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Genetics

Overexpression of the human *BCL-2* gene product results in growth enhancement of Epstein–Barr virus-immortalized B cells

(chromosome translocation/B-cell lymphoma/B-cell growth)

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ABSTRACT **The biological activity of the human *BCL-2* gene product was analyzed in an Epstein–Barr virus (EBV)-infected human lymphoblastoid B-cell line transfected with *BCL-2* sequences driven by the simian virus 40 promoter and enhancer. Overproduction of the BCL-2 protein conferred a selective growth advantage to the EBV-infected B cells as compared with control transfectants in low-serum medium and also after seeding at limiting dilution but did not render the cells tumorigenic in athymic nude mice. This growth enhancement was also seen in cells transfected with the *BCL-2* gene with its own promoter juxtaposed to the immunoglobulin heavy chain gene enhancer, which represents the translocated form of the *BCL-2* gene observed in follicular lymphomas with the t(14;18) translocation. The growth advantage of EBV-infected B cells overproducing the BCL-2 protein is neither due to the enhanced growth factor production nor due to an enhanced sensitivity of the *BCL-2* transfectants to interleukins 1 or 6, although both lymphokines are known to stimulate proliferation of EBV-infected B-cell lines. The growth advantage of EBV-infected B cells by overproduction of the BCL-2 protein suggests the direct involvement of the *BCL-2* gene product in the pathogenesis of follicular lymphoma.**

Chromosome translocations associated with human malignancies are believed to activate cellular genes that play crucial roles in the pathogenesis of these diseases (1–3). About 90% of follicular lymphomas, one of the most common hematopoietic malignancies, carry the specific chromosome translocation, t(14;18)(q32;q21) (4, 5), which directly involves the immunoglobulin heavy chain (IgH) gene on 14q32 and the *BCL-2* gene on 18q21 (6–9). The *BCL-2* gene was the first to be identified by analyzing chromosome translocations. A crucial role for this gene in follicular lymphomagenesis was suggested by the observations that all t(14;18) translocations studied thus far occur within or very near the *BCL-2* gene (6–11) and that the steady-state level of BCL-2 mRNA is elevated by the t(14;18) translocation (9, 12).

The *BCL-2* gene encodes two proteins, α (26 kDa) and β (21 kDa), which are identical except in the carboxyl-terminal portion (13). Use of a rabbit polyclonal antibody against the BCL-2 protein has revealed the presence of the BCL-2 α protein in the membrane fraction (14). Because the amino acid sequences of the BCL-2 α protein show neither a transmembrane domain nor a signal peptide, the BCL-2 α protein is probably localized to the membrane inner surface. Furthermore, the *BCL-2* gene is known to be activated after mitogenic stimulation of normal B and T cells (15). Together these observations suggest the involvement of the BCL-2 protein in signal transduction in B and T cells. However, no direct experimental evidence exists for the involvement of the *BCL-2* gene product in the growth of human B cells. In an effort to provide such evidence, the effect of *BCL-2* gene overexpression on cell growth has been analyzed in a human B-cell line because the *BCL-2* gene is highly expressed in both B and T cells (9, 16), but *BCL-2* gene rearrangement is seen exclusively in B-cell tumors (4, 5).

MATERIALS AND METHODS

Cell lines. Epstein–Barr virus (EBV)-infected human lymphoblastoid B-cell lines GM1500 and GM 607 (17) were obtained from the Human Cell Depository (Camden, NJ), and maintained in RPMI 1640 medium containing 10% fetal calf serum (FCS).

DNA Transfection. The DNA constructs were transfected into EBV B cells by electroporation as described (18, 19) using Bio-Rad Gene Pulser at 260 V, 960 μF. After electroporation, the cells were serially diluted with the above medium supplemented with 20% FCS. Transfectants were selected in the presence of G418 antibiotics at 1 mg/ml (GIBCO).

Immunoprecipitation. The transfectants were labeled with [^{35}S]methionine (New England Nuclear) at 100 μCi/ml (1 Ci = 37 GBq) for 5 hr in the methionine-free medium supplemented with 5% dialyzed FCS. Total proteins were extracted and immunoprecipitated by anti-BCL-2 polyclonal antiserum as described (14).

[^{3}H]Thymidine Incorporation by the Transfectants. The transfectants grown in the medium supplemented with 10% FCS were washed twice to remove FCS, and 10^4 cells were seeded into each well of a 96-well flat-bottomed dish with 0.2 ml of the medium. [^{3}H]Thymidine (1 μCi) was added, and trichloroacetic acid-insoluble radioactivity was measured by the use of glass filters.

RESULTS

Introduction and Expression of the *BCL-2* Gene in EBV B Cells. As the recipient cells for DNA transfection the EBV-immortalized B-cell line GM1500 was chosen because it represents a differentiation stage similar to that of follicular lymphoma cells (17).

Fig. 1 shows the DNA constructs used for transfection. The vector pCΔj (20), which contains the G418 resistance gene (as a selective marker), and the EBV-derived DNA replication origin (*oriP*) was used. The *oriP* element allows independent replication of the DNA constructs in EBV-infected B cells (20). The BCL-2 cDNA sequences are expressed by simian virus 40 enhancer/promoter regulatory elements (pCΔj-bcl-2) or by its own promoter with an immunoglobulin enhancer (pCΔj-989). A DNA construct without BCL-2 cDNA sequences (pCΔj-SV2) was used as a control. GM1500 cells were transfected with these DNA

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Abbreviations: EBV, Epstein–Barr virus; FCS, fetal calf serum; IL, interleukin; SV2, simian virus 40 DNA; *oriP*, EBV-derived DNA replication origin. IgH, immunoglobulin heavy chain gene.

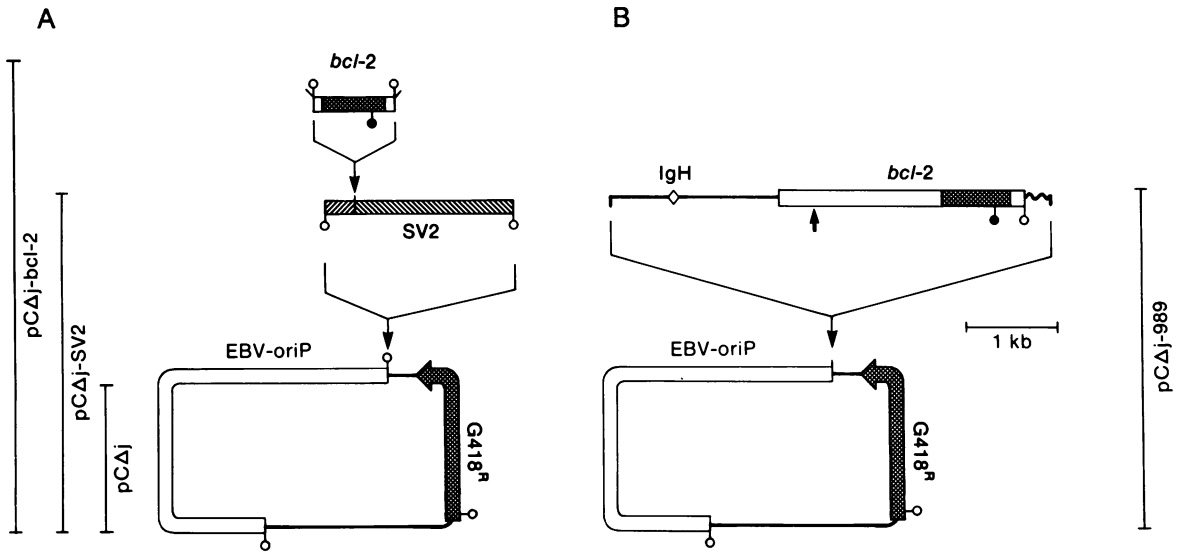

FIG. 1. DNA constructs for DNA transfection. The pCΔj vector (20) was used, which contains EBV-*oriP* and the G418 resistance gene. (*A*) pCΔj-SV2 and pCΔj-bcl-2. Simian virus 40 DNA (SV2) containing promoter/enhancer/poly A site; a *Pvu* II (converted to *Eco*RI in this construction)–*Eco*RI fragment from pSV2CAT (21) with a deletion of a 515-base-pair (bp)-long region (*Hin*dIII to *Bal* I) containing the initiation codon of the *CAT* gene. The BCL-2 cDNA sequences obtained from the pB4 clone (13) containing a full open reading frame sequence for BCL-2 α protein (shown by spotted box). (*B*) pCΔj-989. pCΔj-989 construct consists of three DNA segments joined as an insert in an *Eco*RI site converted to a *Hin*dIII site of pCΔj vector. From left, *Hin*dIII–*Bam*HI containing IgH enhancer (◇) on chromosome 14 juxtaposed to the *BCL-2* gene, *Bam*HI to *Eco*RI segment derived from the 3′ part of BCL-2 cDNA sequences of pB4, *Eco*RI to *Hin*dIII segment derived from *Xba* I (converted to *Eco*RI) to *Nco* I (converted to *Hin*dIII) containing a polyadenylylation site of herpes simplex virus thymidine kinase gene (22). Arrow in *BCL-2* sequences in pCΔj-989 indicates the transcription initiation site. ♀, *Eco*RI; ▮, *Hin*dIII; and ♦, *Bam*HI.

constructs by electroporation (18, 19). DNA transfectants were selected in medium containing G418. DNA transfectants were used for further study without subcloning.

Southern blot analysis of low-molecular weight Hirt DNA (23) from these transfectants revealed the presence of intact extrachromosomal plasmids, and copy number was estimated at five per cell (data not shown). Overexpression of the BCL-2 protein in transfectants was demonstrated by immunoprecipitation with polyclonal anti-BCL-2 antibody (14) as shown in Fig. 2. The BCL-2 protein in transfectants carrying the control construct, pCΔj-SV2, is barely detectable,

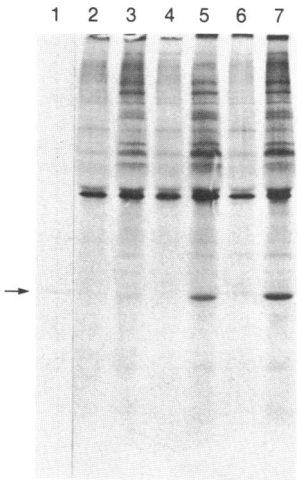

FIG. 2. Immunoprecipitation of BCL-2 protein from transfectants. The *BCL-2* and SV2 transfectants were labeled with [^{35}S]methionine, and proteins were extracted and immunoprecipitated with anti-BCL-2 polyclonal antibodies as described (14). Lanes: 1, [^{35}S]methionine-labeled BCL-2 α protein prepared *in vitro* from mRNA synthesized from cDNA sequences with T7 phage RNA polymerase as described (13); 2 and 3, SV2 transfectants; 4–7, *BCL-2* transfectants; 2, 4, and 6, precipitation with preimmune serum; 3, 5, and 7, precipitation with immune serum. The immunoprecipitated proteins were analyzed by 12.5% polyacrylamide/NaDodSO$_4$ gel electrophoresis. An arrow indicates the BCL-2 α protein.

whereas the protein is present at high levels in pCΔj-bcl-2 transfectants.

Alternation of Growth Properties of EBV B Cells by High Levels of the *BCL-2* Gene Expression. The possibility that overexpression of the *BCL-2* gene product might lead to tumorigenic conversion of GM1500 cells was tested by injecting cells into athymic mice. The *BCL-2* transfectants gave rise to tumors at a slightly higher frequency (three tumors out of 14 injections) than the SV2 transfectants (one tumor out of nine injections) about 1 month after injection. Tumor size did not exceed 1 cm in diameter, and all tumors regressed by 3 months. The comparable result was obtained using athymic mice that had been x-ray-irradiated (250 rad; 1 rad = 0.01 Gy). Tumors developed after injection of transformed NIH 3T3 cells continued to grow until the hosts died. Thus, it was concluded that overproduction of the BCL-2 protein alone does not render the EBV-immortalized B cells tumorigenic in athymic mice. Comparison of the growth profile of *BCL-2* and SV2 transfectants revealed no differences from those of GM1500 cells, and both formed the large aggregates characteristic of the parental cells (data not shown). These contrast sharply with *myc*-transfectants of EBV-immortalized B cells, which grow as single cells and are tumorigenic in athymic mice (24).

The *BCL-2* and SV2 transfectants grew at similar rates when serum concentrations in the medium exceeded 10%. However, at decreased serum concentrations in the medium, the *BCL-2* transfectants were consistently better able to proliferate, forming bigger aggregates than did SV2 transfectants (Fig. 3), although never to a degree seen in the presence of 10% serum (Fig. 4*B*). [^{3}H]Thymidine incorporation by the transfectants grown in 0.2% serum concentration was measured, and [^{3}H]thymidine incorporation by the *BCL-2* transfectants remained higher than that by SV2 transfectants over the 5-day test period (Fig. 4*A*). Moreover, *bcl-2* transfectants were able to maintain a higher rate of DNA synthesis than SV2 transfectants at any serum concentration tested except at 10% serum, in which DNA synthesis was similar for both transfectants (Fig. 4*B*). Comparable results were obtained using human serum and using transfectants derived from a

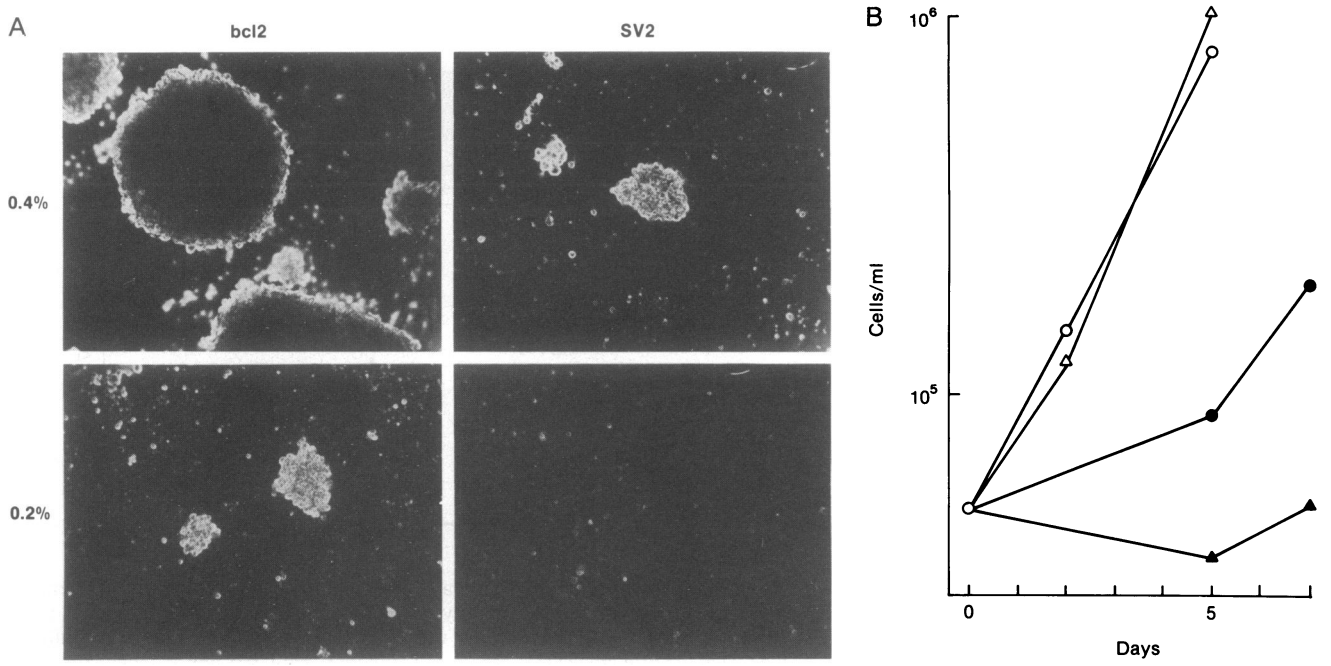

FIG. 3. (*A*) Photographs of *BCL-2* and SV2 transfectants grown at low serum concentration. The transfectants grown in 10% FCS containing RPMI medium were spun and washed twice with serum-free RPMI medium. Cells (2×10^5) were inoculated into 2 ml of RPMI medium containing 0.2% (*Lower*) and 0.4% (*Upper*) FCS in each well of a 24-well dish. Dishes were photographed using phase contrast after 10-day incubation with occasional pipetting to disperse clumps. (*B*) Growth curve of the transfectants. The *BCL-2* (○, ●) and SV2 (△, ▲) transfectants (10^5 cells) were cultured in 2 ml of RPMI medium containing 10% (○, △) and 1% (●, ▲) FCS in each well of a 24-well dish. Number of viable cells was counted.

different EBV-infected lymphoblastoid B-cell line (GM607) (data not shown).

The growth of the transfectants after limiting dilution was also examined. The transfectants grown in the medium containing 10% FCS were serially diluted and seeded in a 96-well dish with the medium supplemented with 10% or 20% FCS. As shown in Fig. 5, the *BCL-2* and SV2 transfectants showed the same viability on the limiting dilution in the

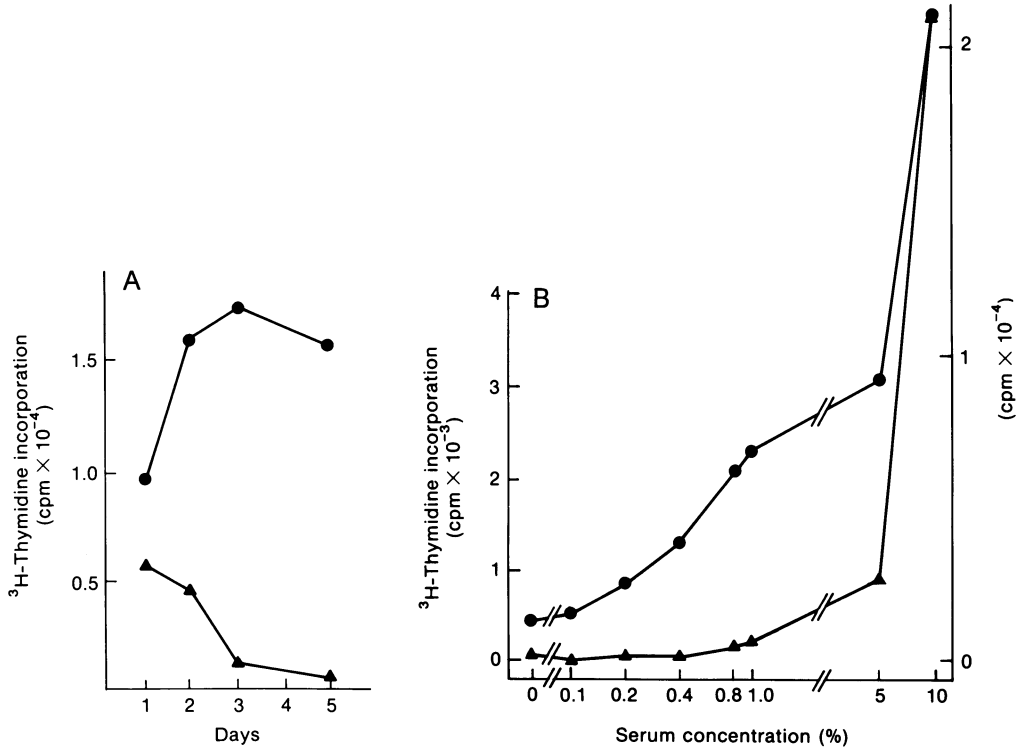

FIG. 4. DNA synthesis by *BCL-2* and SV2 transfectants. (*A*) Transfectants (10^4 cells) were cultured in 0.2 ml of RPMI medium containing 0.2% FCS in each well of a 96-well flat-bottomed dish. [^{3}H]Thymidine (1 μCi) was added to cells at the day indicated in the figure and incubated for another 24 hr. Trichloroacetic acid-insoluble radioactivity was measured. Data are from triplicate cultures. (*B*) Transfectants (10^4 cells) were cultured in 0.2 ml of RPMI medium containing various concentrations of FCS for 3 days and labeled with 1 μCi of [^{3}H]thymidine for 36 hr in 0 to 1% FCS and for the last 1 hr in 5 and 10% FCS. Left and right scales are for data obtained with 0 to 1% FCS and those for 5 to 10%, respectively. Deviations were within 20% for *A* and *B*. ●, *BCL-2* transfectants; ▲, SV2 transfectants.

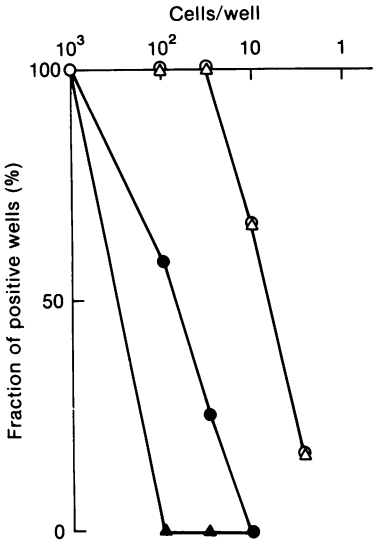

FIG. 5. Viability of the transfectants on the limiting dilutions. The *BCL-2* (○, ●) and SV2 (△, ▲) transfectants were serially diluted with the medium containing 10% (●, ▲) or 20% (○, △) FCS and seeded into 96-well dishes. The fractions of the wells in which the cells grew were counted.

presence of 20% FCS, whereas the *BCL-2* transfectants showed better viability than SV2 transfectants in the presence of 10% FCS.

To determine whether the translocated *BCL-2* gene found in follicular lymphomas with the t(14;18) translocation gives rise to a growth-enhancing effect on B cells, the growth of cells transfected with the DNA construct pCΔj-989 (shown in Fig. 1), which is derived from a case of follicular lymphoma (FL989) in which the 5′-flanking region of the *BCL-2* gene is rearranged with the enhancer of the IgH gene (11). The *BCL-2* gene is transcribed from its own cap site (12, 13) and juxtaposed to the IgH enhancer sequences. Fig. 6 shows that transfectants with the pCΔj-989 construct had the same effect on B-cell growth as that of pCΔj-bcl-2 transfection. Thus, activation of the *BCL-2* gene by juxtaposition to the IgH enhancer without a constant region confers the same growth advantage to the cells as activation by the simian virus 40 regulatory element, suggesting that the growth enhancement by BCL-2 protein seen in tissue culture could reflect the function of the *BCL-2* gene product in follicular lymphoma.

The growth advantage of *BCL-2* transfectants over SV2 transfectants could be explained either by the enhanced production of a relevant growth factor(s) by the *BCL-2* transfectants or by an increased sensitivity to or independence of the growth factor(s) present in the serum. The possibility of enhanced growth factor production by the

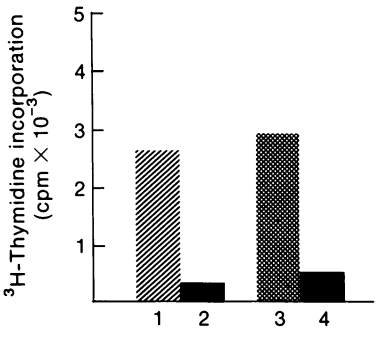

FIG. 6. The pCΔj-SV2 (lanes 2 and 4), pCΔj-bcl-2 (lane 1), and pCΔj-989 (lane 3) transfectants were grown in 0.2% FCS for 3 days and labeled with 1 μCi of [^{3}H]thymidine for 24 hr. Data are from triplicate cultures, and deviations were within 10%.

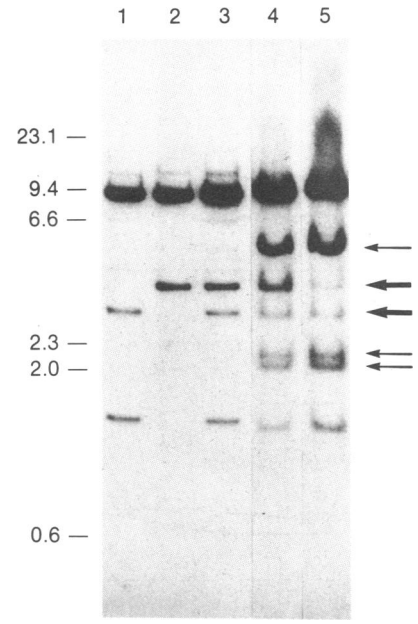

FIG. 7. Cocultivation of the transfectants. An equal number of the *BCL-2* and SV2 transfectants were cocultivated in the medium containing 1% FCS for 7 days. The surviving cells were then grown in the medium containing 20% FCS. The Hirt DNA was extracted and digested with the restriction endonucleases *Bam*HI and *Sac* I. The digested Hirt DNAs, together with the *Bam*HI/*Sac* I-digested pCΔj-bcl-2 and pCΔj-SV2 plasmid DNA, were run on a 1% agarose gel, blotted to nitrocellulose, and hybridized with ^{32}P-labeled pCΔj-bcl-2 DNA. Lanes: 1, pCΔj-bcl-2; 2, pCΔj-SV2; 3, a mixture of pCΔj-bcl-2 and pCΔj-SV2; 4, Hirt DNA from the mixture of two transfectants grown in 20% FCS; 5, Hirt DNA from the surviving cells in 1% FCS medium. The thicker arrows represent the diagnostic fragments. The bands shown by the thinner arrows correspond to the EBV subgenome present in the parental cells. Size is given at left in kb.

BCL-2 transfectants was tested by examining the conditioned medium and also by cocultivation of the transfectants. The conditioned medium from the *BCL-2* transfectants grown in 0.2 and 0.4% serum-containing medium did not enhance growth of the SV2 transfectants (data not shown). Cocultivation of the *BCL-2* and SV2 transfectants was accomplished by cultivating the mixture of an equal number of *BCL-2* and SV2 transfectants in medium containing 1% FCS for 7 days. Surviving cells were then grown in the medium containing 20% FCS, in which the two transfectants grow at the same rate. The population of the two transfectants in the surviving cells in low-serum medium was determined by analyzing the Hirt DNA. As shown in Fig. 7, the intensity of the fragment specific to the *BCL-2* transfectants remains unchanged, whereas the intensity of the fragment specific to SV2 transfectants was reduced, indicating that the *BCL-2* transfectants did not support the growth of the SV2 transfectants in the medium containing the low-serum concentration. These results make it unlikely that the high levels of expression of BCL-2 protein cause enhanced production of a growth factor.

To examine whether *BCL-2* overproduction enhances sensitivity to lymphokines, we tested the effect of the lymphokines, interleukin (IL) 1 and IL-6, which have been shown to play roles in the proliferation of EBV-immortalized B cells (25, 26) on the growth of the transfectants. As shown in Fig. 8, IL-1 and IL-6 showed approximately the same stimulatory effect on *BCL-2* transfectants and on SV2 transfectants examined using Iscove's synthetic medium containing insulin, transferrin, and 2-mercaptoethanol (27) and also RPMI medium containing 10% of FCS (data not shown). Thus, at least IL-1 and IL-6 are not involved in the growth-

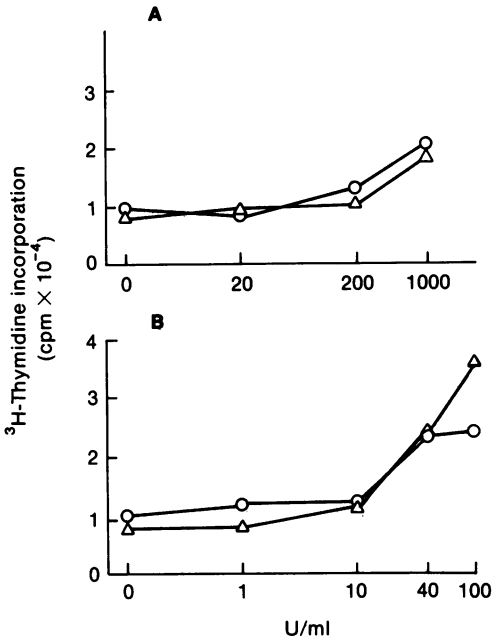

FIG. 8. Stimulatory effect of IL-1 and IL-6 on transfectants *BCL-2* (○) and SV2 (△). Transfectants (10^4) were cultured in 96-well dishes with Iscove's medium supplemented with various amounts of IL-6 (*A*) (provided by T. Hirano and T. Kishimoto) (28) and IL-1 (*B*) (purchased from Genzyme) for 3 days. Cells were then incubated with 1 μCi of [^{3}H]thymidine for 1 day and trichloroacetic acid-insoluble counts were determined. Data were derived from triplicate cultures, and deviations were within 20%.

enhancing effect. The role of other lymphokines remains to be evaluated.

DISCUSSION

Overproduction of the BCL-2 α protein has been shown to confer a growth advantage to EBV-immortalized B cells in the medium with low-serum concentration and also on the limiting dilutions. These observations suggested the possible involvement of the BCL-2 protein in growth factor production, signal transduction of the growth factors, or the signaling by cell-to-cell interaction. The experiment of cocultivation of the *BCL-2* and SV2 transfectants excluded the possibility of enhancement of growth factor production by the high levels of BCL-2 expression. Thus, the BCL-2 protein seems to be involved in the intracellular pathway of signal transductions that are triggered by the growth factor(s) present in the serum and/or by the cell-to-cell interaction. Because cell growth is controlled by cell proliferation and cell death, the signaling pathway affected by the BCL-2 protein could be involved directly in cell proliferation and/or cell survival (29).

It remains to be determined how the BCL-2 protein plays a role in the growth enhancement of human B cells. It is conceivable that the BCL-2 protein plays a cooperative role with other cellular proteins. These might include protooncogene products or viral protein(s), such as EBV-derived protein(s), although EBV has been implicated only in Burkitt lymphoma and not in follicular lymphoma. Both the c-*myc* and BCL-2 genes are involved in consistent chromosomal translocations in human B-cell tumors, and both confer a growth advantage to EBV-infected B cells. The effect of the c-*myc* gene product, however, is more dramatic than that of the *BCL-2* gene product, perhaps reflecting the greater aggressiveness of Burkitt lymphoma than follicular lymphoma.

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