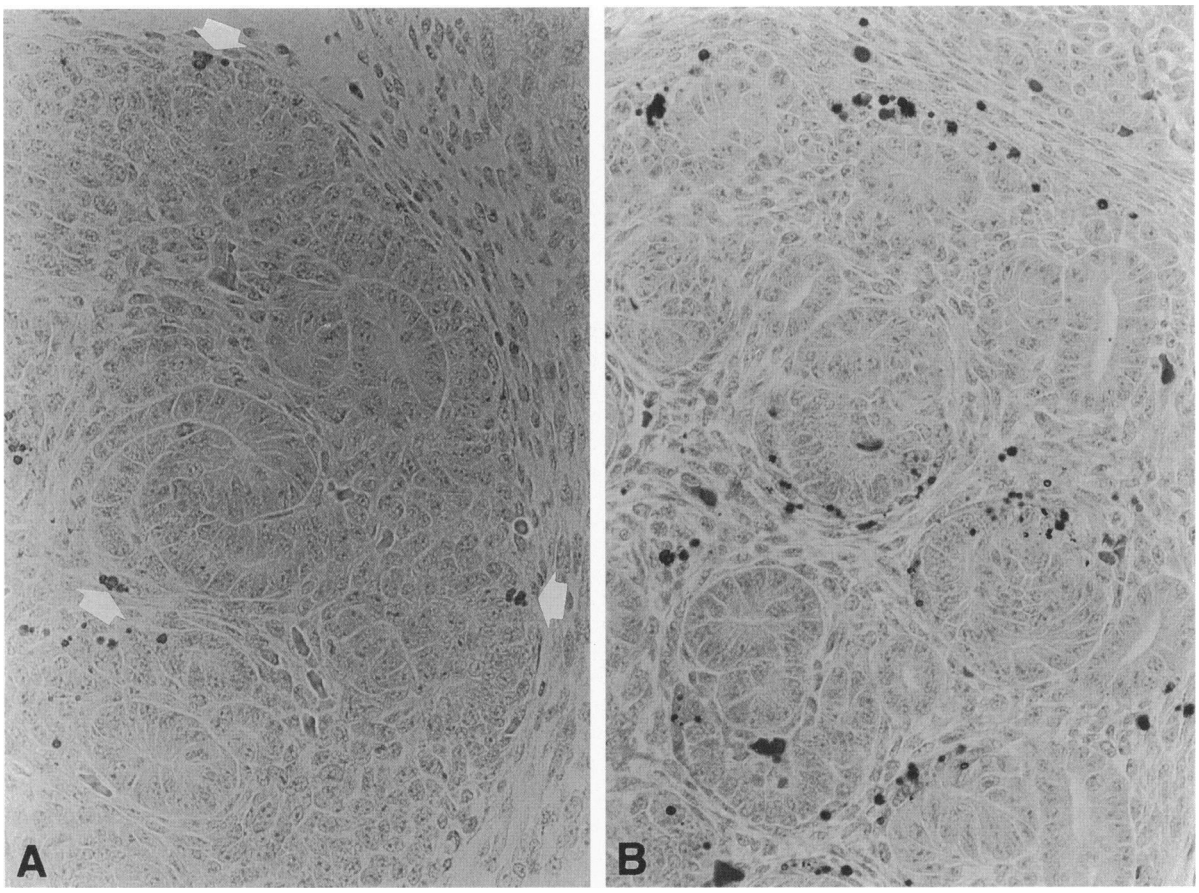

Figure 3. In situ *nick end-labeling in* bcl-*2* +/− **(A)** *and* bcl-*2* −/− **(B)** *mice at E-13.* **A:** *Labeled cells are less frequently noted among mesenchymal cells in* bcl-*2* +/− *mice* **(white arrows). B:** *Labeled cells (black spots) in* bcl-*2* −/− *mice are visible chiefly among subcapsular and peritubular mesenchymal cells. Note that labeled cells and bodies are also observed among tubular cells in a comma-shaped body. Magnification,* ×*450.*

glomeruli, as observed in *bcl-2* +/+ or *bcl-2* −/− mice, was not evident in *bcl-2* −/− mice. Convoluted ureteric buds were rare, but proximal or distal tubuli were found in the deep layer with enlargement. Furthermore, glomeruli were few in number; most of them were noted in the deep cortical layer and were somewhat larger than those in *bcl-2* +/+ or *bcl-2* −/− mice (Figure 6, A and B).

Morphometry confirmed hypoplasia of nephrons in *bcl-2* −/− mice. In *bcl-2* −/− mice, the nephrogenic zone was narrow and contained only approximately 20% of the number of glomeruli found in *bcl-2* +/+ or *bcl-2* +/− mice (Table 1). The overall impression of similar renal development in *bcl-2* +/+ and *bcl-2* +/− mice was confirmed by morphometry. No statistically significant difference in terms of the area of the nephrogenic zone and in number of nephrons were noted. However, the number of apoptotic cells in the nephrogenic zone in *bcl-2* −/− mice was five times higher than that in *bcl-2* +/+ or *bcl-2* +/− mice (Figure 4B). In *bcl-2* −/− mice, apoptotic cells were often seen among mesenchymal cells. In *bcl-2* +/+ and *bcl-2* +/− mice, apoptotic cells were rarely encountered.

Newborn Mice (Day 0 and Day 2)

In newborns, kidneys in *bcl-2* −/− mice were smaller than in *bcl-2* +/+ or *bcl-2* +/− mice (Figure 7). In *bcl-2* −/− mice, the nephrogenic zone was extremely thin and consisted of uninduced mesenchymal cells. In these animals, the margin between the cortex and the medulla, which was clearly visible in *bcl-2* +/− or *bcl-2* +/+ mice, was indistinct. Glomeruli were rarely encountered in the deep cortex in *bcl-2* −/− mice. By contrast, tubuli were hypertrophic and their lumen showed scattered dilatation in several mice. Labeling with *D. biflorus* lectin clearly demonstrated that ureteric buds and collecting ducts were located in the subcapsular region and had a convoluted shape in *bcl-2* +/+ and *bcl-2* +/− mice. In *bcl-2* −/− mice, however, ureteric buds were slender and extended as far as the deep layer of the kidney (Figure 8). Ends of ureteric buds were

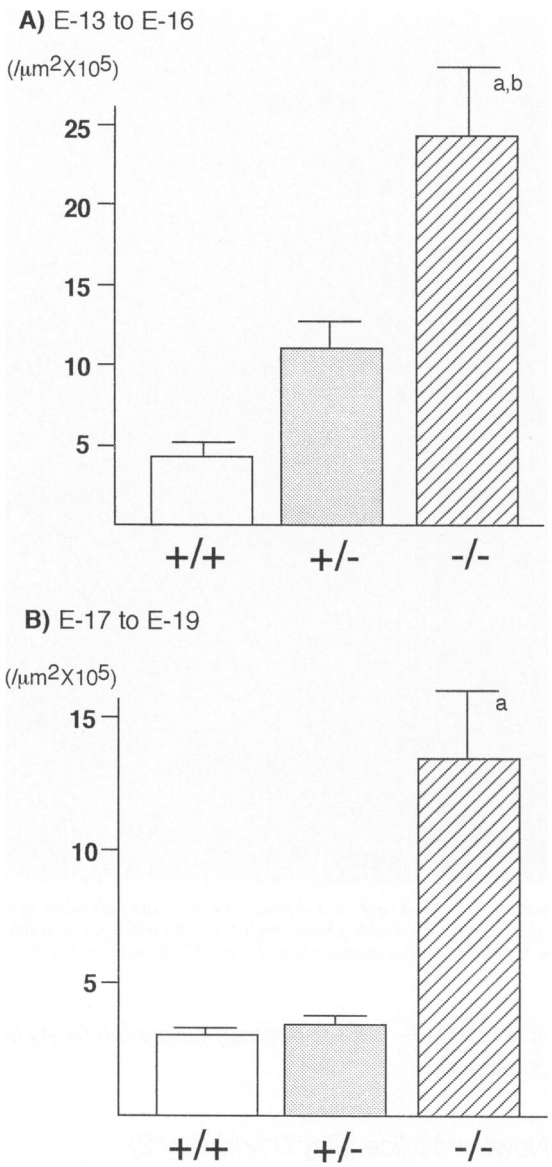

Figure 4. *Numbers of nick end-labeled cells per kidney area at E-13 to E-16* (**A**) *and per nephrogenic zone at E-17 to E-19* (**B**). **A:** *In* bcl-*2 −/− mice, labeled cells were approximately five times as numerous as in* bcl-*2 +/+ mice and twice those in* bcl-*2 +/− mice. Note that* bcl-*2+/− mice tended to have three times more apoptosis compared with* bcl-*2 +/+ mice, but no statistically significant difference was found. n = 24; +/+ = 6, +/− = 11, −/− = 7.* **B:** *Labeled cells were five times more numerous in* bcl-*2 −/− mice than in* bcl-*2 +/+ and* bcl-*2 +/− mice. n = 33; +/+ = 8, +/− = 17, −/− = 8. a,* P < *0.01 in +/+* versus *−/−; b,* P < *0.01 in +/−* versus *−/−.*

occasionally in the subcapsular layer in these animals and were not convoluted.

Discussion

Abnormalities in so-called knockout mice do not always directly reflect the function of the targeted gene.[26] Unidentified redundant compensatory systems might act *in vivo* to generate a normal phenotype. Thus, renal hypoplasia in *bcl-2 −/−* mice seemed to indicate that the *bcl-2* gene product was indispensable for the development of nephrons *in vivo*.

Even in *bcl-2 −/−* mice, the initial induction of nephrons appeared normal. As shown in Figure 2, *bcl-2 −/−* mice at E-13 showed normal tubulogenesis and mice at E-17 to E-19 stage had few but distinct glomeruli that were localized in the deep cortex only. Active tubulogenesis was not observed in nephrogenic zone. As nephron maturation proceeds from deep cortex to subcapsular layer in a centrifugal pattern,[1,3] matured glomeruli in the deep cortex in *bcl-2 −/−* mice suggested derivation from initial tubulogenesis at E-13. This indicates that initial tubulogenesis is accomplished despite the lack of *bcl-2*. Hence, it seems likely that, for the survival of condensed mesenchyme before the interaction between mesenchyme and ureteric buds and after initial tubulogenesis, the function of *bcl-2* is probably not crucial.

At the next stage of nephrogenesis, E-13 to E-16, *bcl-2 −/−* mice displayed extensive apoptosis frequently in mesenchymal cells and less frequent apoptosis in tubular cells. These observations suggest a critical function of *bcl-2* for cell survival at this stage. The pattern of distribution of apoptotic cells in *bcl-2 −/−* mice was almost consistent with that of immunoreactivity of the gene product of *bcl-2* during normal nephrogenesis.[16] Modulation of phenotypes by redundant compensatory systems in knockout mice does raise the question of whether the extensive apoptosis in *bcl-2 −/−* mice was caused by the *bcl-2* deficiency. Sorenson and colleagues[22] demonstrated *in vitro* the fulminant apoptosis and hypoplasia in metanephroi harvested from *bcl-2 −/−* mice at E-12 and cultured for 3 days. Their results tend to support the hypothesis that *bcl-2* deficiency was responsible for the fulminant apoptosis that we observed at E-13 to E-16 *in vivo*. Therefore, it appears that *bcl-2* acts as a repressor of apoptosis at this early stage of nephrogenesis (E-13 to E-16).

At the next stage of nephrogenesis, E-17 to E-19 (the late stage of nephrogenesis), abnormalities in *bcl-2 −/−* mice became morphologically apparent. These abnormalities were characterized by the absence of centrifugal maturation of glomeruli and failure of the development of ureteric buds. Simultaneously, a higher frequency of apoptotic cells was seen in *bcl-2 −/−* mice. With respect to these observations, two questions need to be answered. First, was inactive nephrogenesis in *bcl-2 −/−* mice

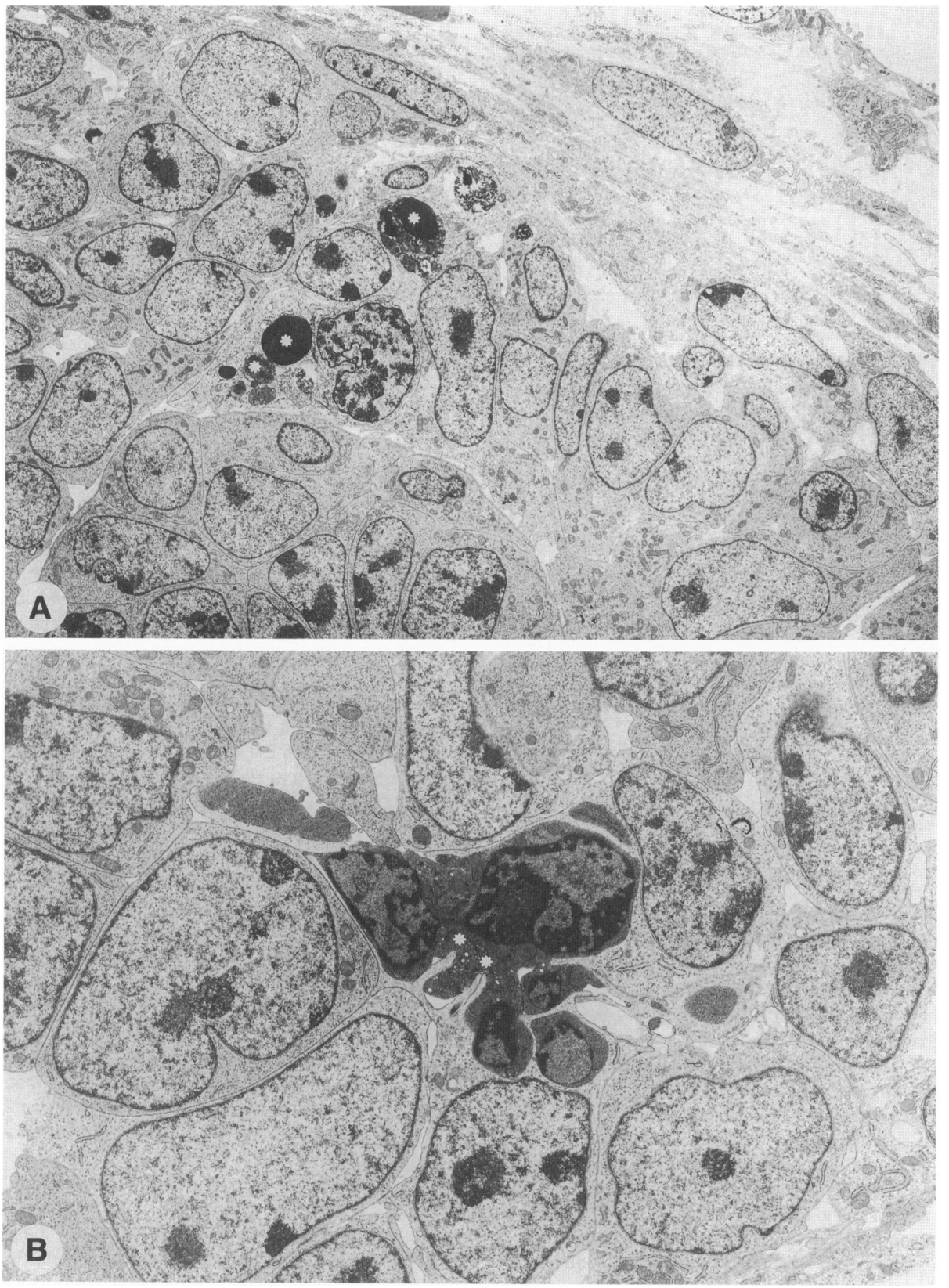

Figure 5. *Electron micrographs at E-13 of samples from* bcl-2 −/− *mice.* **A:** *An apoptotic cell and phagocytosed apoptotic bodies are seen in the subcapsular mesenchyme* (white asterisks). *Magnification, ×3000.* **B:** *Projections from intact mesenchymal cells are in contact with the cell membrane of an isolated apoptotic cell* (white asterisks). *Magnification, ×5000.*

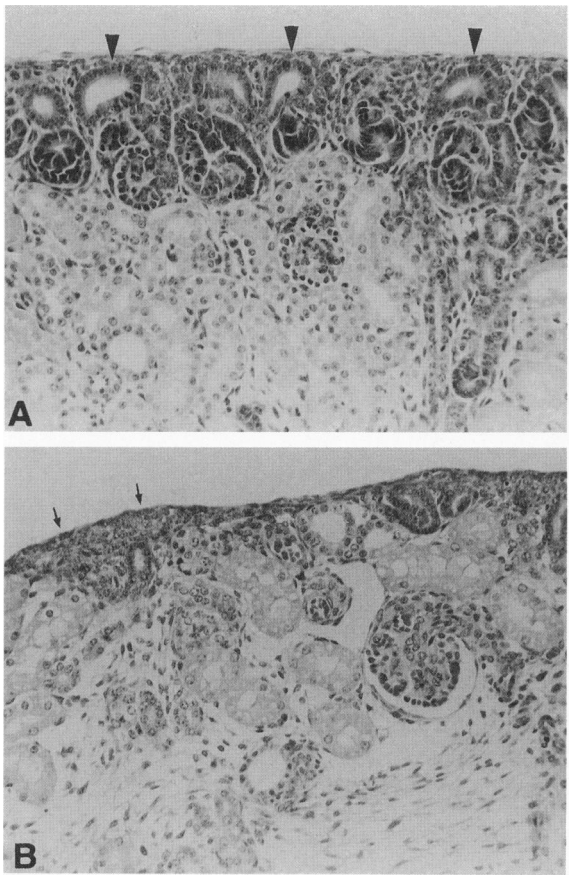

Figure 6. *Kidneys at E-18 showing inactive tubulogenesis in* bcl-2 −/− *mice.* **A:** *A sample from a* bcl-2 +/− *mouse shows centrifugal maturation of the nephrogenic zone. Ureteric buds with convolutions are clearly seen in the subcapsular area* (**arrowheads**). **B:** *A sample from the* bcl-2 −/− *mouse shows residual uninduced mesenchymal cells* (**arrows**) *with a few, but large, glomeruli in the deep cortex. Tubuli are also enlarged. Note that no convolution of ureteric buds is seen in the* bcl-2 −/− *mouse. Magnification, ×340.*

at this stage caused by the fulminant apoptosis from E-13 to E-16, and second, does *bcl-2* function as a repressor of apoptosis from E-17 to E-19 as well?

Morphometry demonstrated that *bcl-2* −/− mice had a five times higher incidence of apoptotic cells than that of *bcl-2* +/+ mice from E-13 to E-16 and approximately one-fifth of the nephron number from E-17 to E-19. Moreover, apoptotic cells at E-13 to E-16 were often located among mesenchymal cells (progenitor of the glomeruli) and less frequently among tubular cells. These findings imply that inactive nephrogenesis from E-17 to E-19 was probably caused by the fulminant apoptosis from E-13 to E-16 among uninduced mesenchymal cells and to some extent was accelerated by apoptosis of tubular cells. Together, the findings suggest that *bcl-2* deficiency-dependent apoptosis at the early stage of nephrogenesis (E-13 to E-16) resulted in inactive nephrogenesis at the late stage of nephrogenesis (E-17 to E-19).

The question of whether the product of *bcl-2* acts as an inhibitor of apoptosis at the late stage of nephrogenesis requires some discussion. Nick end-labeling provided evidence that apoptosis occurred frequently from E-17 to E-19 in *bcl-2* −/− mice. However, this result does not directly lead to the conclusion that *bcl-2* functions in cell survival at this stage. As is well known, nephrogenesis is initiated by mesenchymal-epithelial interactions. Survival and differentiation of mesenchymal cells are regulated by unidentified soluble factors generated by the ureteric buds. Conversely, the branching and development of ureteric buds are regulated by mesenchymal cells.[1,3] Koseki and colleagues[11] showed that mesenchymal cells underwent apoptosis *in vivo* in the absence of inducer, spinal cord. Their findings *in vitro* may reflect the situation *in vivo* wherein mesenchymal cells die if adequate development of ureteric buds does not occur. Indeed, ureteric buds of *bcl-2* −/− mice at this stage failed to branch normally and seldom reached the subcapsular layer. As a result, uninduced mesenchymal cells remained in the subcapsular region. Furthermore, the number of uninduced mesenchymal cells was reduced in newborn mice. These reductions in cell number in the mesenchyme were consistent with excessive apoptosis at this stage. It seems likely that fulminant mesenchymal apoptosis from E-17 to E-19 in *bcl-2* −/− mice was induced by the defect in the development of ureteric buds due to dramatic mesenchymal apoptosis in the foregoing E-13 to E-16. The role of *bcl-2* for cell survival in the late stage of nephrogenesis requires additional investigation.

It remains unclear what fraction of the mesenchymal cells is protected by *bcl-2* and also whether the proliferation and differentiation of surviving mesenchymal cells can modulate renal morphology in *bcl-2* −/− mice. As *bcl-2* −/− mice exclusively had severe renal hypoplasia, considerable numbers of cells must have died in the absence of gene production of *bcl-2*. In preliminary studies, we have found that, when ES cells bearing homozygous mutation (*bcl-2* −/−) at the *bcl-2* locus are injected into normal mice blastocysts (*bcl-2* +/+), chimeric mice are generated.[27] The resultant renal mesenchyme is composed of a mixture of *bcl-2* −/− and *bcl-2* +/+ mesenchymal cells. However, the kidneys in these mice exhibit no apparent abnormalities at birth (K. Nakayama, unpublished observation). Thus, *bcl-2* might protect substantial numbers of cells from apoptosis during nephrogenesis.

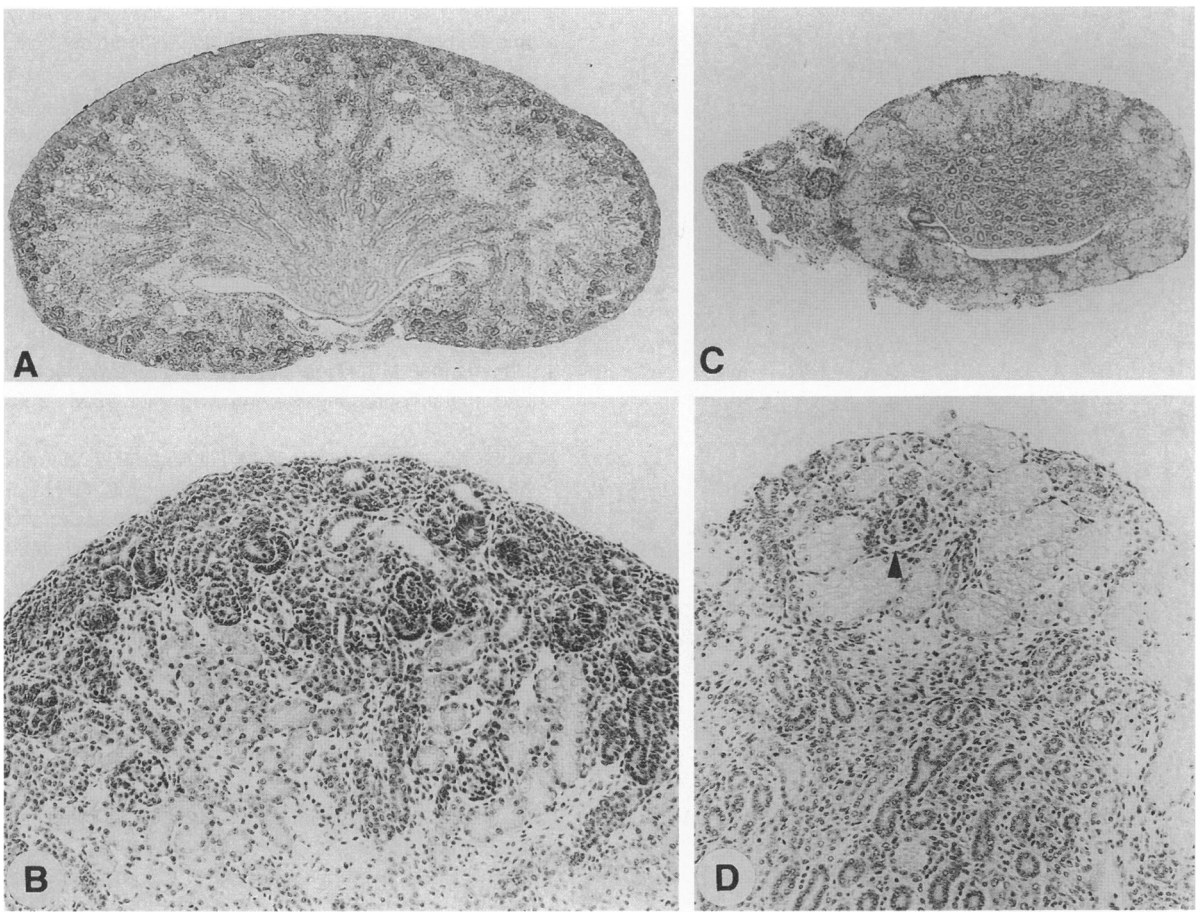

Figure 7. *Representative example of the kidneys at birth at the same magnification from* bcl-2 +/− *mouse* (**A** *and* **B**) *and a* bcl-2 −/− *mouse* (**C** *and* **D**). *The size difference is apparent.* **A** *and* **B**: *Kidney from a* bcl-2 +/− *mouse shows active centrifugal nephrogenesis.* **C** *and* **D**: *Kidney from a* bcl-2 −/− *mouse lacks a nephrogenic zone and exhibits tubular hypertrophy. One glomerulus is marked by an* arrowhead. **B** *and* **D** (*magnification,* ×*170*) *are higher-magnification views of* **A** *and* **C** (×*64*), *respectively.*

Among the three genotypes that we examined, the number of apoptotic cells from E-13 to E-16 was highest in *bcl-2* −/− mice. The number of apoptotic cells in *bcl-2* +/− mice was approximately one-half that in *bcl-2* −/− mice, yet, although the difference did not reach statistical significance, it tended to be three times higher than that in *bcl-2* +/+ mice. This difference was consistent with the differences in levels of *bcl-2* protein among these mice.[20] The *bcl-2* −/− mice exclusively displayed severe hypoplasia. However, morphometry indicated identical renal phenotypes in *bcl-2* +/+ and *bcl-2* +/− mice, despite the threefold higher incidence of apoptosis in *bcl-2* +/− mice than in *bcl-2* +/+ mice. These findings imply that the nephron number was not linearly correlated with the number of cells that died, but they suggest that there is a threshold in terms of cell number for normal nephrogenesis. In other words, when the number of renal stem cells is greater than a certain number as a consequence of the proliferation of remaining mesenchymal cells, normal nephrogenesis can occur. Perhaps renal hypoplasia in *bcl-2* −/− mice could be prevented by some growth factors that stimulate the proliferation of remaining mesenchymal cells. Characteristics of pre-induced mesenchymal cells as multipotential to differentiate might suggest this idea.[28]

In nephron development, the induction of and protection from apoptosis occur separately, in terms of both growth stage and distribution. The mechanisms of regulation of these opposite phenomena are far from understood. Recently, Oltivai et al[29] reported the promotion of apoptosis by *bax*, a heterodimer of the production of *bcl-2* . They proposed that the relative levels of these two gene products might determine cell fate, ie, survival or death. Furthermore, *bax* was suggested to be involved in nephron development by its distribution in developing kidneys.[30] Thus, a balance between activities of *bcl-2* and *bax* might regulate cell fate during nephrogenesis. Additional studies are needed to determine the temporal and spatial patterns of the expression of

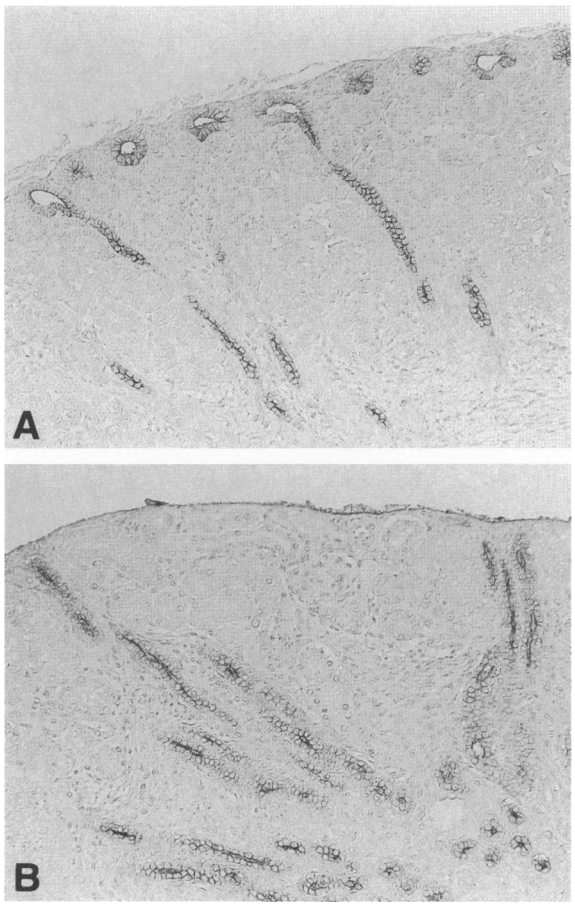

Figure 8. *Labeling with* D. biflorus *lectin of ureteric buds at birth.* **A:** *Note the elongation and convolution of the ureteric buds that extend to the subcapsular area in a* bcl-2 *+/− mouse.* **B:** *In a* bcl-2 *−/− mouse, ureteric buds are rare in the subcapsular region and ends are slim (not convoluted). Magnification, ×160.*

bcl-2 and *bax* during nephrogenesis and the mechanism by which *bcl-2* converts to *bax* or *vice versa*.

In conclusion, *bcl-2* is indispensable for inhibition of programmed cell death in uninduced mesenchymal cells and sometimes in tubular cells, in particular during the early stage of nephrogenesis *in vivo*.

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