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Bcl-2 Modulates Telomerase Activity*

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Mahitosh Mandal and Rakesh Kumar‡

From the Cell Growth Regulation Laboratory, Department of Clinical Investigation, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

Apoptosis is a physiological mechanism of cell death that plays an important role in the regulation of tissue homeostasis. The regulation of apoptosis is a complex process and involves a number of gene products including the survival factor Bcl-2, which has been found to be frequently deregulated in human cancers. In addition to deregulation of apoptosis, the process of neoplasia is also believed to be driven by the activation of telomerase, a ribonucleoprotein complex that adds telomeric repeats (hexanucleotide 5′-TTAGGG-3′) to the ends of replicating chromosomes. Activation of telomerase has been detected in a vast majority of human cancer cells. Although recent studies have demonstrated the wide occurrence of telomerase activation and Bcl-2 deregulation in human cancer cells, it remains unclear whether there is any linkage between the deregulation of Bcl-2 and telomerase activity in cancer cells. In the studies presented here, we report that the stable overexpression of Bcl-2 in human cancer cells with low Bcl-2 expression was accompanied by increased levels of telomerase activity. In addition, using an IL-2-dependent cytotoxic T-cell line, CTLL-2, we demonstrated that IL-2 deprivation (8 h), which is known to down-regulate Bcl-2 expression, also resulted in concurrent inhibition of telomerase activity in the absence of any detectable apoptosis and accumulation of cells in the G_0/G_1 phase of the cell cycle. Re-exposure of IL-2-deprived CTLL-2 cells to the recombinant IL-2 led to the up-regulation of both Bcl-2 expression and telomerase activity. Taken together, these findings establish a close linkage between the modulation of telomerase activity by survival factor Bcl-2, and provide a model to study regulation of telomerase activity by an anti-apoptotic pathway that is widely deregulated in cancer cells.

In recent years, it has become accepted that apoptosis or programmed cell death plays an important role in the regulation of tissue development and homeostasis (1, 2). Deregulation of apoptosis has been shown to contribute to the pathogenesis of a number of human diseases including cancer (3, 4). Regulation of apoptosis is a complex process, which involves a number of cellular genes including that for B cell leukemia/lymphoma 2 (Bcl-2)[1] and related family members (5). The *bcl-2* gene was first identified at the breakpoint of a chromosomal translocation t(14:18) in B follicular lymphoma (1). A *bcl-2* gene encodes a protein of 26 kDa that protects cells against apoptosis in a variety of experimental systems. Overexpression of Bcl-2 has been shown to suppress the initiation of apoptosis in response to a number of stimuli including anticancer drugs (1, 3, 5–9). Recent studies have indicated that cells from a variety of human cancer may have a decreased ability to undergo apoptosis in response to some physiologic stimuli (4), and a defect in apoptosis may be involved in the aberrant survival and/or development of cancer.

In addition to deregulation of apoptosis, it is increasingly clear that the process of neoplasia is characterized by the activation of telomerase, a ribonucleoprotein enzyme complex that adds telomeric repeats (hexanucleotide 5′-TTAGGG-3′) to the ends of replicating chromosomes, telomeres (10, 11). Telomeres play an important role in chromosome structural integrity and functions including protection against the activation of DNA-damage checkpoints, and to counter the loss of terminal DNA segments that occurs when linear DNA is replicated (12). Recent reports have shown the involvement of telomerase function in acquisition of immortality in cancer cells as telomerase activity has been detected in the vast majority of human cancer cell lines and tumors tested, but is either absent or expressed at very low levels in most (but not all) somatic cells (13–15). The mechanism of telomerase activation during immortalization is not known at the moment. It has been proposed (16) that telomerase activity is repressed in somatic cells; during cell division, telomeres continue to shorten until they reach a critical point at which some cellular factor(s) detect the shortened telomere, resulting in the exit from the cell cycle (M1 crisis) and the cell's senescence. Mutations in genes detecting genetic damage allow a clonal population to continue to divide and escape senescence, resulting in further telomeric loss. Further cell division leads to M2 crisis, and most cells die. However, a rare cell somehow activates its telomerase activity, resulting in stabilization of telomere length and immortalization, which may represent an essential requirement for the expansion of human cancer cells. Thus, activation of telomerase during immortalization may be also linked with the proliferation of cancer cells (16, 17).

Although both activation of telomerase activity and Bcl-2 deregulation have been widely detected in human tumor cells, it remains unclear whether there is any linkage between the deregulation of Bcl-2 and telomerase activity in cancer cells. In the studies presented here, we investigated the influence of Bcl-2 expression on the levels of telomerase activity. We report that the overexpression of Bcl-2 in human cancer cells with low levels of Bcl-2 such as cervical carcinoma HeLa cells and colorectal carcinoma DiFi cells was accompanied by enhanced lev-

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‡ To whom correspondence should be addressed: Cell Growth Regulation Section, Box 36, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030.

[1] The abbreviations used are: Bcl-2, B cell leukemia/lymphoma 2 gene product; IL-2, interleukin-2; rIL-2, recombinant interleukin-2; CTLL-2, cytotoxic T-cell line; mAb, monoclonal antibody; PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; TRAP, telomeric repeat amplification protocol; bp, base pair(s); TRF, terminal restriction fragment.

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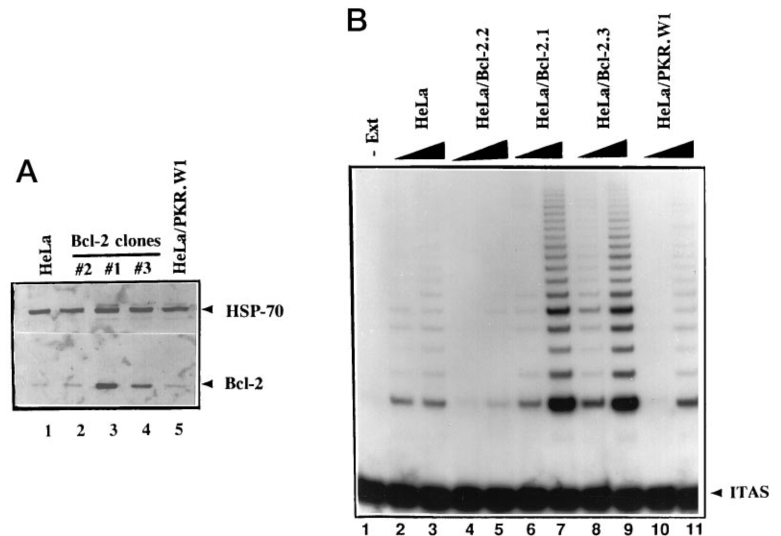

FIG. 1. **Overexpression of Bcl-2 enhances telomerase activity in HeLa cells.** *A*, levels of Bcl-2 in HeLa clones. *Lane 1*, control HeLa; *lane 2*, clone 2; *lane 3*, clone 1; *lane 4*, clone 3; *lane 5*, HeLa/PKR.W1 cells. As an internal control, the upper portion of the Bcl-2 blot was reprobed with an unrelated HSP-70 mAb. *B*, CHAPS extracts were analyzed for telomerase activity by TRAP assay as described under "Materials and Methods." For each sample, 0.01 and 0.1 μg of protein was used. *Lane 1*, reaction without any extract. The observed telomerase activity was RNase A-sensitive, as it could be inactivated completely by the pretreatment of extracts with RNase A (200 μg/ml). Results shown are representative of four experiments.

els of telomerase activity compared with the levels of a telomerase activity present in control cells. In contrast, down-regulation of Bcl-2 expression in an IL-2-dependent cytotoxic T-cell line, CTLL-2, by IL-2 deprivation (8 h) resulted in concurrent inhibition of telomerase activity, and both of these phenotypes (down-regulation of Bcl-2 and telomerase activity in IL-2-deprived CTLL-2 cells) could be effectively reversed by the addition of rIL-2. Taken together, these results demonstrate a close linkage between the deregulation of survival factor Bcl-2 and the telomerase activity in human cancer cells.

MATERIALS AND METHODS

Cell Lines—The cell lines used were human cervical carcinoma HeLa cells (8), human colorectal carcinoma DiFi cells (9, 19), and mouse cytotoxic T-cell line CTLL-2 (20). HeLa cells were cultured in modified Eagle's medium. DiFi cells were maintained in Dulbecco's modified Eagle's medium/F-12 (1:1), and CTLL-2 cells were cultured in RPMI 1640 supplemented with 0.5 ng/ml rIL-2 (Immunex). All complete culture medium contained 10% fetal bovine serum.

Stable Expression of Bcl-2—Cells at a density of 10^6 cells/100-mm diameter plate were transfected with plasmid DNA containing the full-length human Bcl-2 cDNA and a selectable marker, a neomycin phosphotransferase gene (21), by calcium phosphate precipitation procedures as described (6–9). Expression of Bcl-2 in individually isolated clones was determined by immunoblotting with Bcl-2 mAb. Once a stable cell line from each clone had been established, the drug was removed from the culture medium. The clonal lines have been maintained in drug-free medium since then, and expression of Bcl-2 was periodically examined. As a control, cells were transfected either with an unrelated PKR-plasmid DNA containing neomycin marker or with plasmid DNA containing neomycin phosphotransferase gene.

Cell Extracts and Immunoblotting—All experiments were performed with cells in a logarithmic phase by controlling the plating density. Cell extracts were prepared as described (26). Cell lysate containing equal amounts of total protein (15–25 μg) were resolved on a 10% SDS-polyacrylamide gel electrophoresis, followed by probing with an anti-Bcl-2 mAb (Neomarkers Inc.) using alkaline phosphatase-conjugated second antibody (7, 8). As an internal control, the same blot was cut into two pieces, and the upper portion was probed with an unrelated heat shock protein (Hsp)-70 mAb (Neomarkers Inc.) or actin antibody (Sigma). Low molecular mass colored markers (Amersham Corp.) were used as molecular weight standards. Quantitation of specific protein bands was performed by using a protein data base scanner (Molecular Dynamics).

Telomerase Assay—Subconfluent cultures were used to prepare the detergent CHAPS extracts (13). Telomerase enzyme activity was measured by using a PCR-based telomeric repeat amplification protocol (TRAP) kit from the Oncor Inc. as described (22, 23). Each reaction product was amplified in the presence of an internal TRAP assay standard (36 bp). The TRAP reaction products were separated by 10% polyacrylamide gel electrophoresis, dried, and autoradiographed. The basal level of telomerase activity (ladder formation) was measured by serial dilution of the protein extracts, and an appropriate range of protein concentration was selected that produced a linear response. Each set of TRAP assay included control reaction tubes without any extract, and with extracts treated with RNase A (200 μg/ml). To quantitate the levels of telomerase activity, the average densitometric optical density of first six TRAP bands after a primer band was presented as a ratio to the internal TRAP assay standard band.

hTR Expression by PCR—Total RNA was prepared from subconfluent cells by TRIzol reagent (Life Technologies, Inc.), and 1 μg of RNA was subjected to RT-PCR using a RNA PCR core kit (Perkin Elmer) as per manufacturer's instruction (24). Amplification was carried out for 30 cycles. Equal aliquots of reaction product were analyzed by 2% agarose gels containing ethidium bromide. Primers for hTR were synthesized using the TRC3 sequence (24, 25). As PCR reaction product size markers, we used glyceraldehyde-3-phosphate dehydrogenase primers (reaction product about 300 bp), β_2-microglobulin primers (reaction product about 550 bp), and hTR product (about 150 bp) from SW-480 positive control cells.

Cell Cycle Analysis—Cell cycle distribution was determined by staining with propidium iodide (Sigma) as described (26). Briefly, cells were stained with propidium iodide and passed through the beam of an argon ion laser turned to 514 (FAC/Scan; Becton Dickinson). The resulting fluorescent signal was amplified, recorded in the memory, and analyzed in the form of a DNA histogram, by using a computer program interfaced with the integrator.

RESULTS

To examine a possible relationship between Bcl-2 expression and telomerase activity, we investigated whether deregulation of Bcl-2 in HeLa cells would modulate the levels of telomerase activity. For these studies, HeLa cells were transfected with a Bcl-2 expression vector (21) and G418-resistant clonal cell lines stably expressing Bcl-2 were generated (8). Fig. 1*A* shows the levels of Bcl-2 protein in three out of seven such clones, HeLa/Bcl-2.2 (*lane 2*), HeLa/Bcl-2.1 (*lane 3*), and HeLa/Bcl-2.3 (*lane 4*). Clones 1 and 3 express 4–6-fold higher levels of Bcl-2, compared with the levels detected in the parental HeLa cells (*lane 1*) or in the clone expressing an unrelated *PKR/neo* gene (*lane 5*). Results in *panel B* show the effect of Bcl-2 overexpression on the levels of telomerase activity. It was noted that overexpression of Bcl-2 in HeLa cells leads to a significant enhancement (5–10-fold) in the levels of telomerase activity. The observed increase in telomerase activity resulted from overexpression of Bcl-2, since it was detected in both clone 1 and clone 3 (Fig. 1*B*) but not in control HeLa cells or HeLa cells overexpressing an unrelated *PKR/neo* gene (HeLa/PKR.w1 cells). The observed increase in the levels of telomerase activity in Bcl-2-overexpressing cells was not due to elongation of telomere terminal restriction fragments (TRFs) as determined by the Southern hybridization of *Hin*fI-digested DNAs with telo-

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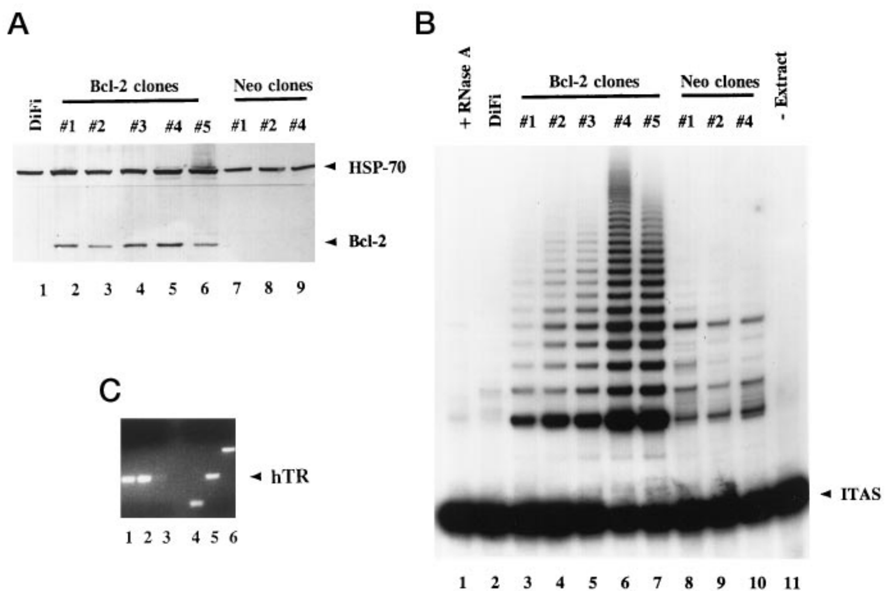

FIG. 2. **Effect of Bcl-2 deregulation on telomerase activity and hTR expression in DiFi cells.** *A*, cells lysate (20 μg) from control DiFi cells (*lane 1*), 5 independent Bcl-2-overexpressing clones (*lanes 2–6*), and three control neo clones (*lanes 7–9*) were immunoblotted with an anti-Bcl-2 mAb. As an internal control, the upper portion of the blot was reprobed with a HSP-70 mAb. *B*, CHAPS extracts from DiFi cells and its clones were analyzed for telomerase activity by TRAP assay using 0.01 μg of protein. *Lane 1*, cell extract (0.01 μg) from Bcl-2-overexpressing clone 4 was pretreated with RNase A (200 μg/ml). *Lane 11*, reaction without cell extract. *C*, hTR expression. Total RNAs (1 μg) from DiFi/Neo#2 cells (*lane 1*) and DiFi/Bcl-2.5 cells (*lane 2*) were subjected to RT-PCR as described under "Materials and Methods." *Lane 3*, 1 μg of RNA from DiFi/Neo#2 cells without reverse-transcriptase enzyme; *lane 4*, glyceraldehyde-3-phosphate dehydrogenase product; *lane 5*, positive control hTR product; *lane 6*, β_2-microglobulin product.

meric probe (data not shown). Lack of association between telomerase activity and TRF length has been shown previously (27, 28).

To determine whether the observed relationship between Bcl-2 deregulation and enhanced telomerase activity is a phenomena restricted to HeLa cells or whether they can show it with other cells with low levels of Bcl-2, we examined the influence of Bcl-2 deregulation on the levels of telomerase activity in human colorectal carcinoma DiFi cells. Fig. 2 shows the levels of Bcl-2 expression in DiFi cells, its five Bcl-2-overexpressing clones, and three control neo transfectants. Overall, there was a 6–10-fold overexpression of Bcl-2 in different clone with highest being in clone 4 compared with parental DiFi cells or control vector transfectants. Results in Fig. 2*B* illustrate that all the Bcl-2-overexpressing clones have significantly elevated levels (8–12-fold) of telomerase activity compared with either control DiFi cells or neo clones. The observed differences in the levels of telomerase activity were not due to the presence of a potential telomerase inhibitor in extracts as we obtained similar results when assayed the levels of telomerase activity in Bcl-2-overexpressing DiFi clones as a function of protein concentration (Fig. 3). The observed increase in the levels of telomerase activity in DiFi/Bcl-2.5 cells compared with the levels in control cells was not due to enhanced expression of hTR component as determined by a quantitative RT-PCR assay (Fig. 2*C*).

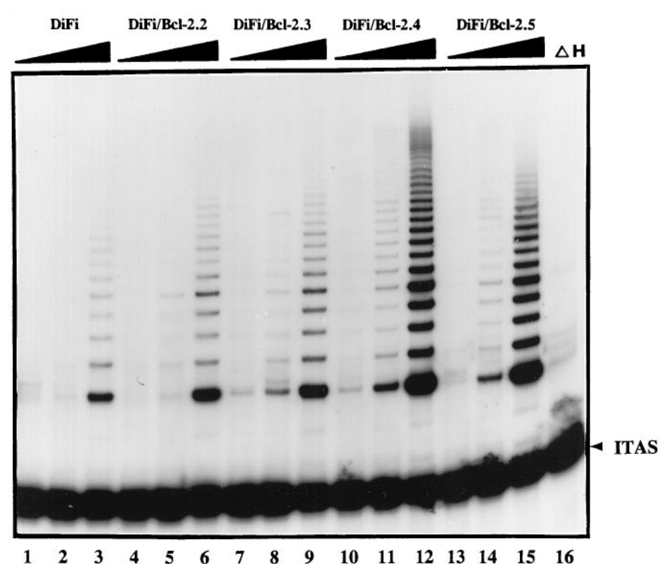

FIG. 3. **Telomerase activity in DiFi and its Bcl-2-overexpressing clones as a function of protein extracts.** CHAPS extracts from DiFi cells and its clones were analyzed for telomerase activity by TRAP assay. For DiFi cells (*lanes 1–3*), TRAP assays were performed using 0.001, 0.01 and 0.1 μg of protein; for its Bcl-2 clones (*lanes 4–15*), TRAP assays were performed using 0.0001, 0.001, and 0.01 μg of protein. *Lane 16*, cell extract (0.1 μg) from Bcl-2-overexpressing clone 4 was inactivated by pretreatment with RNase A (200 μg/ml). Results shown are representative of two experiments.

We next determined the effect of bi-directional regulation of Bcl-2 expression on the status of telomerase activity in IL-2-dependent CTLL-2 cells (29). As expected from the data in literature (29), IL-2 deprivation of CTLL-2 cells for as little as 8 h resulted in the down-regulation (40% inhibition compared with control cells) of Bcl-2 expression compared with the levels present in control cells (Fig. 4*B*, compare *lane 2* with *lane 1*), and this was associated with down-regulation of telomerase activity starting at 8 h post-IL-2 deprivation (Fig. 4*A*). The observed early down-regulation in the levels of telomerase activity was not due to IL-2 deprivation-associated G_0/G_1 cell cycle arrest and/or apoptosis, as these phenotypic changes became evident only at 24 h and not 8 h post-IL-2 deprivation (Fig. 4*C*). This view was further supported by the data in Fig. 5*A*, demonstrating that there was no significant effect of 8 h of IL-2 deprivation on the expression of apoptotic targets such as PARP and lamin B. Since IL-2 is a known inducer of Bcl-2 (29), we examined whether exposure of IL-2-deprived CTLL-2 cells to rIL-2 will up-regulate telomerase activity. Results in Fig. 5 show that the down-regulation of both Bcl-2 expression and telomerase activity could be reversed by the addition of rIL-2 to IL-2-deprived (24 h) CTLL-2 cells. Other experiments indicated that apoptosis triggered by IL-2 deprivation (24 h) was reversible by the addition of IL-2, as expected from the published data (29). Taken together, these results suggested that IL-2 regulation of Bcl-2 expression may be closely related with modulation of telomerase activity in CTLL-2 model system.

DISCUSSION

It is now well accepted that apoptosis is a physiological phenomenon that plays an important role in the maintenance of tissue homeostasis. Apoptosis is regulated by specific cellular pathways including Bcl-2, deregulation of apoptosis contrib-

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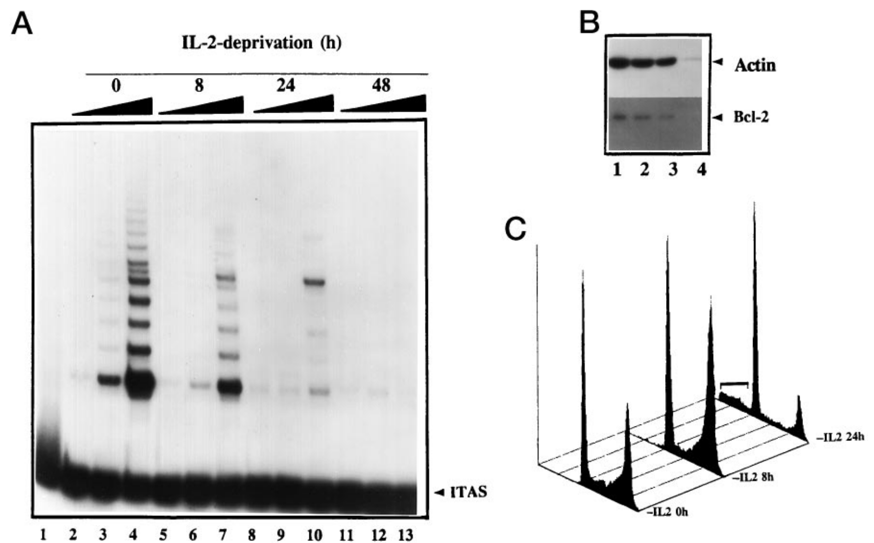

FIG. 4. **IL-2 deprivation leads to inhibition of both Bcl-2 expression and telomerase activity.** IL-2-dependent CTLL-2 cells were cultured in IL-2-deprived medium for 8, 24, and 48 h. CHAPS extracts were assayed for telomerase activity (*panel A*) and Bcl-2 expression (*panel B*). *Black wedges*, 0.002, 0.01, and 0.1 μg of protein. *Lane 1*, reaction without cell extract. Results shown are representative of four experiments. *C*, FACS profile of CTLL-2 cells cultured in the absence of IL-2 (*−IL-2*) for 0, 8, and 24 h. Cell cycle distribution at 0-h point, 35.3% G_0/G_1, 37% S, and 27.8% G_2/M; 8-h point, 24.6% G_0/G_1, 24% S, and 41.5% G_2/M; and 24-h point, 62.41% G_0/G_1, 16.6% S, and 21% G_2/M. *Bar* at 24-h point shows apoptotic cells.

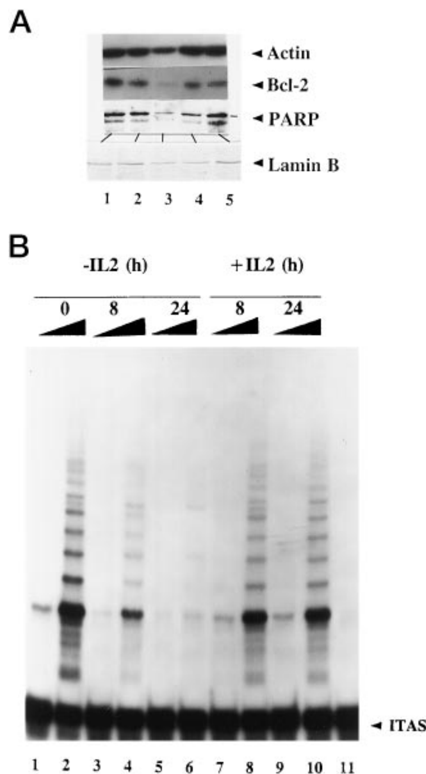

FIG. 5. **Regulation of telomerase activity by IL-2 in CTLL-2 cells.** *A*, CTLL-2 cells were cultured in IL-2-deprived medium for 8 h (*lane 2*) or 24 h (*lane 3*). At 24 h post-IL-2 deprivation, CTLL-2 culture was supplemented by recombinant IL-2 (0.5 ng/ml) for 24 h (*lane 4*) or 48 h (*lane 5*). CHAPS extracts were analyzed for the expression of Bcl-2, PARP, and lamin B by immunoblotting. *B*, CHAPS extracts from the above samples were assayed for the levels of telomerase activity. *Black wedges*, 0.01 and 0.1 μg of protein. *Lane 11*, reaction without cell extract. Results shown are representative of three experiments.

utes to the pathogenesis of a number of human diseases including cancer, and Bcl-2 deregulation is frequently associated with human cancer. Emerging lines of evidence suggest that in addition to deregulation of apoptosis, the process of neoplasia may be also driven by the activation of telomerase, a ribonucleoprotein enzyme complex that adds telomeric repeats to the ends of replicating chromosomes. Although activation of telomerase activity has been detected in human cancer cells, it remains unclear whether there is any linkage between the deregulation of Bcl-2 and telomerase activity. The present study was undertaken to investigate the possible relationship between the modulation of Bcl-2 expression and telomerase activity.

We have now demonstrated that the deregulation of Bcl-2 expression in human cancer cells with low levels of Bcl-2 is closely linked with the increased levels of telomerase activity as overexpression of Bcl-2 in HeLa and DiFi cells was accompanied by increased telomerase activity compared with control transfectants and/or parental cells. Since the activation of telomerase activity has been shown to be associated with the development of human cancer (16, 17), our finding of potential involvement of survival factor Bcl-2 in the deregulation of telomerase activity may provide an important insight into the molecular mechanism involved in the initiation of cellular malignancies. The finding that Bcl-2 enhances telomerase activity without elongation of telomeric TRFs in HeLa cells is interesting, as it raises the possibility that additional pathway(s) such as Bcl-2 deregulation that may influence telomerase activity in cancer cells. The observation that HeLa clones with variable levels of Bcl-2 expression (Fig. 1*A*) exhibited comparable enhanced levels of telomerase activity would suggest that the deregulation of Bcl-2 over the presumptive threshold levels may not further up-regulate telomerase activity, and this threshold level could differ from one cell type to another. Our finding that IL-2 deprivation of CTLL-2 cells for 8 h was sufficient to down-regulate both Bcl-2 expression and telomerase activity (Fig. 4) without any detectable apoptosis (Fig. 4*C*) is important, as it suggests a close relationship between the levels of survival factor Bcl-2 and telomerase activity in cancer cells. Since IL-2 signal was sufficient to concurrently influence the levels of both Bcl-2 and telomerase activity in CTLL-2 cells, it remains to be resolved whether the observed modulation of telomerase activity by IL-2 is mediated via changes in Bcl-2 expression and/or a direct effect of IL-2 and/or both Bcl-2 expression and telomerase activity are regulated via a IL-2-responsive common pathway(s). Taken together, these findings provide new evidence in support of possible association between the deregulation of Bcl-2 and telomerase activity, and are consistent with the predicted role of telomerase in maintaining the integrity of chromosomes against DNA damage. It remains to be seen whether the observed increase in the levels of telomerase activity is a phenomenon restricted to Bcl-2 or a general event associated with other anti-apoptotic gene products such as $Bcl\text{-}X_L$.

Recent studies have indicated that the cells from a variety of human cancer may have a decreased ability to undergo apo-

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ptosis in response to physiologic stimuli, and a defect in apoptosis has been proposed to be involved in the aberrant survival and/or development of cancer (4). Therefore, in recent years, approaches such as identification of agent(s) that can modulate Bcl-2 have become the subject of active investigation to control cancer cell growth. In addition to apoptosis, telomerase has also attracted a great deal of interest as a possible target in cancer biology. The apparent lower levels of measurable telomerase activity in many normal cells and its widespread detection in human cancer cells have raised the possibility that telomerase may also serve an important target to control cell growth. In spite of wide occurrence of deregulation of Bcl-2 and telomerase activity in cancer cells, to date, to the best of our knowledge, no close linkage between these two phenotypes has been reported. Our findings of the modulation of telomerase activity by a widely deregulated survival factor, Bcl-2, may serve an important model to study the regulation of telomerase activity by an apoptotic pathway and could open new possibilities to develop novel strategies to control cancer cell growth by co-targeting both pathways.

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