Isolation and characterization of the chicken *bcl-2* gene: expression in a variety of tissues including lymphoid and neuronal organs in adult and embryo

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ABSTRACT

The expression of human *bcl-2* gene is de-regulated by t(14;18) translocation in most of follicular lymphoma. Recent studies indicated that the *bcl-2* gene product has an ability to block apoptosis of hematopoietic cells. To facilitate the analysis of the role of this gene in normal development using an animal model, we have isolated and partially characterized the chicken homologue of human *bcl-2* gene. The analysis of nucleotide sequence showed that the organization of the chicken *bcl-2* gene is very similar to that of human *bcl-2* gene. The primary transcript is spliced to encode a 25,687 dalton (233 a.a.) protein. The chicken Bcl-2 protein has two regions highly homologous to human Bcl-2 protein surrounding a totally non-homologous region. The expression of the chicken *bcl-2* gene was analyzed in various chicken tissues. In the adult chicken, *bcl-2* transcripts were detected in thymus, spleen, kidney, heart, ovary and brain, with the highest levels being detected in the thymus. However, the bursa of Fabricius, which is the site of early B cell development, expressed much less amounts of *bcl-2* RNA. On the other hand, in embryo, the gene is extensively expressed in the bursa, as well as in muscle and the above tissues. Our findings indicate that a homologue of the human *bcl-2* gene does exist in the chicken and that its expression is developmentally regulated in some tissues.

INTRODUCTION

The *bcl-2* gene was originally identified through the studies of the t(14;18) chromosome translocation associated with human follicular lymphoma (1, 2, 3). The breakpoints of the translocation consistently cluster within or in close vicinity of the *bcl-2* gene (1). It has been demonstrated by several groups including ours that a dominant role of the *bcl-2* gene product is to prolong the survival of hematopoietic cells in the absence of required growth factors and also in the presence of various stimuli inducing cellular death. This death-sparing activity has been shown to specifically

result from blocking apoptosis, a mechanism of programmed cell death (4, 5, 6). This unique biological activity, together with its main localization in mitochondrial inner membrane (5), makes the *bcl-2* gene distinct from other oncogenes. The promotion of the long-term survival of cells conferred by the *bcl-2* gene could set a stage for secondary events required for malignant transformation. Accordingly, *bcl-2* transgenic mice over-expressing *bcl-2* gene developed lymphoproliferative disorder with an increased number of polyclonal IgM/IgD B lymphocytes (7, 8). In a significant subset of these animals, high-grade clonal lymphomas emerged, indicating the acquisition of additional genetic events, such as *c-myc* gene activation for fully malignant transformation (9). It is, however, not clear whether the promotion of cell survival is a sole role of the *bcl-2* gene in tumorigenesis. There have been reports which demonstrated that the *bcl-2* gene provided cells with the modest yet significant growth advantage (6, 10).

The *bcl-2* gene is expressed in variety of tissues (11, 12), with the highest level found in lymphoid organs where the *bcl-2* gene seems to play an important role by blocking apoptosis in subset of lymphocytes (13). However, in other tissues such as neuronal ones, a role of the *bcl-2* gene has not been experimentally determined. Furthermore, it is not known how the *bcl-2* gene expression is regulated in embryos versus adults. Therefore, to understand the role of the *bcl-2* gene product in tumorigenesis of lymphoid and in non-lymphoid organs as well as its role in embryogenesis, we attempted to utilize an avian system as a model.

In this paper, we have described the molecular cloning and characterization of the chicken *bcl-2* gene, and also shown that the *bcl-2* gene is expressed in a variety of tissues in embryos and adults.

MATERIALS AND METHODS

Screening of Chicken Genomic Library

Human cDNA probe pBp4-2 (2.4 kb cDNA probe covering all protein coding region of the human *bcl-2* gene; unpublished) were used for screening chicken genomic library. Chicken genomic

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library constructed in the phage vector λ EMBL3 (14) from partially Sau3AI-digested chicken thymocyte DNA was obtained from Dr. L. Showe. The library was screened by plaque filter hybridization procedure (15) with some modifications as follows; hybridization was performed at 37°C in 6×SSC in the presence of 40% formamide and filters were washed with 6×SSC at 65°C. The six positive phage clones obtained were then classified by hybridization with 5' probe, pB16 (1.6 kb cDNA probe covering exons I and II of the human *bcl-2* gene; 16) and 3' probe, p18-4H (human genomic probe covering exon III; probe A in reference 16).

DNA Sequencing

DNA sequencing was performed mainly by the dideoxynucleotide chain termination method (17). Both strands of DNA were sequenced. Chemical modification method (18) was also used for confirmation of the sequence in regions where the sequence was hardly determined by the chain termination method.

RNA Analysis

All tissues were obtained from line SC White Leghorn chickens (gs⁻, Chf⁻, c/o; HY-line International, Dallas center, IA USA). Tissues were collected from a 12-week-old female chicken, except for the testis which was from a male chicken of the same age. Embryo tissues were obtained from 17-day-old embryos, except for the bone marrow which was from one day post-hatching chicks. Hematopoietic cells were harvested from yolk sac of 13-day-old embryonated eggs. RNAs were extracted by homogenizing each tissue in the presence of guanidine thiocyanate, and were purified by centrifugation in cesium chloride solution as described in (19). Poly(A) RNAs were obtained from the total RNAs using oligo(dT)-cellulose (Collaborative Research), analyzed by agarose gel containing 6% formaldehyde, and transferred to a nitrocellulose filter (Schleicher & Schuell). The filters were hybridized with ³²P-labeled probes as follows; pCBL73 (3' probe) bearing SphI-HindIII 2.8 kb fragment from λ CBL-4, including the 3' coding frame, pCBL911 (intron probe) bearing the sequence from 1456 to the SacI site shown at the right end of Figure 1, which was derived from λ CBL-2 and including part of the intron sequence, and pCBL8 (5' probe) bearing SacI-SacI 1.1 kb fragment from λ CBL-2, including the 5' coding frame. Plasmid pKSGapDH, a subclone of pGAD-28 (20), contained about 1.1 kb fragment from GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) gene at PstI site of pBluescriptKS⁺, which was used for verifying the amount of mRNA in Northern blot analysis and was obtained from Dr. P. Suttrave.

Determination of Splicing Site

Junction sequence at the splicing site was preferentially amplified by reverse PCR reaction using primers 1 (5'-CGGAATTCGAGTACCTGAACCGGCACCTGCAC-3', including the sequence from 1250 to 1274, which locates just upstream of the splicing site) and 2 (5'-CGCGGATCCATGGAATTCTGATATTTCC-TTTGGCAGTAATAGC-3', including the sequence from 2842 to 2818, which locates about 300 bp downstream of the splicing site). A reverse PCR reaction was carried out according to the manufacturer's instruction. In brief, the 40 ng poly(A) RNA isolated from 12-week-old chicken thymus was reverse-transcribed with primer 2 using rTth DNA polymerase to make cDNA. The cDNA was then used as a template for PCR amplification using primers 1 and 2 with the same enzyme. The sequence of the PCR product was determined by chain termination method after cloning it into pUC19 (21).

RESULTS

Cloning of the chicken *bcl-2* Gene

Southern blot hybridization of chicken genomic DNA with human *bcl-2* gene probes under a stringent condition revealed specific hybridization bands, indicating the presence of a gene homologous to human *bcl-2* in the chicken genome (data not shown).

To isolate the chicken *bcl-2* gene, a genomic library was screened with human *bcl-2* cDNA probe, pBp4-2 (see Materials and Methods). Human *bcl-2* gene consists of 3 exons with the 1st intron of 220 bp and 2nd intron of approximately 370 kb (22). For convenience, we use terminology of the 5' and 3' regions of the *bcl-2* gene, to indicate the 1st + 2nd exon region and 3rd exon region, respectively. The probe pBp4-2 has both 5' and 3' regions. Six positive clones were classified into two groups, according to their restriction maps and hybridization pattern to the 5' and 3' regions of human *bcl-2* gene. The first group included four overlapping clones which hybridized to the 5' region of the human *bcl-2* gene and the 2nd group included two overlapping clones hybridizing to the 3' region of the *bcl-2* gene. The representative clones, λ CBL-2 (5' clone) and λ CBL-4 (3' clone) were used for further characterization, and the restriction maps of the protein coding and surrounding regions are shown in Figure 1.

Sequence of chicken *bcl-2* gene

The nucleotide sequences of the chicken *bcl-2* genomic locus were determined using the 5' clone and 3' clone described above (nucleotide sequences are available from the data base). A region whose sequence is highly homologous to that of human *bcl-2* gene was found in each clone. Comparisons of nucleotide sequences and putative amino acid sequences of the genes from chicken and human (discussed later), together with the information of splicing site determination (23, 24), suggests that the splicing occurs at the position 1302 as a donor site and the position 2565 as an acceptor site to generate the mRNA of the chicken *bcl-2* gene (see Figure 1 for these positions). To confirm this splicing event, we analyzed the sequence of the junction formed by splicing using reverse PCR as described in Materials and Methods. Poly(A) RNA isolated from 12-week-old chicken thymus was reverse-transcribed using primer 2, and the resulting cDNA was then used as a template for PCR amplification using primers 1 and 2. The reaction generated a single product with an expected size of 354 bp (data not shown). Analysis of the sequence of the amplified fragment revealed the existence of mRNA in which the sequence from 1302 to 2565 was removed from primary transcripts (Figure 2).

Coding Regions

Analysis of the 5' region of chicken *bcl-2* gene showed that a large open reading frame started at the position 735. Because RNA splicing removes the intron sequence from 1302 to 2565 as described above, the reading frame jumps from 1301 to 2566 and ends at 2697. The open reading frame can encode a protein consisting of 233 amino acid residues with a calculated molecular weight 25,687 daltons. This protein has amino acid sequences homologous to those of human and mouse Bcl-2 α proteins as shown in Figure 3.

On the basis of the degree of homology among Bcl-2 α proteins of chicken, human and mouse, the protein can be divided into five regions (Figure 3). Region I is from the first residue to the

Figure 1. Restriction Map of the Chicken *bcl-2* Gene. Shown are schematic structures of the 5' (upper) and 3' (lower) regions of chicken genomic *bcl-2* DNA. Each of them is a part of the fragment cloned in λ CBL-2 (5' clone) or λ CBL-4 (3' clone), respectively. Thick lines show the region whose nucleotide sequences were determined (available from the data base). The closed boxes on the top of the restriction maps indicate the protein coding regions highly homologous to human *bcl-2* gene (see text). Shaded bars indicate the positions of probes, pCBL8 (5' probe), pCBL911 (intron probe), and pCBL73 (3' probe). The restriction sites shown are BamHI (Bm), BglII (Bg), HincII (Hc), HindIII (Hd), NcoI (Nc), PstI (Ps), PvuII (Pv), SacI (Sc), SmaI (Sm), and SphI (Sp). Relevant nucleotide numbers are also shown (see text).

33rd, region II from the 34th to the 79th, region III from the 80th to the 126th, region IV from the 127th to the 189th and the region V from the 190th to the 233rd residues of the chicken Bcl-2 α protein, respectively. The extent of homology in each region was summarized in Table 1. The region IV is most highly conserved among these species (identical for human and mouse). The regions I and III are also highly conserved between human and mouse, differing only one and two residues, respectively. These regions of chicken are less conserved, having 84% and 77% homology to human sequence in regions I and III, respectively. Surprisingly, the region II of chicken Bcl-2 α protein has no significant homology to those of human and mouse, while residues in the region of human and mouse Bcl-2 α proteins are conserved with 64% homology. Therefore, this region does not seem to be responsible for the function common to all species of the protein. The region V encoded by 3' exon is highly conserved like region IV, showing more than 90% homology among the three species. This region has a stretch of hydrophobic residues as described previously (16), and is probably responsible for localization of the Bcl-2 protein in the membrane fraction (5, 25).

In human and mouse, the presence of Bcl-2 β protein has been suggested, which can be translated from unspliced mRNA (11, 16). On the putative unspliced mRNA of chicken *bcl-2* gene, the open reading frame started from the position 735 is terminated by the termination codon at the position 1383, and can code a protein of 216 amino acid residues with a calculated molecular weight 23,492 daltons. This protein (chicken Bcl-2 β protein) shares the same amino acid sequence with the chicken Bcl-2 α protein in the regions I to IV, but the region V comes from the intron sequence in the case for Bcl-2 α protein. The residues

Figure 2. Nucleotide Sequence of the PCR-amplified Fragment Containing the Junction Formed by Splicing. The region containing the splice junction is reverse-transcribed and amplified by PCR reaction using primers 1 and 2 (see Materials and Methods). After cloning the product, its nucleotide sequence was determined. Genomic sequences from the splicing donor and acceptor sites are shown for comparison. The identical regions are boxed.

downstream from the position 190 (region V) are not conserved in the Bcl-2 β proteins of three species, although the first three residues are similar probably because of the signal for splicing.

Non-coding Regions

The 5' untranslated region from the position 211 to 734 of the chicken *bcl-2* gene are significantly homologous to those of human and mouse genes, although the extent of the similarity is much less than that observed for the coding region. In this region, sequences between 211 and 284, between 315 and 322, and between 410 and 490 are highly conserved. They include the sequences GAGTAAGT (from 245 to 252) and TTCCGACAGCCAGG (from 419 to 432), which are almost identical to the consensus sequences of the splicing sites (24), and the sequence CGTTGAGA (from 315 to 322), which match the consensus sequence of the branch site (26). Because the human *bcl-2* mRNA in which the corresponding sequence was

Figure 3. Comparison of Amino Acid Sequences of Bcl-2 Proteins from Various Animals. The amino acid sequences of Bcl-2 proteins from chicken, human and mouse, deduced from their nucleotide sequences, are aligned to obtain a maximum homology. Numbers are the positions of amino acid residues from the amino terminus of the chicken Bcl-2 protein. Residues shown by hyphens in human and mouse sequences are identical to those of chicken at the corresponding positions. Asterisks indicate the stop codon. From residues 190, shown are the sequences of Bcl-2 α and Bcl-2 β proteins derived from spliced and unspliced transcripts, respectively. Roman numerals are the names of regions according to the extent of homology among three proteins (see text and Table 1). Sources of the sequences of the human Bcl-2 protein are Tsujimoto & Croce (16) and Cleary et al. (37) and that of mouse Bcl-2 protein is Negrini et al. (11). Some parts of human and mouse *bcl-2* genes were re-sequenced. The following residues were found to be incorrect in our previous reports; the 96th, 110th and 237th residues in human Bcl-2 protein (corresponding to the 90th, 104th and 231st residues in chicken Bcl-2 protein, respectively) and the 221st and 222nd residues in mouse Bcl-2 protein (corresponding to the 218th and 219th residues in chicken Bcl-2 protein, respectively). The corrected sequences are shown.

removed from precursor RNA by splicing was actually detected (22), the sequence from 257 to 431 could act as an intron also in chicken. The distance from this intron to the initiation codon for Bcl-2 protein is very similar among the three species. The functional motifs or units of sequences other than splicing signals were not found in this region. The region upstream from the position 210 does not show any significant similarity to those of human and mouse *bcl-2* genes.

The 3' untranslated region also showed some similarity to human and mouse *bcl-2* genes. Although chicken *bcl-2* gene lacks the (CA)_n cluster whose existence just downstream of the termination codon was previously reported for human and mouse *bcl-2* genes (11, 16), the similarity downstream of the sequence suggests that the chicken *bcl-2* mRNA also has a long untranslated sequence downstream of the coding region as in the cases of human and mouse. Biological significance of this region is not known.

The intron sequences from 1302 to 1684 and from 1685 to 2565 do not overlap with each other. Because we could not obtain the phage clone containing both sequences of 5' region and of 3' region, the intron should be larger than 3 kb.

Expression of *bcl-2* Gene in Various Tissues of Chicken

We analyzed transcripts from chicken *bcl-2* gene by Northern blot analysis. Because the human and mouse *bcl-2* genes are reported to be transcribed into at least two mRNAs, namely spliced and unspliced transcripts (11, 16), two different probes were used for blot hybridization to detect the chicken *bcl-2* mRNA. One is a 3' probe, pCBL73 (see Figure 1), bearing SphI-HindIII 2.8 kb fragment from the 3' clone λ CBL-4, and including the 3' coding frame. The other probe is an intron probe, pCBL911

Table 1. The Extent of Homology in Amino Acid Sequences Among Bcl-2 α Proteins from Various Species.

Region	Residues	Chicken Human	Chicken Mouse	Mouse Human
I	1-33	84	82	97
II	34-79	26	15	64
III	80-126	77	75	96
IV	127-189	97	97	100
V	190-233	91	91	100

The total number of the identical residues was divided by the total residues present in each region of Bcl-2 α protein of the species shown in upper column, and the ratio was shown in percentage. Positions of the regions are shown in Figure 4. Numberings of the residues are those of chicken Bcl-2 α protein.

(see Figure 1), bearing the intron sequence from the position 1456 to the SacI site at the right end of the 5' region shown in Figure 1. The 3' probe detected a major transcript of 6.5 kb and a minor one of 4.5 kb (Figure 4A and C). Because these transcripts were also detected using a 5' probe, pCBL8 (data not shown; see Figure 1 and Materials and Methods for the probe), they should be the spliced products and correspond to the 8.5 kb and 5.5 kb transcripts of the human *bcl-2* gene, respectively, and to the 7.5 kb transcript of the mouse *bcl-2* gene. On the other hand, the intron probe detected no transcript under the same condition (data not shown), and the 5' probe did not detect any transcripts other than those detected by the 3' probe (data not shown). Therefore, we concluded that most transcripts from chicken *bcl-2* gene are spliced.

We examined the expression of the chicken *bcl-2* gene in various tissues by Northern blot analysis of the poly(A) RNA

Figure 4. Analysis of Chicken *bcl-2* Transcripts in Various Tissues. Poly(A) RNAs (2 μ g) isolated from various tissues of line SC chickens (12-week-old) and embryos were electrophoresed, transferred to a nitrocellulose filter, and hybridized with 32 P-labeled DNA probes. Poly(A) RNAs from adult tissues were hybridized with (A) a 3' probe pCBL73, and then rehybridized with (B) a GAPDH gene probe to verify the amount of mRNA loaded in all lanes. Poly(A) RNAs from embryo tissues were hybridized with (C) a 3' probe pCBL73 and with (D) a GAPDH gene probe. Figures (C) and (D) also include the poly(A) RNAs from adult tissues loaded on the same filter for comparison. Sources of poly(A) RNAs indicated at the top of the figures are testis (Te), ovary (Ov), bursa (Bu), spleen (Sp), thymus (Th), bone marrow (Bm), liver (Li), kidney (Ki), cerebrum (Cr), cerebellum (Cl), optic lobe (Ol), retina (Re), heart (He), muscle (Mu), and yolk sac (Ys). Positions of RNA size markers (kb) were shown on the left of the Figure (A). For probes, see Materials and Methods. Adult muscle expressed GAPDH gene much higher than other tissues as reported previously (20). The bands for *bcl-2* transcripts were diffuse probably because of the heterogeneity in the transcription initiation sites as previously described for human and mouse *bcl-2* genes (11,22).

from each tissue. In adult (12-week-old) chicken, the *bcl-2* gene is expressed in a variety of tissues; with the highest levels of the *bcl-2* transcripts being detected in thymus, lower levels in spleen, kidney, heart, ovary and nervous systems including cerebrum, cerebellum, optic lobe and retina, and the lowest levels in the bone marrow and bursa (Figures 4A). *Bcl-2* transcripts were not detected in the liver, muscle, and testis. This expression pattern is very similar to those of the mouse *bcl-2* gene (11). In embryo, thymus, spleen, kidney and nervous systems expressed the *bcl-2* gene, and bone marrow and liver expressed a much lesser amount, as seen in adult tissues (Figure 4C). Bursa and muscle of embryo chicken, on the other hand, expressed the gene (Figure 4C), which is in a contrast to its repression in the same tissues of adult chicken (Figures 4A and C).

DISCUSSION

We have isolated the *bcl-2* gene from chicken and have determined its nucleotide sequence. The organization of the genomic sequence of the chicken *bcl-2* gene is very similar to that of human and mouse *bcl-2* genes. The coding region of the gene is divided into two portions by an intron. Splicing in chicken *bcl-2* gene occurs at the site exactly corresponding to those of other species. The northern blot analysis showed that the 6.4 kb

transcripts are derived from the processing of the 5' and 3' exons, encoding a protein of 233 amino acid residues with deduced molecular weight 25,687 daltons, which is highly homologous to human and mouse Bcl-2 α proteins with molecular weights of 26,170 daltons (239 amino acid residues; 16, 22) and 26,476 daltons (236 amino acid residues; 11), respectively. On the contrary to the existence of the *bcl-2* β transcripts derived only from 5' exon and a part of the intron in human and mouse (11, 16), chicken *bcl-2* gene seems to produce virtually no unspliced *bcl-2* β mRNA.

In the human, the chromosomal translocation t(14;18) associated with most follicular lymphomas juxtaposes the *bcl-2* gene with the IgH gene. This chromosomal configuration results in the constitutive expression of the *bcl-2* gene in B lymphoma cells. High level expression of the *bcl-2* gene resulted in blocking the apoptotic cell death of human and mouse B or T cells, which, in lymphomagenesis, may play a role to increase the probability of additional genetic changes required for neoplastic transformation. In normal development of lymphocytes, the Bcl-2 protein appears to be a critical regulator in selection of subset of immune cells (13). Because of the similarity of the amino acid sequences, it is very likely that the chicken Bcl-2 protein has similar biological functions. We are currently analyzing the death-sparing activity of the chicken Bcl-2 protein in chicken and mouse lymphocytes.

Analysis of chicken *bcl-2* transcripts in different tissues shows a high level expression in some lymphoid tissues, including thymus and spleen in both adult and embryo. The requirement to maintain a long lived pool of antigen reactive cells may be mediated by *bcl-2* expression and the blocking of programmed cell death. In contrast to these tissues, expression of *bcl-2* gene in the bursa of Fabricius, which is the site of early B cell development, was detected in the embryo and much less in adult chicken. These findings can be interpreted to correlate with the changing role of the bursa in B cell maturation during development. In the embryo, pre-bursal stem cells enter the bursa between day 8 and day 14 (27) and undergo rapid proliferation to form a pool of cells (post-bursal stem cells), that migrate to distal lymphoid tissues shortly after hatching (28). These post-bursal stem cells are responsible for maintaining the peripheral B lymphocyte population, independent of the bursa of Fabricius (28). Antibody diversity in the chicken is directly affected by the size of this stem cell pool (29, 30). It would therefore be advantageous to maximize cell proliferation and to minimize cell death of B cells to allow expansion of this stem cell pool in the embryonic bursa. The processes of gene conversion and somatic mutation would be allowed to increase immunoglobulin gene diversity in an expanding B cell pool (31). Once the post bursal stem cells have completed seeding to peripheral tissues about 2 weeks after hatching, the bursa is no longer required to maintain humoral immunity (32, 33). The Bcl-2 protein may function as a regulator of B cell longevity in the chicken, by blocking apoptosis, allowing expansion of B cell pool and immunoglobulin diversity during embryonic development. Around the time of hatching, *bcl-2* expression may decrease in B cells in the bursa and programmed cell death may take over, as suggested by the fact that late embryonic and post hatch bursal cells that are removed from the bursal environment undergo spontaneous apoptosis (34). In human and mouse, B cells in germinal centers and T cells in thymus cortex, most of which undergo apoptosis, express very low amounts of the Bcl-2 protein (12, 13, 35), although most other B cells or T cells express the gene. These observations are consistent with the idea that the expression of the *bcl-2* gene is suppressed to eliminate unnecessary lymphocytes during their development in chicken as well as in mammalian.

Some tissues other than the lymphoid tissues, namely nerve systems, kidney, heart and ovary were found to express the *bcl-2* gene, whereas almost no detectable expression was observed in liver and testis regardless of developmental stage. It should be noted that the gene was expressed in embryo muscle, but not in adult muscle. This result indicates that all long-lived cells do not necessarily express the *bcl-2* gene. Biological function of Bcl-2 protein in these non-lymphoid tissues is not understood. It is known that a significant fraction of nerve cells undergoes apoptosis during their development, and survived cells have a long span of life (36, 37). It is possible that the level of Bcl-2 protein in nerve cells determines their fate as well as in lymphoid cells.

In this study, we showed that even in the embryo, the expression of the *bcl-2* gene is somewhat restricted to certain tissues, and differences are evident between its expressions in adults and embryos. Further characterization would help us to understand a role of *bcl-2* gene in a variety of tissues during development. The similarity in genetic sequence and tissue-specificity of the expression of the *bcl-2* gene in the chicken and mammals suggest that the chicken can provide a useful model for the analysis of the role of the *bcl-2* gene in normal and neoplastic development.

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