intact virus and/or the purified hemagglutinin can now be used as a powerful tool for the direct investigation of 9-*O*-acetylation in intact cells and tissues.

REFERENCES AND NOTES

1. J. M. Levine, L. Beasley, W. B. Stallcup, *J. Neurosci.* **4**, 820 (1984).
2. R. Guidoni, S. Sonnino, V. Chigorno, A. Malesci, G. Tettamanti, *Adv. Exp. Med.* **152**, 532 (1980).
3. E. A. Muchmore, N. Varki, M. Fukuda, A. Varki, in preparation.
4. A. Varki and S. Kornfeld, *J. Exp. Med.* **152**, 532 (1980).
5. A. Varki and S. Diaz, *J. Biol. Chem.* **258**, 12465 (1983).
6. D. A. Cheresh, R. A. Reisfeld, A. Varki, *Science* **225**, 844 (1984).
7. A. Varki and S. Diaz, *Anal. Biochem.* **137**, 236 (1984).
8. G. N. Rogers, G. Herrler, J. C. Paulson, H. D. Klenk, *J. Biol. Chem.* **261**, 5947 (1986).
9. G. Herrler *et al.*, *EMBO J.* **4**, 1503 (1985).
10. A. K. Shukla and R. Schauer, *Hoppe-Seyler's Z. Physiol. Chem.* **363**, 255 (1982).
11. J. B. Pfeifer and R. W. Compans, *Virus Research* **1**, 281 (1984).
12. K. Kirsch, in *The Enzymes*, P. Boyer, Ed. (Academic Press, New York, 1971), p. 62.
13. C. M. Jackson and Y. Nemerson, *Annu. Rev. Biochem.* **49**, 765 (1980).
14. S. Nakada *et al.*, *J. Virol.* **50**, 118 (1984).
15. M. Ohuchi, R. Ohuchi, K. Mifune, *ibid.* **42**, 1076 (1982).
16. R. T. C. Huang, R. Rott, K. Wahn, H. D. Klenk, T. Kohama, *Virology* **107**, 313 (1980).
17. J. A. Griffin, S. Basak, R. W. Compans, *ibid.* **125**, 324 (1983).
18. P. Palese, K. Tobita, and M. Ueda, *ibid.* **61**, 397 (1974).
19. G. Herrler, R. W. Compans, H. Meier-Ewert, *ibid.* **99**, 49 (1979).
20. H. Higa, S. Diaz, A. Varki, *Biochem. Biophys. Res. Commun.*, in press.
21. U. K. Laemmli, *Nature (London)* **227**, 680 (1970).

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Regulation of *bcl*-2 Proto-Oncogene Expression During Normal Human Lymphocyte Proliferation

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The *bcl*-2 and c-*myc* proto-oncogenes are brought into juxtaposition with the immunoglobulin heavy chain locus in particular B-cell lymphomas, resulting in high levels of constitutive accumulation of their messenger RNAs. Precisely how the products of the *bcl*-2 and c-*myc* genes contribute to tumorigenesis is unknown, but observations that c-*myc* expression is rapidly induced in nonneoplastic lymphocytes upon stimulation of proliferation raise the possibility that this proto-oncogene is involved in the control of normal cellular growth. In addition to c-*myc*, the *bcl*-2 proto-oncogene also was expressed in normal human B and T lymphocytes after stimulation with appropriate mitogens. Comparison of the regulation of the expression of these proto-oncogenes demonstrated marked differences and provided evidence that, in contrast to c-*myc*, levels of *bcl*-2 messenger RNA are regulated primarily through transcriptional mechanisms.

NONRANDOM CHROMOSOMAL TRANSlocations are found in a variety of leukemias and lymphomas, suggesting that these specific genetic changes impart a selective growth advantage. In some cases these translocations involve a known proto-oncogene and result in the "activation" of the gene through alterations either in the regulation of its expression or in its coding sequences (*1*). Perhaps the best studied examples of translocations associated with a lymphoid neoplasm are those involving the c-*myc* proto-oncogene in Burkitt lymphoma, wherein the c-*myc* gene on chromosome 8 becomes juxtaposed to sequences in the immunoglobulin heavy [t(8;14)], κ [t(2;8)], or λ [t(8;22)] chain loci. Translocations involving the c-*myc* gene result in constitutive accumulation of high levels of c-*myc* messenger RNA (mRNA) through mechanisms that are poorly understood but that probably involve enhancer-like elements within the transcriptionally active immunoglobulin loci in these B-cell neoplasms (*2*).

In addition to translocations involving c-*myc* on chromosome 8 in Burkitt lymphoma, other B-cell neoplasms harbor translocations involving immunoglobulin loci, such as the t(11q13;14q32) found in a variety of acute and chronic B lymphocytic leukemias and the t(14q32;18q21) seen in most follicular B-cell lymphomas (2). Although none of the known proto-oncogenes appears directly involved in these translocations, it has been postulated that such genes may reside at 11q13 and 18q21 (termed *bcl*-1 and *bcl*-2, respectively, for B-cell lymphoma/leukemia 1 and 2), and that they may become activated by analogy to the c-*myc* gene in Burkitt lymphoma.

By means of chromosomal walking techniques, the *bcl*-2 gene has recently been cloned and sequenced (*3, 4*). The predicted products of human *bcl*-2 appear to be 26-kD (*bcl*-2-α) and 22-kD (*bcl*-2-β) proteins that (i) differ in their carboxyl termini as a result of alternative splice site selection; (ii) lack transmembrane, leader, or kinase domains; and (iii) share no significant homology with other proteins whose sequences are known (*4*). In all lymphomas with t(14q32;18q21) thus far examined, the rearranged *bcl*-2 gene is expressed at high levels and shows no evidence of gross alterations in its coding sequences (*4, 5*). Hence, aberrant expression of the *bcl*-2 gene, rather than an abnormal gene product, most likely accounts for any selective growth advantage that the t(14q32;18q21) may impart.

Given the possibility that expression of the *bcl*-2 gene may contribute to the altered growth characteristics of malignant lymphocytes harboring a t(14;18), we wondered whether expression of this gene occurs during normal lymphocyte proliferation. Several proto-oncogenes, including c-*myc*, become expressed in normal lymphocytes after stimulation with appropriate mitogens (*6, 7*). We therefore sought to determine whether the *bcl*-2 gene is expressed in these cells. Though oncogenic activity has yet to be demonstrated for *bcl*-2, we henceforth refer to the normal form of this gene as a proto-oncogene.

Figure 1A shows Northern blot data derived from human peripheral blood lymphocytes (PBL) that had been stimulated for various times with the mitogenic lectin phytohemagglutinin (PHA). Because levels of ribosomal RNA (rRNA) increase in PBL after stimulation with PHA, we compared equal amounts of total cellular RNA rather than RNA from equal numbers of cells. Accumulation of 8.5- and 5.5-kb mRNAs for *bcl*-2-α and of a less abundant 3.5-kb mRNA for *bcl*-2-β rose from undetectable to maximal levels within 6 to 14 hours after stimulation of PBL with PHA. These three mature transcripts result from alternative polyadenylation and splice site selections

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(*4*). In contrast to *bcl*-2 mRNAs, accumulation of a 2.4-kb c-*myc* mRNA began within minutes of exposure of PBL to PHA, as reported previously (*6, 7*). Increased expression of *bcl*-2 and c-*myc* occurred prior to the initiation of DNA synthesis in PHA-stimulated PBL, since accumulation of histone H3 mRNA did not become detectable until 24 hours after stimulation and peaked at 48 to 72 hours. Unlike *bcl*-2 and c-*myc*, mRNA for c-*fgr* was constitutively present in resting PBL and was relatively unaffected by stimulation with mitogenic lectin, indicating that PHA specifically increases the expression of some but not all proto-oncogenes in PBL. The time courses of mRNA accumulation were similar in purified populations of B cells and of T cells after stimulation with appropriate mitogens for *bcl*-2, c-*myc*, and H3 (Fig. 1B).

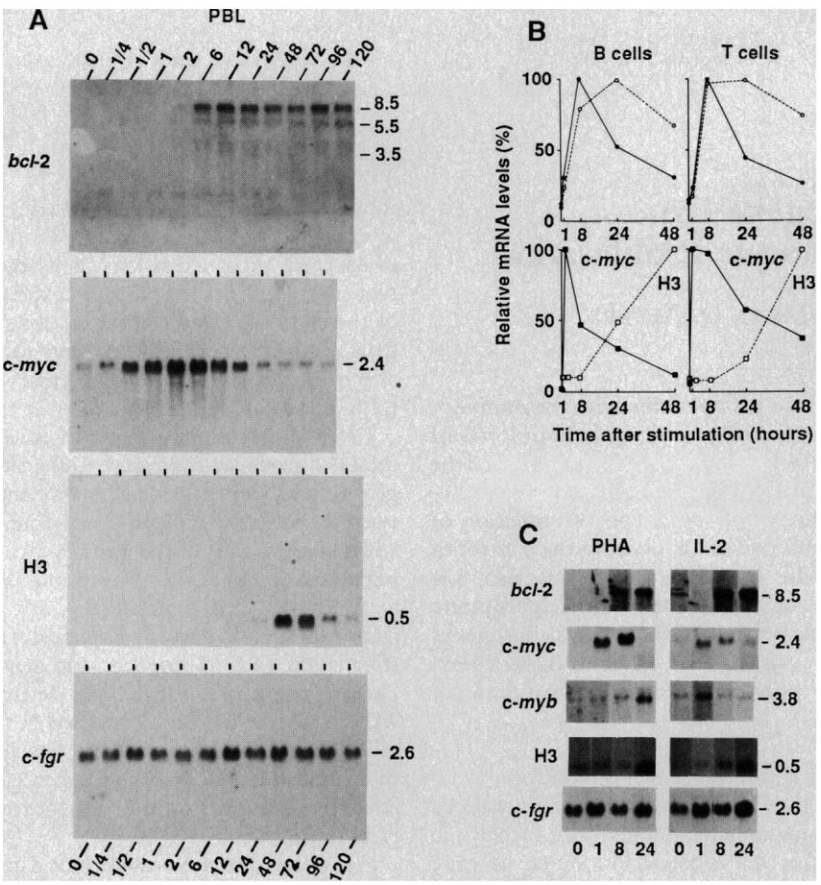

Fig. 1. Measurement of steady-state levels of accumulated mRNAs in lymphocytes following stimulation with mitogens. For (A) and (C), time is shown at the top or bottom (in hours); size is shown at the side (in kilobases). (**A**) Human peripheral blood lymphocytes (PBL), isolated from whole blood by Ficoll density gradient centrifugation, were cultured (2×10^6 per milliliter) in RPMI 1640 and 10% (v/v) fetal bovine serum (complete medium, CM) and stimulated for various times with phytohemagglutinin at 1 μg/ml (PHA-P; Difco). (**B**) B and T lymphocytes, purified from freshly excised human tonsils by two sequential rosettings with sheep erythrocytes, were cultured (10^6 per milliliter) in CM and stimulated for various times with phorbol myristic acid (10 ng/ml; PMA) and either 0.1% (v/v) Formalin-fixed *Staphylococcus*-A suspension (Pansorbin; Calbiochem) for B cells or PHA-P (1 μg/ml) for T cells. Data are expressed relative to each maximum. (**C**) Preactivated T lymphocytes, derived by stimulation of PBL for 3 days with PHA-P followed by 2 days of culture in CM containing IL-2 (5 to 10 unit/ml) (*8*), were washed three times then incubated for 16 to 24 hours in CM (2×10^6 per milliliter) without mitogens or IL-2 to achieve quiescence. Cells were then restimulated for various times with either PHA-P (1 μg/ml) or purified recombinant IL-2 (100 unit/ml; Cetus) (*14*). RNA blotting was performed as described (*7, 15*). RNA blots were hybridized at high stringency with ^{32}P-labeled DNA probes for *bcl*-2 (pBp-4.2; cDNA encoding all translated sequences for *bcl*-2-α), c-*myc* (pRYC7.4; cDNA encoding second and third exons), histone H3, or c-*fgr* (840-bp Sma I–Bam HI fragment from pV-*fgr*-1700 specific for c-*fgr*–like sequences), washed, and exposed to XAR film (Kodak) with intensifying screens for various times (*4, 16–18*). Data are representative of at least three experiments; lanes were reordered for clarity of presentation. In (A), *bcl*-2 and c-*fgr* data were derived from a single RNA blot, and c-*myc* and H3 data from another, by eluting the previous ^{32}P-labeled probe prior to rehybridization. Data in (B) were derived from a single blot by sequential hybridization and elution of ^{32}P-labeled probes and represent scanning densitometric quantification of RNA blot data (LKB Ultrascan), wherein relative areas under the tracings were expressed as a percentage of the maximal value for each specific mRNA. (○, 8.5-kb *bcl*-2; ●, 5.5-kb *bcl*-2; ■, c-*myc*; □, H3.) Data in (C) were derived from the same RNA blot.

Because production of interleukin-2 (IL-2) by PBL begins approximately 6 hours after stimulation with PHA, we wondered whether this growth factor contributes to the induction of *bcl*-2 expression. PBL were activated with PHA for 3 days, grown in IL-2 for 2 more days, and then incubated for 1 day in the absence of growth factor or mitogen to achieve quiescence. In contrast to primary cultures of PBL, these preactivated cells are >95% T lymphocytes, express low levels of IL-2 receptors, and respond to stimulation with either PHA or IL-2. Stimulation of these preactivated T cells with PHA or IL-2, at concentrations that yielded equivalent proliferative responses (on the basis of [^{3}H]thymidine incorporation), resulted in similar time courses of *bcl*-2 and c-*myc* mRNA accumulation with expression of c-*myc* preceding that of *bcl*-2 (Fig. 1C). IL-2, however, induced quantitatively more accumulation of mRNAs for *bcl*-2 than did PHA, whereas PHA stimulated greater increases in the levels of c-*myc* mRNA than did IL-2.

In contrast to *bcl*-2 and c-*myc*, PHA and IL-2 stimulated different time courses of expression of another proto-oncogene, c-*myb*, with peak levels of accumulated c-*myb* mRNA occurring earlier in preactivated T cells treated with IL-2 (1 to 8 hours) than in cells exposed to PHA (≅24 hours). Different time courses of c-*myb* mRNA accumulation result because IL-2 directly stimulates c-*myb* expression, whereas PHA indirectly induces its expression by stimulating endogenous IL-2 production (*8*).

By comparison to c-*myb*, therefore, both PHA and IL-2 appear capable of directly inducing accumulation of mRNAs for *bcl*-2 and c-*myc*. IL-2, however, is a more potent inducer of *bcl*-2 expression in these cells than is PHA. Consequently, the time course of mRNA accumulation observed in PHA-stimulated PBL (Fig. 1A) reflects the combined effects of mitogenic lectin and of endogenously produced IL-2 on the expression of the *bcl*-2 and c-*myc* proto-oncogenes.

Previous investigations of the c-*myc* gene in normal lymphocytes have suggested that expression of this gene is controlled, at least partially, by a labile inhibitory protein, since treatment of resting cells with the protein synthesis inhibitor cycloheximide (CHX) results in c-*myc* mRNA accumulation (*6*). Inhibition of protein synthesis in PBL, and in purified B and T cells, caused increased accumulation of *bcl*-2 mRNAs (Fig. 2A). The effects of CHX on *bcl*-2 mRNA levels were probably specific, since addition of this inhibitor diminished levels of c-*fgr* mRNA and produced no change in the levels of another mRNA, He7, which encodes an unknown protein and is constitutively pre-

sent in unstimulated lymphocytes (*9*). Treatment of resting lymphocytes with CHX for 16 to 24 hours also induced marked increases in the accumulation of c-*myc* mRNA and slight elevations in the levels of β-actin mRNA (Fig. 2A).

There were more rapid elevations in the levels of c-*myc* mRNA than of *bcl*-2 mRNA in CHX-treated cells (Fig. 2B). Increased accumulation of c-*myc* mRNA occurred within 30 minutes after addition of CHX to cultures, whereas elevations of *bcl*-2 mRNAs were not detected until 4 hours after cessation of protein synthesis. These findings suggest that distinct proteins with different degradation rates negatively regulate the levels of *bcl*-2 and c-*myc* mRNAs in lymphocytes.

We then determined the degradation rates of mature mRNAs for *bcl*-2, c-*myc*, and β-actin in lectin-stimulated PBL in the presence or absence of CHX (Fig. 2C). The 8.5-kb and 5.5-kb *bcl*-2 mRNAs had half-lives ($t_{1/2}$'s) of roughly 2.5 to 3.0 hours, which increased to 3.5 to 4.5 hours in the presence of CHX. Levels of the 3.5-kb *bcl*-2 mRNA were too low for accurate measurement. The $t_{1/2}$ of the 5.5-kb *bcl*-2 mRNA appeared to be slightly longer than that of the 8.5-kb message, but this may be an artifact attributable to cross-hybridization of the ^{32}P-labeled *bcl*-2 complementary DNA (cDNA) probe to 28*S* rRNA. In contrast to *bcl*-2 mRNAs, the $t_{1/2}$ of c-*myc* mRNA in lectin-stimulated lymphocytes was considerably shorter, measuring roughly 30 to 45 minutes in the absence of CHX and increasing to approximately 1.3 to 1.5 hours in CHX-treated cells. CHX thus decreased the rates of degradation of both *bcl*-2 and c-*myc* mRNAs in lectin-stimulated PBL. The percentage increase in mRNA half-life, however, was greater for c-*myc* (average ≅125% increase) than for *bcl*-2 mRNAs (average ≅35% increase). The CHX-induced stabilization of *bcl*-2 and c-*myc* mRNAs appeared to be specific, since levels of β-actin mRNA were unaffected, at least over the 4-hour period of these experiments.

The data in Fig. 2C suggested that increased mRNA stability was not enough to explain the CHX-induced accumulation of *bcl*-2 mRNAs. Because steady-state levels of accumulated mRNAs (Figs. 1 and 2) reflect, at least in part, the net effects of mRNA synthesis and degradation, we next investigated relative rates of *bcl*-2 gene transcription by nuclear "run-off" assays (Fig. 3). Stimulation of resting PBL induced rapid increases in the rate of *bcl*-2 transcription, beginning within 1 hour and reaching peak levels approximately 6 hours after addition of mitogens to cultures (Fig. 3A). Comparison of the kinetics of *bcl*-2 gene transcription with the time course of *bcl*-2 mRNA accumulation (Fig. 1A) revealed that *bcl*-2 transcription precedes accumulation of mature *bcl*-2 mRNAs by a few hours. This delay between the onset of transcription and the accumulation of stable *bcl*-2 mRNAs may reflect the time needed for processing of primary transcripts of *bcl*-2 to mature 8.5-, 5.5-, and 3.5-kb mRNAs.

In contrast to the 15- to 20-fold increase in *bcl*-2 transcription (0.08 to 1.35 relative transcription units) relative rates of c-*myc* proto-oncogene transcription increased only three- to fourfold (0.10 to 0.35 relative transcription units) after stimulation of PBL (Fig. 3A). Given that steady-state levels of c-*myc* mRNA rose 10- to 40-fold in mitogen-stimulated lymphocytes (Fig. 1), these data support previous reports indicating that c-*myc* mRNA levels are regulated primarily by post-transcriptional mechanisms (*10*). Transcription of the He7 gene increased gradually after stimulation of PBL with mitogens (Fig. 3A). Furthermore, transcription of the genes for *bcl*-2, c-*myc*, and He7 was probably mediated by RNA polymerase II, since addition of α-amanitin (2 μg/ml) to nuclear "run-off" assays specifically inhibited the transcription of these genes but did not impair the RNA polymerase I–dependent

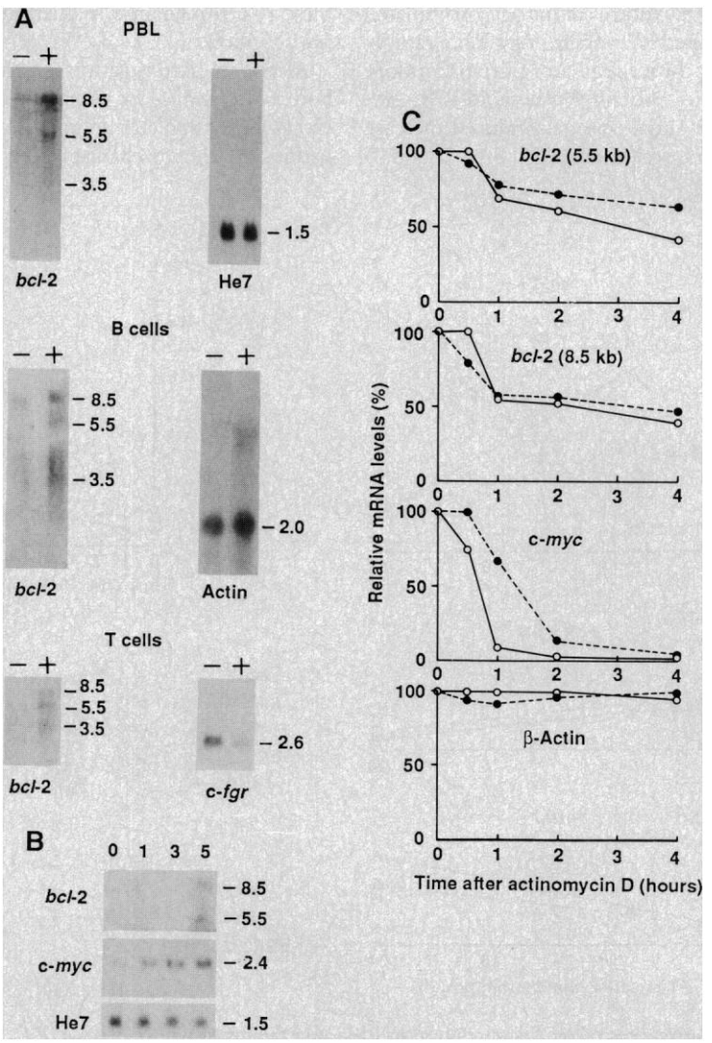

Fig. 2. Effects of cycloheximide (CHX) on *bcl*-2 mRNA accumulation and stability in normal lymphocytes. (**A**) Resting PBL, purified B cells (>90% OKB7+), and purified T cells (>90% OKT3+) were cultured with (+) or without (−) CHX (15 to 20 μg/ml) (Sigma) for 16 to 24 hours and relative levels of *bcl*-2 and other mRNAs analyzed by blotting (10 μg per lane) of total cellular RNA. (**B**) Quiescent preactivated T cells were cultured for 0, 1, 3, or 5 hours with cycloheximide before performing RNA blot analysis. (**C**) Degradation rates of *bcl*-2, c-*myc*, and β-actin mRNA were analyzed in PBL that had been stimulated for 8 hours with PHA and PMA by addition of actinomycin D (5 μg/ml) to cultures and removal of aliquots of cells 0, 0.5, 1, 2, or 4 hours later for isolation of RNA. The densitometric quantification of RNA blot data is shown (average of two experiments; standard deviations ≤15%), relative to 0-hour mRNA levels (100%). Comparison is made between PBL either treated (●) or not treated (○) with CHX 0.5 hours prior to blocking RNA synthesis with actinomycin D. DNAs used as hybridization probes were pBp-4.2 (*bcl*-2), pRYC7.4 (*myc*), purified Sma I–Bam HI v-*fgr* fragment, pHe7 (control probe) (*9*), and pA1 (β-actin) (*19*). Sizes are shown in kilobases.

transcription of 28S rRNA genes in mitogen-stimulated PBL (Fig. 3E).

Investigation of the effects of CHX on transcription of genes for *bcl*-2, c-*myc*, and He7 in resting PBL provided evidence that *bcl*-2 is negatively regulated at the transcriptional level by a labile repressor protein. Inhibition of protein synthesis in unstimulated PBL produced a marked increase in the relative rate of *bcl*-2 transcription, beginning approximately 4 hours after addition of CHX to cultures (Fig. 3B). In contrast, levels of c-*myc* and He7 gene transcription were unaltered by treatment of resting PBL with CHX.

In addition, experiments in which CHX was added to cultures of mitogen-stimulated cells indicated that there may be a rapidly inducible positive regulator of transcription. Inhibition of protein synthesis in PBL prevented the initial mitogen-induced increase in *bcl*-2 transcription (Fig. 3A; 1 and 6 hours). At later times (24 hours), however, high relative rates of *bcl*-2 transcription were observed, presumably resulting from loss of the putative short-lived repressor of *bcl*-2 transcription. Unlike its effects on *bcl*-2, CHX only minimally altered mitogen-induced transcription of the c-*myc* and He7 genes in these experiments (Fig. 3A).

Though highly speculative, the finding that *bcl*-2 transcription is dependent, at least in part, on new protein synthesis raises the intriguing possibility that the c-*myc* gene product might be required for expression of the *bcl*-2 proto-oncogene in mitogen-stimulated PBL. However, inhibition of IL-2 production also probably contributes to these CHX-mediated reductions in *bcl*-2 transcription.

Although accumulation of mRNAs for both *bcl*-2 and c-*myc* occurred in lymphocytes when stimulated to proliferate, investigations of the mechanisms regulating the increase in these proto-oncogene mRNAs revealed striking differences. In contrast to c-*myc* gene expression, which is subject to considerable post-transcriptional regulation in normal cells (*10*) (Fig. 3A), expression of the *bcl*-2 gene appeared to be controlled predominantly at the transcriptional level. [We observed comparable (10- to 20-fold) mitogen-induced increases in *bcl*-2 mRNA accumulation (Fig. 1A) and in *bcl*-2 transcription (Fig. 2A) as measured by densitometry.]

Experiments with CHX demonstrated additional differences in the regulation of the *bcl*-2 and c-*myc* genes and provided presumptive evidence for (i) an inducible stimulator of *bcl*-2 transcription; (ii) a constitutive repressor of *bcl*-2 transcription; and (iii) CHX-sensitive proteins that destabilize or degrade *bcl*-2 and c-*myc* mRNAs. Regulation of the *bcl*-2 gene thus appears complex but is not without precedent, since expression of histone genes is similarly regulated at the transcriptional level by both stimulatory and inhibitory factors, and at the post-transcriptional level by a labile repressor (*11*).

Further work will be needed to investigate the possibilities of antisense transcription and of blockage of transcript elongation, as has been recently described for c-*myc* (*12, 13*). Furthermore, we cannot presently exclude the possibility of regulation of *bcl*-2 expression through additional mechanisms such as RNA processing, transport, or translation.

It remains to be determined precisely how the regulation of *bcl*-2 expression differs in leukemic cells with a t(14;18) translocation. We have not observed greater levels of *bcl*-2 gene transcription (Fig. 3D) or of *bcl*-2 mRNA accumulation in a lymphocytic leukemia cell line (380) possessing this translocation than in normal lymphocytes after stimulation with mitogen. Thus, it is possible that deregulated continuous expression of the *bcl*-2 gene, rather than the absolute levels of transcripts, represents the major difference in the control of this gene in normal and neoplastic lymphocytes.

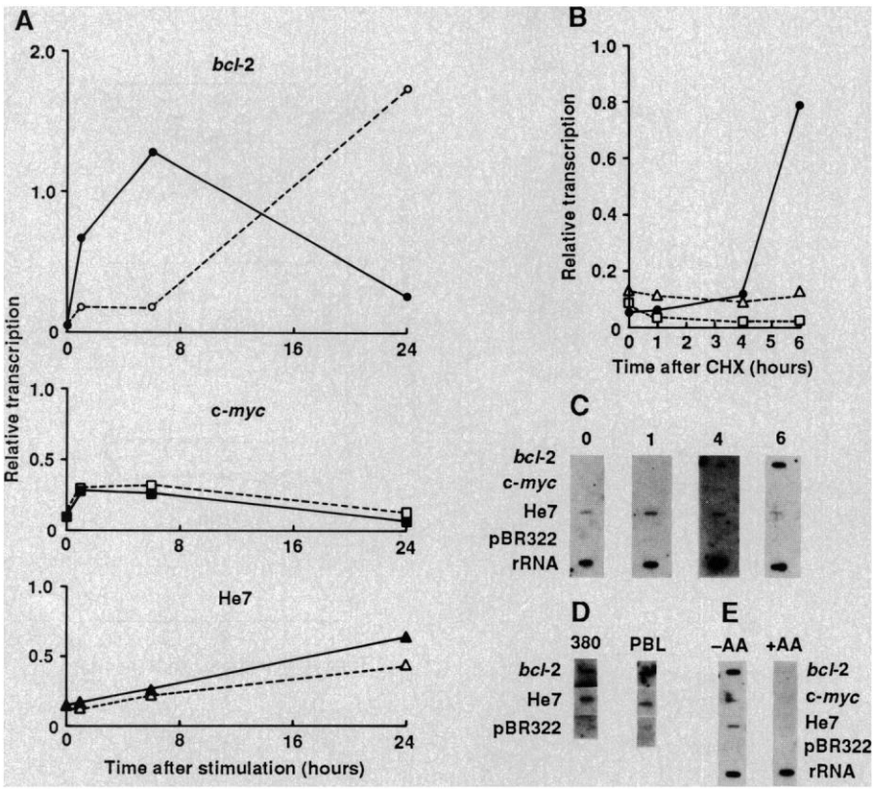

Fig. 3. Relative rates of *bcl*-2 transcription in PBL after treatment with mitogens or CHX. (**A**) PBL were stimulated for 0, 1, 6, or 24 hours with 1 μg/ml PHA-P and PMA (10 ng/ml), in the absence (solid lines) or presence (dashed lines) of CHX, before analyzing relative transcription rates in isolated nuclei by nuclear "run-off" assays as described (*20, 21*). Shown is densitometric quantification of data, averaged from two experiments (standard deviations <15%) expressed relative to levels of transcription in unstimulated PBL. To correct for inequalities in the amounts of ^{32}P-labeled transcripts hybridized to filters, values were expressed relative to levels of 28S rRNA (A1 probe) (*22*) after subtraction of background hybridization (pBR322). (**B**) CHX was added to cultures of resting PBL and nuclei were isolated after 0, 1, 4, or 6 hours for transcriptional analysis. Densitometric quantification of the data (average of two experiments) was performed as in (A). Shown in (**C**) are typical autoradiograms from experiments presented in (B) where ●, □, and △ correspond to *bcl*-2, c-*myc*, and He7, respectively. (**D**) Relative transcription rates of *bcl*-2 gene were compared in 380 acute lymphocytic leukemia cells and in mitogen-stimulated PBL (4 hours). (**E**) Transcription assays were conducted in the presence (+AA) or absence (−AA) of α-amanitin (2 μg/ml) comparing equal numbers (5×10^7) of nuclei obtained from mitogen-stimulated PBL.

REFERENCES AND NOTES

1. P. Nowell and C. Croce, *Am. J. Pathol.* **125**, 8 (1986).
2. C. Croce, Y. Tsujimoto, J. Erikson, P. Nowell, *Lab. Invest.* **51**, 258 (1984).
3. Y. Tsujimoto *et al.*, *Science* **226**, 1097 (1984).
4. Y. Tsujimoto and C. M. Croce, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 5214 (1986).
5. M. L. Cleary, S. D. Smith, J. Sklar, *Cell* **47**, 19 (1986).
6. K. Kelly, B. H. Cochran, C. D. Stiles, P. Leder, *ibid.* **35**, 603 (1983).
7. J. C. Reed, J. D. Alpers, P. C. Nowell, R. G. Hoover, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 3982 (1986).
8. J. Reed, in *Induction and Recognition of the Transformed State*, M. Greene, Ed., in press.
9. H.-T. Kao and J. Nevins, *Mol. Cell. Biol.* **3**, 2058 (1983).
10. J.-M. Blanchard *et al.*, *Nature (London)* **317**, 443

(1985); E. Knight, Jr. *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 1151 (1985); M. Piechaczyk *et al.*, *Cell* **42**, 589 (1985).
11. M. Osley *et al.*, *Cell* **45**, 537 (1986).
12. D. L. Bentley and M. Groudine, *Nature (London)* **321**, 702 (1986); E. Nepveu and K. Marcu, *EMBO J.* **5**, 2859 (1986).
13. In our investigations, we purposely chose a c-*myc* cDNA probe containing only second and third exon sequences (pRYC7.4) to avoid measuring prematurely terminated transcripts. Because of problems in identifying suitable 5′ probes lacking GC-rich regions that caused nonspecific hybridization we were unable to assess whether a similar mechanism exists for the *bcl*-2 proto-oncogene. Our studies also do not address the possibility of antisense transcription in the *bcl*-2 gene, but the excellent agreement between transcription data (Fig. 3A) and RNA blot data (Fig. 1A) indirectly suggests that antisense transcription does not represent a significant portion of the signal measured by our transcription assays.
14. S. A. Rosenberg *et al.*, *Science* **223**, 1412 (1984).
15. Total cellular RNA was isolated from lymphocytes at various times after stimulation by a guanidinium-isothiocyanate procedure with cesium chloride gradient centrifugation. RNA was quantified by measuring absorbance at 260 nm and equal amounts of RNA (10 μg) were size-fractionated by electrophoresis through 6% formaldehyde–1% agarose gels prior to transfer to nylon membranes (GeneScreen Plus; New England Nuclear).
16. A. ar-Rushdi *et al.*, *Science* **222**, 390 (1983).
17. M. Plumb, J. Stein, G. Stein, *Nucleic Acids Res.* **11**, 2391 (1983).
18. G. Naharro *et al.*, *J. Virol.* **47**, 611 (1983).
19. D. Cleveland *et al.*, *Cell* **20**, 95 (1980).
20. M. Krönke, W. Leonard, J. Depper, W. Greene, *J. Exp. Med.* **161**, 1593 (1985).
21. Nuclei were isolated from PBL for transcriptional ("run off") analysis by lysis in 10 m*M* tris (*p*H 7.5), 2 m*M* $MgCl_2$, 3 m*M* $CaCl_2$, 3 m*M* dithiothreitol (DTT), and 0.02% NP40, with subsequent centrifugation through 2*M* sucrose. After resuspension in 100 μl of 50% glycerol, 50 m*M* tris (*p*H 7.5), 5 m*M* $MgCl_2$, and 0.1 m*M* EDTA; 5×10^7 nuclei were either frozen for later use or added immediately to an equal volume of transcription buffer containing 0.2*M* KCl; 4 m*M* $MgCl_2$; 4 m*M* DTT; 0.8 m*M* of ATP, CTP, and GTP; and 200 units RNasin (Promega). Nuclei were incubated at 26 to 28°C for 20 to 25 minutes. (Freshly isolated and thawed nuclei yielded comparable results.) Reactions were stopped by two sequential 15-minute incubations with 20 μg DNase I (BRL). The mixture was then adjusted to 1% SDS and 5 m*M* EDTA, treated with proteinase K (1 mg/ml) at 42°C for 30 minutes, and extracted with chloroform/phenol. The resulting aqueous solutions were centrifuged through Sephadex G-50 (Pharmacia) 1-ml spin columns and precipitated in ethanol. Equal amounts of ^{32}P-labeled material (count/min) were then resuspended in 3-ml hybridization buffer (50% formamide, 0.75*M* NaCl, 0.5% SDS, 2 m*M* EDTA, 50 m*M* Hepes (*p*H 7), 10× Denhardt's solution, polyadenylate (20 μg/ml) (Pharmacia), and denatured salmon sperm DNA (500 μg/ml). The suspension was added to plastic bags containing prehybridized nitrocellulose filters onto which linearized and denatured p18-4 [*bcl*-2 genomic clone encompassing second exon and approximately 200 bp of intron sequences (5)], pRYC7.4 (c-*myc*), pHe7 (+ control), pBR322 (− control), and A1 (28*S* rRNA) plasmid DNAs (equivalent to 0.5 μg of insert DNA) had been slot-blotted in a manifold apparatus (BRL). These amounts of plasmid DNAs were found to be in excess over ^{32}P-labeled RNA in preliminary experiments. After hybridization for 3 days, filters were washed [three times at 25°C in 2× SSC (0.3*M* NaCl, 30 m*M* sodium citrate, *p*H 7) and 0.1% SDS, then twice at 50°C in 0.1× SSC and 0.1% SDS], dried, and exposed to Kodak XAR film with intensifying screens at −70°C for 4 to 7 days. Data from autoradiograms were quantified by scanning laser densitometry.
22. I. L. Gonzalez *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 7666 (1985).
23. We thank W. Greene and K. Marcu for nuclear transcription protocols; A. ar-Rushdi and L. Showe for helpful comments and critical reading of the manuscript; K. Bradley and S. Epstein for technical assistance; M. J. Larsen, K. Ray, and W. Fore for photographic artwork; and L. Delpino for manuscript preparation. Supported in part by NIH grant CA-42232.

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Duplication of CaMV 35*S* Promoter Sequences Creates a Strong Enhancer for Plant Genes

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A variant of the cauliflower mosaic virus 35*S* promoter with transcriptional activity approximately tenfold higher than that of the natural promoter was constructed by tandem duplication of 250 base pairs of upstream sequences. The duplicated region also acted as a strong enhancer of heterologous promoters, increasing the activity of an adjacent and divergently transcribed transferred DNA gene several hundredfold, and to a lesser extent, that of another transferred DNA gene from a remote downstream position. This optimized enhancer element should be very useful for obtaining high levels of expression of foreign genes in transgenic plants.

DICOTYLEDONOUS PLANTS CAN BE genetically transformed by the transfer of DNA from *Agrobacterium tumefaciens* through the mediation of modified Ti plasmids (*1–4*). Genetic transformation can be used to study the molecular details of plant biology and to create plants with improved or novel characteristics. For many of these purposes, high levels of expression of the transferred genes are necessary. Cauliflower mosaic virus (CaMV) has a double-stranded DNA genome within which two distinct promoters, producing 19*S* and 35*S* transcripts, have been identified (*5, 6*). The 35*S* promoter is constitutively active in several different species (*7*) and so has been used to express a number of foreign genes in transgenic plants (*8–11*). Correct initiation of transcription from the 35*S* promoter is dependent on proximal sequences that include a TATA element, whereas the rate of transcription is determined by sequences that are dispersed over 300 bp of upstream DNA (*7*). We sought to raise the efficiency of transcription of the natural CaMV 35*S* promoter by duplicating the transcription-activating sequences upstream of the TATA element (Fig. 1). We also investigated the effect of the CaMV 35*S* upstream sequences on the expression of several genes and showed that both the natural and duplicated regions act as transcriptional enhancers.

The natural and duplicated 35*S* promoters were transferred to tobacco plants for functional analysis by the use of intermediate vectors derived from pMON178. This vector is a modification of pMON129 (*12*) that contains a nopaline synthase (NOS) gene; a chimeric neomycin phosphotransferase type II (NPTII) gene, which permits selective growth of transformed plant tissue in the presence of kanamycin; and an 1800-bp fragment from the transferrable region of an octopine-type Ti plasmid (Fig. 1). The NPTII-encoding sequences of pMON178 are linked to a promoter that was derived from the transferred DNA (T-DNA) NOS gene. This promoter was excised and replaced by 35*S* promoters with single or double copies of the upstream region to make the intermediate vectors pCKR1 or pCKR2 as shown in Fig. 1.

The intermediate vectors were established in *A. tumefaciens* by homologous recombination with resident copies of the disarmed octopine-type Ti plasmid pTiB6S3-SE as described previously (*13*). The recombinant plasmids contain the octopine-type T-DNA genes 5 and 7 (*14*) and the NPTII and NOS genes of the intermediate vector, all of which are flanked by right and left T-DNA border sequences, which delineate the region of the Ti plasmid that will be integrated into the genome of transformed plant cells (Fig. 1).

Leaf disks of *Nicotiana tabacum* cv. Xanthi H38 were infected with cultures of recombinant *A. tumefaciens* and cultured in vitro as described (*13*). Tobacco cells carrying stably integrated copies of the NPTII gene and associated DNA were selected by growth of calli and subsequent shoot development in the presence of kanamycin (300 μg/ml). Individual shootlets were excised from the transformed tissue and grown to a height of 40 to 60 cm prior to nucleic acid extraction from several leaves (four per plant) at approximately the same stage of development.

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