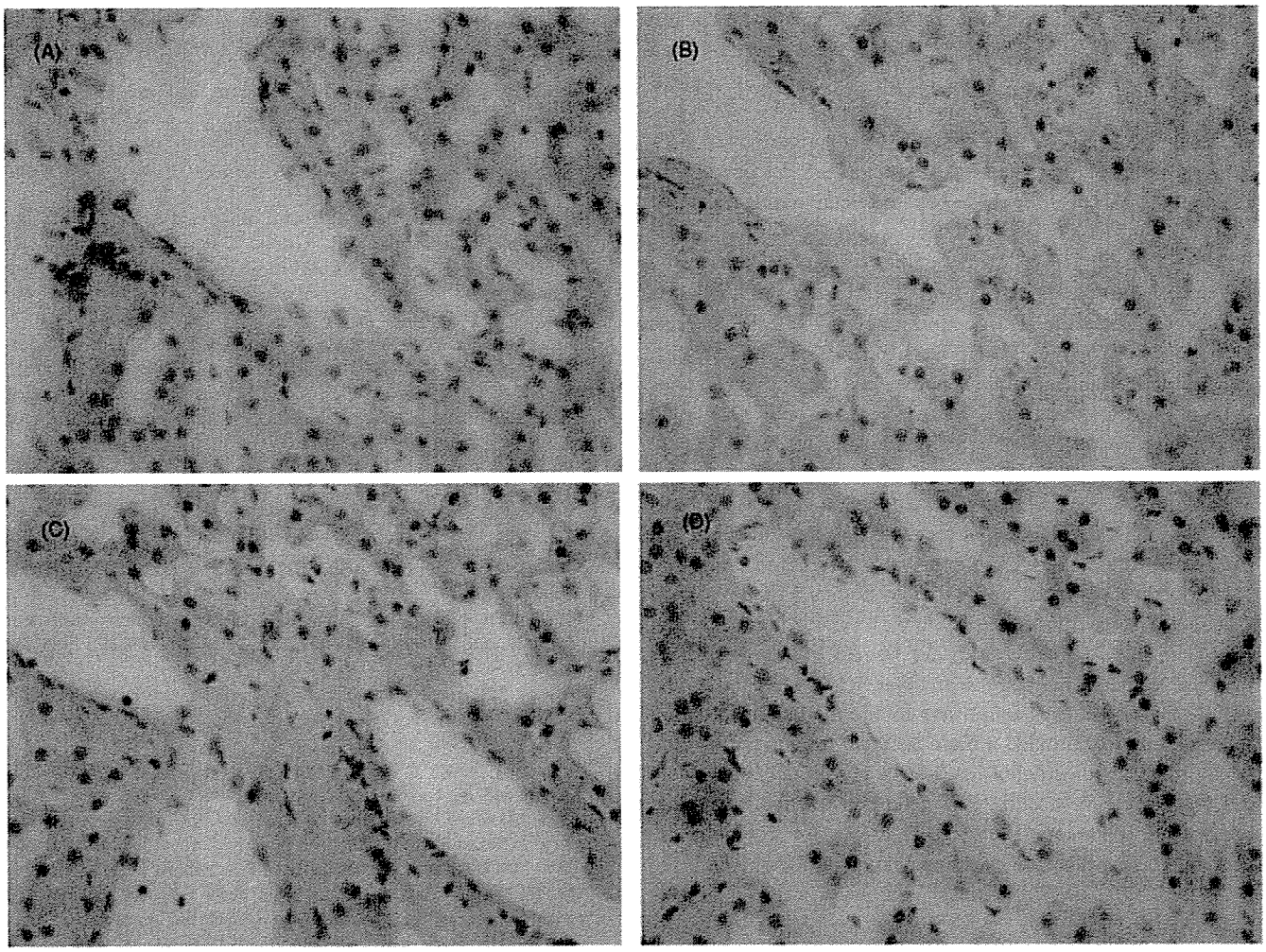


**Fig. 4.** Immunohistochemical study of the macaque liver. Frozen sections of the macaque liver on glass slides were incubated with monoclonal antibody 3A6 (A), normal mouse IgG (B), sheep polyclonal antibodies to human factor IX (C), or normal sheep IgG (D) and bound antibodies were detected by the avidin–biotin complex method as described in Materials and methods.

mice. However, there are obvious species differences between humans and mice, making it difficult under some circumstances to extrapolate data obtained in mice to human patients. Hemophilia B mice (FIX knock-out mice) and hemophilia B dogs have been used to study gene therapy approaches for treatment of hemophilia B [3–6]. Better animal models may be required, however, because there may be significant differences in transduction efficiency of skeletal muscles with AAV2 vectors between mice and dogs. Primates are used successfully as models in other disease applications, but there are as yet no hemophilic primates available. If one can distinguish human molecules from primate molecules *in vivo*, primates may be used for hemophilia gene therapy research, despite the fact that the genetic abnormality is not indigenous to the species.

Rhesus macaques are proposed to be a good primate model for studying hemophilia B gene therapy because of the amino acid sequence similarity between human FIX and macaque FIX and low immunogenicity of human FIX to rhesus macaque [13]. However, quantification of the human FIX expressed was difficult due to cross-reactivity of the rabbit anti-FIX antibodies. Rhesus macaques also developed antibodies to human FIX upon receiving viral vectors carrying the human FIX gene despite the high amino acid sequence homology [17]. A human FIX-specific EIA was developed using macaque antihuman FIX antibodies and EIA quantified human FIX levels in macaque plasma at 30 ng $mL^{-1}$ (1% of the normal FIX level). The amino acid sequence of cynomolgus macaque FIX is identical to that of rhesus macaque FIX [14], raising the possibility that distinguishing the recombinant human molecule expressed in cynomolgus macaques *in vivo* from the endogenous macaque FIX molecule may be difficult. Thus, seven antihuman FIX mAbs available in our facilities were screened for their inability to bind to simian FIX. The 3A6 antibody did not bind to macaque FIX and an EIA was developed with this antibody to quantify human FIX in macaque plasma. The EIA was approximately 20-fold more sensitive than that used in the previous study [13], detecting human FIX at 1.79 ng $mL^{-1}$, or 0.06% of the normal plasma levels in cynomolgus macaques. The advantage of mAb 3A6 was also confirmed by the data that 3A6 did not react with macaque FIX in the liver by immunohistochemistry (Fig. 4).

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Type 2 AAV vectors were initially considered for expression of transgenes in skeletal muscle, although there appears to be a significant difference in transduction efficiency of skeletal muscles with AAV vectors among different AAV serotypes. In mice, type 1 AAV vectors appear to be superior to other AAV serotypes, since types 3 and 5 AAV vectors are less potent than the type 1 AAV vector but have better skeletal muscle transduction efficiency than type 2 AAV vectors [18]. It remains possible that the transduction efficiency of human skeletal muscle by different AAV vectors differs from that in mice and dogs. It is also possible that the transduction efficiency of other organs, such as the liver, by AAV vectors has significant species specificity.

The cynomolgus macaque is native to southern Asia and has been used as a simian model in medical research, such as in Parkinson's disease. As reported previously, human FIX could be immunogenic to rhesus macaques transduced with adenoviral vectors or AAV vectors carrying the human FIX gene and to cynomolgus macaques that received repeated subcutaneous injections of human FIX in the presence of Freund's adjuvant [13,15]. Thus, as far as antibodies to human FIX develop in macaques during transduction with vectors carrying the human FIX gene, the long-term study for human FIX expression is impossible, but if antibody development to expressed human FIX is suppressed, macaques may mimic the human situation more closely and provide a more accurate assessment of viral vector transduction efficiencies, and we may be able to evaluate the efficacy of therapeutic FIX gene construction and select appropriate promoters, vectors, and organs for transgene expression, taking advantage of monoclonal antibody 3A6.

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## References

1 Kay MA, High K. Gene therapy for the hemophilias. *Proc Natl Acad Sci USA* 1999; **96**: 9973–5.
2 High KA. Gene transfer as an approach to treating hemophilia. *Circ Res* 2001; **88**: 137–44.
3 Kay MA, Rothenberg S, Landen CN, Bellinger DA, Leland F, Toman C, Finegold M, Thompson AR, Read MS, Brinkhous KM. *In vivo* gene therapy of hemophilia B: sustained partial correction in factor IX-deficient dogs. *Science* 1993; **262**: 117–9.
4 Snyder RO, Miao CH, Patijn GA, Spratt SK, Danos O, Nagy D, Gown AM, Winther B, Meuse L, Cohen LK, Thompson AR, Kay MA. Persistent and therapeutic concentrations of human factor IX in mice after hepatic gene transfer of recombinant AAV vectors. *Nat Genet* 1997; **16**: 270–6.
5 Herzog RW, Yang EY, Couto LB, Hagstrom JN, Elwell D, Fields PA, Burton M, Bellinger DA, Read MS, Brinkhous KM, Podsakoff GM, Nichols TC, Kurtzman GJ, High KA. Long-term correction of canine hemophilia B by gene transfer of blood coagulation factor IX mediated by adeno-associated viral vector. *Nat Med* 1999; **5**: 56–63.
6 Snyder RO, Miao C, Meuse L, Tubb J, Donahue BA, Lin HF, Stafford DW, Patel S, Thompson AR, Nichols T, Read MS, Bellinger DA, Brinkhous KM, Kay MA. Correction of hemophilia B in canine and murine models using recombinant adeno-associated viral vectors. *Nat Med* 1999; **5**: 64–70.
7 Kay MA, Manno CS, Ragni MV, Larson PJ, Couto LB, McClelland A, Glader B, Chew AJ, Tai SJ, Herzog RW, Arruda V, Johnson F, Scallan C, Skarsgard E, Flake AW, High KA. Evidence for gene transfer and expression of factor IX in haemophilia B patients treated with an AAV vector. *Nat Genet* 2000; **24**: 257–61.
8 Manno CS, Chew AJ, Hutchison S, Larson PJ, Herzog RW, Arruda VR, Jen Tai S, Ragni MV, Thompson A, Ozelo M, Couto LB, Leonard DGB, Johnson FA, McClelland A, Scallan C, Skarsgard E, Flake AW, Kay MA, High HA, Glader B. AAV-mediated factor IX gene transfer to skeletal muscle in patients with severe hemophilia B. *Blood* 2003; **101**: 2963–72.
9 Yoshioka A, Giddings JC, Fujimura TY, Bloom AL. Immunoassays of factor IX antigen using monoclonal antibodies. *Br J Hematol* 1985; **59**: 265–75.
10 Sugo T, Mizuguchi J, Kamikubo Y, Matsuda M. Anti-human factor IX monoclonal antibodies specific for calcium ion-induced conformations. *Thromb Res* 1990; **58**: 603–14.
11 Mimuro J, Sakata Y, Wakabayashi K, Matsuda M. Level of protein C determined by combined assays during disseminated intravascular coagulation and oral anticoagulation. *Blood* 1987; **69**: 1704–11.
12 Sugo T, Nakamikawa C, Takano H, Mimuro J, Yamaguchi S, Mosesson MW, Meh DA, DiOrio JP, Takahashi N, Takahashi H, Nagai K, Matsuda M. Fibrinogen Niigata with impaired fibrin assembly: an inherited dysfibrinogen with a Bβ Asn-160 to Ser substitution associated with extra glycosylation at Bβ Asn-158. *Blood* 1999; **94**: 3806–13.
13 Lozier JN, Metzger ME, Donahue RE, Morgan RA. The rhesus macaque as an animal model for hemophilia B gene therapy. *Blood* 1999; **93**: 1875–81.
14 Tomokiyo K, Teshima K, Nakatomi Y, Wtanabe T, Mizuguchi J, Nozaki C, Nakagaki T, Miyamoto S, Funatsu A, Iwanaga S. Induction of acquired factor IX inhibitors in cynomolgus monkey (*Macaca fascicularis*): a new primate model of hemophilia B. *Thromb Res* 2001; **102**: 363–74.
15 Zauber PN, Levin J. Factor IX levels in patients with hemophilia B (Christmas disease) following transfusion with concentrates of factor IX or fresh frozen plasma. *Medicine* 1977; **56**: 213–24.
16 MacCarthy K, Stewart P, Sigman J, Read M, Keith JC Jr, Brinkhous KM, Nichols TC, Schaub RG. Pharmacokinetics of recombinant factor IX after inravenous and subcutaneous administration in dogs and cynomolgus monkey. *Thromb Haemost* 2002; **87**: 824–30.
17 Nathwani AC, Davidoff AD, Hanawa H, Hu Y, Hoffer FA, Nikanorov A, Slaughter C, Ng C, Zhou J, Lozier JN, Mandrell TD, Vanin EF, Nienhuis W. Sustained high-level expression of human factor IX (hFIX) after liver-targeted delivery of recombinant adeno-associated virus encoding the hFIX gene in rhesus macaques. *Blood* 2002; **100**: 1662–9.
18 Chao H, Liu Y, Rabinowitz J, Li C, Samulski RJ, Walsh CE. Several log increase in therapeutic transgene delivery by distinct adeno-associated viral serotype vectors. *Mol Ther* 2000; **2**: 619–23.

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Short Communication

# The adenovirus E1A and E1B19K genes provide a helper function for transfection-based adeno-associated virus vector production

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Although the adenoviral E1, E2A, E4 and VA RNA regions are required for efficient adeno-associated virus (AAV) vector production, the role that the individual E1 genes (*E1A*, *E1B19K*, *E1B55K* and *protein IX*) play in AAV vector production has not been clearly determined. E1 mutants were analysed for their ability to mediate AAV vector production in HeLa or KB cells, when cotransfected with plasmids encoding all other packaging functions. Disruption of *E1A* and *E1B19K* genes resulted in vector yield reduction by up to 10- and 100-fold, respectively, relative to the wild-type E1. Interruption of the *E1B55K* and *protein IX* genes had a modest effect on vector production. Interestingly, expression of anti-apoptotic E1B19K cellular homologues such as Bcl-2 or Bcl-$x_L$ fully complemented E1B19K mutants for AAV vector production. These findings may be valuable for the future development of packaging cell lines for AAV vector production.

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Adeno-associated virus (AAV)-based vector systems are particularly attractive vehicles for clinical applications requiring long-term *in vivo* gene expression from post-mitotic tissues. AAV vectors have been shown to promote stable expression of a wide variety of transgenes in numerous tissues, including skeletal and cardiac muscle, liver, the central nervous system and retina (Rabinowitz & Samulski, 1998). Overt evidence of inflammation is either minimal or non-existent in target tissues immediately following AAV vector administration. Furthermore, cytotoxic T-lymphocyte responses are not normally elicited to transgene products delivered by AAV vectors, even when such proteins are foreign to the host (Jooss *et al.*, 1998). AAV vectors are considered to be relatively safe because the parental virus is non-pathogenic and unable to replicate in the absence of a co-infecting helper virus. Additionally, current production methods have reduced the regeneration of replication competent wild-type AAV during vector production to undetectable levels (Allen *et al.*, 1997). Finally, the robust protein capsid of AAV makes AAV vectors particularly amenable to existing production methods for protein pharmaceuticals (Gao *et al.*, 2000) and confers upon them desirable drug stability characteristics.

AAV2, the parent virus from which the vector system is derived, is replication defective and requires co-infection of helper viruses to propagate. Adenovirus (Atchinson *et al.*, 1965) and herpes virus (Buller *et al.*, 1981) act as complete helpers and vaccinia virus (Schlehofer *et al.*, 1986) acts as a partial helper. The set of adenoviral (type 2 or 5) genes that facilitate AAV2 propagation has been defined and consists of E1A, E1B55K, the VA RNAs, E2A and E4orf6 (Samulski & Shenk, 1988). E1A acts as a cue to begin virus replication by up-regulating transcription from the *rep* gene promoters, P5 and P19 (Tratschin *et al.*, 1984) and by activating the early adenovirus promoters. E1A is also required to drive the host cell into the S-phase of the cell cycle for viral DNA replication because the AAV encoded proteins are not capable of this function. An adverse effect of E1A is that it stabilizes p53, which leads to apoptosis (Lowe *et al.*, 1993). To prevent this, the E1B55K and the E4orf6 proteins form a complex with p53 and cause it to be degraded through ubiquitin-mediated proteolysis (Querido *et al.*, 1997; Steegenga *et al.*, 1998). Later in infection, E1B55K and E4orf6 form a heterodimer that causes the preferential export of AAV and adenoviral late mRNAs from the nucleus while inhibiting the transit of adenoviral early and cellular mRNAs (Pilder *et al.*, 1986). The 72 kDa DNA-binding protein encoded by E2A has functions in viral DNA replication, viral mRNA processing and export, and AAV promoter regulation (Carter *et al.*, 1992; Ward *et al.*, 1998; Chang & Shenk, 1990). It causes an increase in the intracellular levels of the

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single- and double-stranded forms of the AAV genome, the spliced forms of the rep proteins, and dramatically increases capsid protein production. Lastly, the VA RNAs inhibit the interferon-inducible eIF-2 protein kinase, thereby circumventing this cellular anti-viral mechanism from blocking viral protein translation (West *et al.*, 1987).

With respect to E2A, E4orf6 and the VA RNAs, the helper gene requirement for AAV vector and virus production is identical. We and others, have shown that plasmids encoding these genes, when cotransfected into 293 cells along with plasmids encoding rep/cap and a vector, mediate higher levels of vector production than that produced by adenovirus infection (Xiao *et al.*, 1998; Matsushita *et al.*, 1998). This so-called 'triple plasmid' transfection method forms the basis of the current scale-up vector production effort at Avigen and has a respectable mean production efficiency of $1 \times 10^{13}$ vector genomes produced per 850 $cm^2$ roller bottle. A report was published describing a method for producing AAV vector in 293 cells using only E4orf6 as the helper gene (Allen *et al.*, 2000). This method requires the use of a heterologous promoter to drive the capsid gene and is about 10-fold less productive than methods using a plasmid encoding all three adenoviral helper genes (unpublished data).

The genes of the E1 region have not been analysed for their contribution to AAV vector production. In this study, we have investigated the role of the *E1A* and *E1B* genes in AAV vector production by using a series of E1 mutant plasmids and cell lines that lack adenoviral genes. E1A was required for efficient vector production. In contrast to the helper requirements for AAV production, our data indicated that *E1B19K* gene greatly augmented vector production, however, *E1B55K* gene did not.

The contributions of each of the component genes from the E1 region to AAV helper function was assessed by creating a set of plasmids with mutations in the *E1A*, *E1B19K*, *E1B55K* or *protein IX* genes and then testing them for their ability to support transfection-based AAV vector production. At least one truncation or one deletion mutation was made for each gene (Fig. 1).

For vector construction the plasmid pE1, which encodes the *E1A*, *E1B19K*, *E1B55K* and *protein IX* genes, was created from Ad2 DNA (Invitrogen). Briefly, the *Afl*III fragment (nt positions 142–5927) of Ad2 was cloned into the *Afl*III site of pBR322 (New England Biolabs) to generate pE1. pE1A-825stop was constructed by the insertion of an adapter (CCGGACTAATTAACTAGT), which includes a stop codon and an *Spe*I site, into the *Bsp*EI site of pE1. Similarly, pE1B19K-1912stop, pE1B55K-2243stop,

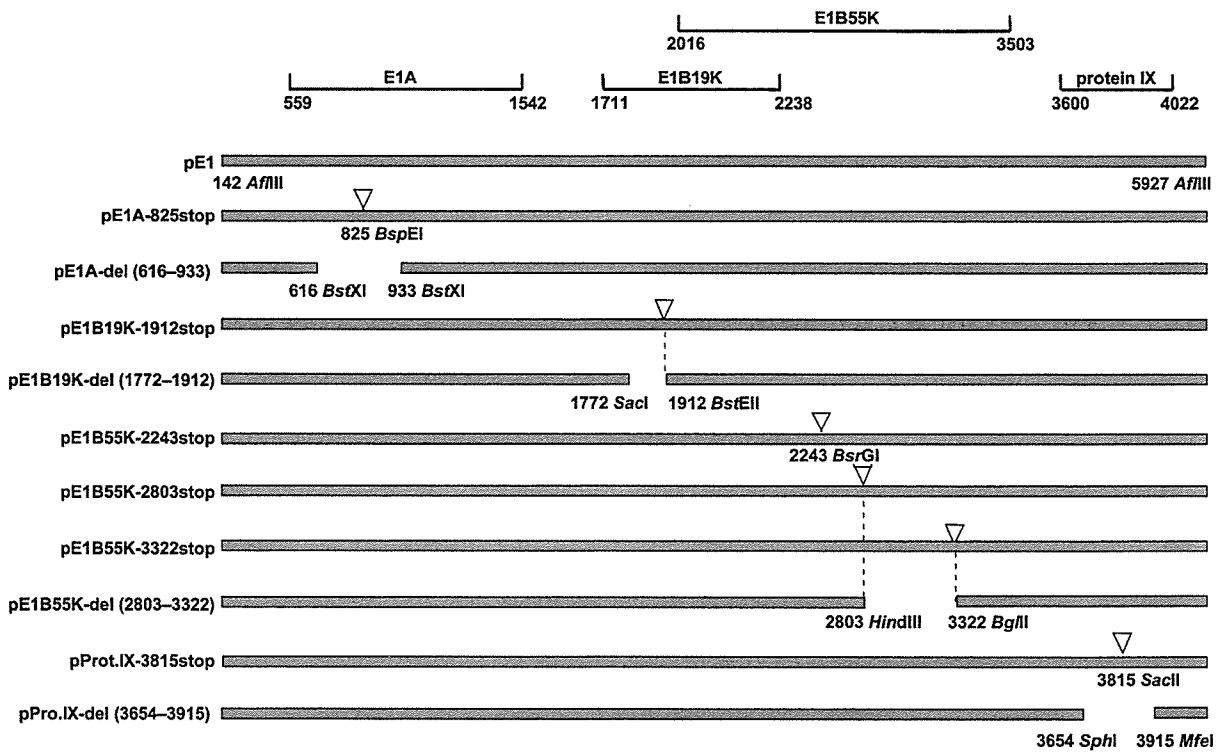


**Fig. 1.** Schematic representation of plasmids harbouring adenoviral E1 mutants used in this study. A 5·8 kb DNA fragment of adenovirus type 2 was cloned into the *Afl*III site of pBR322. pE1 encodes the entire E1 region, and the E1 mutant plasmids shown here were derived from it. The vertical flags mark the positions of inserted stop codons. The gaps in pE1A, pE1B19K, pE1B55K or pProt.IX constructs represent deletions.

pE1B55K-2803stop and pE1B55K-3322stop were made by the insertion of oligonucleotides into the *Bst*EII, *Bsr*GI, *Hin*dIII and *Bgl*II sites of pE1, respectively. pE1A-del (616–933) has a deletion of a 318 bp segment (positions 616–933 in Ad2). pE1B19K-del (1772–1912) and pE1B55K-del (2803–3322) have the same deletions as *dl337* (Pilder *et al.*, 1984) and *dl338* (Pilder *et al.*, 1986), respectively, used by Samulski & Shenk (1988) to examine E1 helper function for AAV2 production. Briefly, pE1B19K-del (1772–1912) lacks sequences between positions 1772 and 1912, and pE1B55K-del (2803–3322) lacks sequences between positions 2803 and 3322. pProt.IX-3815stop was constructed by the insertion of oligonucleotides into a *Sac*II site. pProt.IX-del (3654–3915) lacks a 262 bp segment (between positions 3654 and 3915 of Ad2).

The helper activities of the various E1 plasmids were assayed by cotransfecting them with a plasmid encoding both an AAV CMV*lacZ* vector and rep/cap (pW4389LacZ), and a plasmid encoding the adenovirus-2 VA RNA, E2A and E4 regions (Pladeno5), into KB or HeLa cells, and then quantifying *lacZ* vector production as described previously (Matsushita *et al.*, 1998). AAV vector was harvested 40 or 72 h after transfection and stocks were prepared by the freeze-thaw method. AAV vector production was quantified by titration of the vector stocks in 293 cells in the presence of adenovirus, followed by X-Gal staining and manual counting by light microscopy. For each experiment, all constructs were tested using triplicate production cultures, and all experiments were conducted at least three times, independently.

Elimination of the entire E1 region resulted in 2 (HeLa cells) to 3 log (KB cells) reduction in vector production relative to production in the presence of pE1, a plasmid encoding the entire E1 region ($P<0{\cdot}01$ by Student's *t*-test) (Fig. 2a, b). Disruption of the *E1A* genes, whether by truncation or deletion, caused 1 (HeLa cells) to 1·5 log (KB cells) reduction in vector production ($P<0{\cdot}01$). Truncations or deletions in the *E1B19K* gene also resulted in substantial reduction in vector production, 1 log in HeLa cells and greater than 2 logs in KB cells ($P<0{\cdot}01$). The lesser severity of the E1B19K mutant in HeLa cells, relative to KB cells, may be due to the relatively high level of Bcl-2 expression in HeLa cells (Liang *et al.*, 1995), or the human papilloma virus E6/E7 genes they harbour. The E6/E7 genes have been shown to facilitate some of the processes in AAV replication (Walz *et al.*, 1997). In most cases, disruption of the *E1B55K* and *protein IX* genes had a modest effect on vector production in either HeLa or KB cells. Two constructs, pE1B55K-2243stop and pProt.IX-3815stop showed fivefold reduction in vector yield in KB cells but little reduction in HeLa cells.

Our results differ substantially from those of Samulski & Shenk (1988) who examined the effect of E1B adenovirus mutants on AAV2 production, DNA replication, and mRNA and protein expression. This group found that an E1B19K adenovirus-2 mutant (*dl337*) mediated efficient AAV production from HeLa cells transfected with a plasmid encoding an AAV wild-type provirus (pSM620) but that

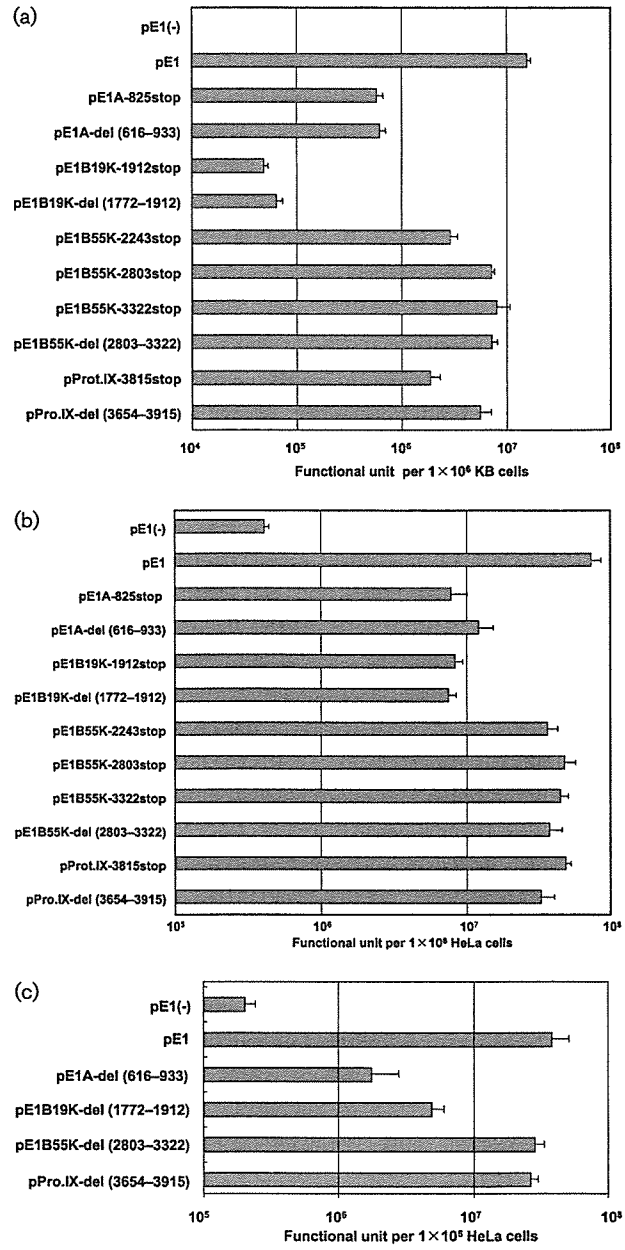


**Fig. 2.** Comparison of E1 mutant plasmids with respect to AAV helper function in KB (a) and HeLa cells (b) at 72 h after transfection, or in HeLa cells (c) at 40 h after the transfection. AAV *lacZ* vector was produced by the transfection of HeLa or KB cells with pW4389lacZ (encodes rep/cap and an AAV *lacZ* vector) and pladeno 5 (encodes the E2A, E4 and VA RNA regions), in the presence and absence of the indicated E1 plasmids. AAV vector production was assessed by titration of *lacZ* vector in 293 cells. pE1 (-) is identical to pBR322 without the expression cassette. Each bar represents the mean value obtained from triplicate cultures, and the error bar represents the standard deviation.

E1B55K (*dl338*) and E4orf6 (*dl355*) adenovirus mutants did not. AAV virion production was measured at a 40 h time point. The E1B55K and E4orf6 defects were caused by a delay in AAV mRNA accumulation that resulted in delays in viral DNA replication, capsid expression and ultimately virus production. AAV mRNA, DNA and capsid protein concentrations in cultures infected with E1B55K and E4orf6 mutants eventually reached levels seen in cultures infected by wild-type adenovirus but at longer time points, 72–96 h for adenovirus mutants compared with 24–40 h for wild-type adenovirus.

An important difference between our study and that of Samulski & Shenk (1988) was the timing of AAV/AAV vector harvest, 40 h in our study versus 72 h in theirs. Therefore, we examined a subset of the E1 region plasmids in transfection experiments using the same 40 h time point for vector harvest (Fig. 2c). The results were essentially similar to those at the 72 h time point and still differed from those produced by the adenovirus mutants. This observed difference in helper gene requirement may be attributable to technical factors associated with using virus infection or DNA transfection. A possible explanation for the conclusions reached by Samulski & Shenk (1988) might be the differences in the growth rates of the adenovirus mutants tested. The E1B55k mutant, *dl338*, was reported to grow inefficiently (100-fold reduced relative to wild-type) in HeLa cells (Pilder *et al.*, 1986) while the E1B19K mutant, *dl337*, was reported to be less defective (about 10-fold reduced relative to wild-type) (Pilder *et al.*, 1984). The lag in AAV mRNA, DNA and virus production seen with the E1B55K mutant may be simply because of a slow growing helper virus, resulting in low copy numbers of all of the adenovirus helper genes, and may not be directly due to the lack of the mutated gene. The observation that E1B19K is apparently not required for adenovirus mediated AAV production is harder to explain. It is tempting to speculate that the transfection-based production system benefits from additional anti-apoptitic activity provided by E1B19K. If this is true, this requirement does not appear to be cell-type or transfection-reagent specific (calcium phosphate and polycation-based transfection reagents both show an E1B19K effect, data not shown), and may have something to do with the adenoviral helper gene dose or kinetics of expression. Other differences between the two methods of identifying AAV helper function include: transfection method, the packaging of AAV virus versus a vector, and the use of replicating helper (AAV) versus non-replicating plasmid helpers. Full resolution of these issues will require further experimentation.

The adenovirus *E1B19K* gene, and its cellular homologues Bcl-2 and Bcl-$x_L$, encode anti-apoptotic proteins that function by inhibiting proapoptotic Bcl-2 homologues, such as Bax and Bak, by forming inactive heterodimers with them. To determine whether other anti-apoptotic members of the Bcl-2 family could augment AAV vector production, plasmid vectors expressing the *E1B19K*, *Bcl-2* or *Bcl-$x_L$* gene

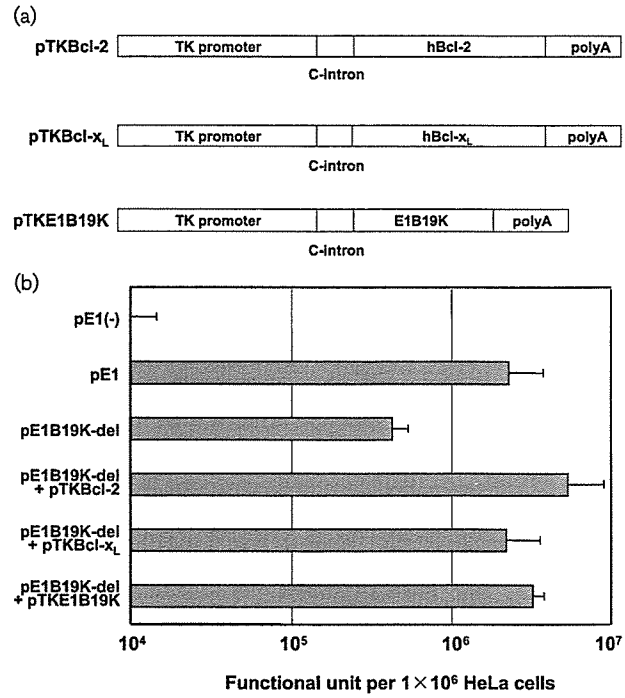


**Fig. 3.** (a) Schematic representation of Bcl-2, Bcl-$x_L$ and E1B19K expression plasmids. TK promoter, HSV-*tk* promoter; C-intron, chimeric CMV/$\beta$-globin intron; polyA, SV40 late polyadenylation signal; hBcl-2, human Bcl-2 cDNA; hBcl-$x_L$, human Bcl-$x_L$ cDNA; and E1B19K, adenovirus type 2 early region 1B 19 kDa protein gene. (b) Bcl-2 family members complement the vector production defect of an E1B19K mutant in HeLa cells. AAV *lacZ* vector was produced by the transfection of HeLa cells with pW4389lacZ (encodes rep/cap and an AAV *lacZ* vector), pladeno 5 (encodes the E2A, E4 and VA RNA regions), and pE1B19K-del (1772–1912), in the presence and absence of the indicated plasmids expressing Bcl-2 family genes, including E1B19K. AAV vector production was assessed by titration of *lacZ* vector in 293 cells. Each bar represents the mean value of triplicate cultures and the error bar represents the standard deviation.

products were tested for their ability to complement the vector production defect of the E1B19K deletion mutant, pE1B19K-del (1772–1912) (Fig. 3a). pTKPRMCS was assembled by the removal of a *Renilla* luciferase (R*luc*) reporter gene from pRL-TK (Promega) (between the *Nhe*I and *Xba*I sites) and insertion of a multiple cloning site (between the *Kpn*I and *Xba*I sites) from pBluescript II (Stratagene). pTK-Bcl-2 and pTK-Bcl-$x_L$ were created by the insertion of human Bcl-2 and Bcl-$x_L$ cDNA sequences, respectively, into pTKPRMCS. pTK-E1B19K was constructed by the insertion of the E1B19K fragment into pTKPRMCS. As shown in Fig. 3(b), plasmids expressing E1B19K, Bcl-2 or Bcl-$x_L$ restored vector production of the E1B19K deletion mutant to levels equivalent to that produced by the wild-type pE1 plasmid. The use of the medium strength HSV-*tk* promoter to drive the expression

of the Bcl-2 homologues was essential for helper function. CMV-driven constructs produced low vector yields in a dominant fashion and caused a substantial increase in apoptosis (data not shown).

The fact that E1B19K mutants can be complemented by similarly anti-apoptotic cellular homologues such as Bcl-2 or Bcl-$x_L$ suggests a common mechanism, the inhibition of Bak/Bax-mediated apoptosis. Interestingly, no increase in DNA ladder formation is seen in HeLa cells when transfected with E1B19K mutant plasmids relative to wild-type plasmids (data not shown). Consequently, the mechanism of vector production augmentation is not clear.

Current transfection-based AAV vector production methods are sufficient to commercially support gene therapy applications with large doses and small patient populations (e.g. haemophilia, other genetic diseases) or applications with small doses and large patient populations (e.g. Parkinson's disease). Applications with large doses and large patient populations (e.g. heart failure) will be a challenge for transfection-based production methods that scale linearly. Consequently, the construction of a producer cell line that is both helper virus-free, and suspension culture-adaptable, is of great interest. This is a formidable task since many of the viral helper proteins are toxic to the cell either alone (e.g. E2A) or in combination with other helper functions (e.g. E4orf6 and E1B55K, E1A and *rep*). The task is further complicated by genes such as E1B19K that must be expressed in a rather precise manner. Packaging cell lines containing inducible E1 genes, along with the E2a, VA and E4 regions, and an integrated AAV vector have been produced but were found to suffer from relatively low vector yield and substantial production instability (Qiao *et al.*, 2002). Both of these problems were likely due to, or exacerbated by, helper gene toxicity. Our data indicates that one source of toxicity, the inhibition of host mRNA nuclear export mediated by the E4orf6/E1B55K heterodimer, could be eliminated by not including the E1B55K gene in packaging cell lines.

Defining the minimum set of helper genes necessary for efficient vector production is the first step in creating suitable packaging cell lines for AAV vectors. Using our transfection-based assay, we define that set to be E1A, E1B19K, the VA RNAs, E2A and E4orf6 genes.

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## References

**Allen, J. M., Debelak, D. J., Reynolds, T. C. & Miller, A. D. (1997).** Identification and elimination of replication-competent adeno-associated virus (AAV) that can arise by nonhomologous recombination during AAV vector production. *J Virol* **71**, 6816–6822.

**Allen, J. M., Halbert, C. L. & Miller, A. D. (2000).** Improved adeno-associated virus vector production with transfection of a single helper adenovirus gene, E4orf6. *Mol Ther* **1**, 88–95.

**Atchinson, R. W., Casto, B. C. & Hammon, W. M. (1965).** Adenovirus-associated defective virus particles. *Science* **149**, 754–756.

**Buller, R. M., Janik, J. E., Sebring, E. D. & Rose, J. A. (1981).** Herpes simplex virus types 1 and 2 completely help adenovirus-associated virus replication. *J Virol* **40**, 241–247.

**Carter, B. J., Antoni, B. A. & Klessig, D. F. (1992).** Adenovirus containing a deletion of the early region 2A gene allows growth of adeno-associated virus with decreased efficiency. *Virology* **191**, 473–476.

**Chang, L. S. & Shenk, T. (1990).** The adenovirus DNA-binding protein stimulates the rate of transcription directed by adenovirus and adeno-associated virus promoters. *J Virol* **64**, 2103–2109.

**Gao, G., Qu, G., Burnham, M. S. & 7 other authors (2000).** Purification of recombinant adeno-associated virus vectors by column chromatography and its performance *in vivo*. *Hum Gene Ther* **11**, 2079–2091.

**Jooss, K., Yang, Y., Fisher, K. J. & Wilson, J. M. (1998).** Transduction of dendritic cells by DNA viral vectors directs the immune response to transgene products in muscle fibers. *J Virol* **72**, 4212–4223.

**Liang, X. H., Mungal, S., Ayscue, A., Meissner, J. D., Wodnicki, P., Hockenbery, D., Lockett, S. & Herman, B. (1995).** Bcl-2 proto-oncogene expression in cervical carcinoma cell lines containing inactive p53. *J Cell Biochem* **57**, 509–521.

**Lowe, S. W., Ruley, H. E., Jacks, T. & Housman, D. E. (1993).** p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. *Cell* **74**, 957–967.

**Matsushita, T., Elliger, S., Elliger, C., Podsakoff, G., Villarreal, L., Kurtzman, G. J., Iwaki, Y. & Colosi, P. (1998).** Adeno-associated virus vectors can be efficiently produced without helper virus. *Gene Ther* **5**, 938–945.

**Pilder, S., Logan, J. & Shenk, T. (1984).** Deletion of the gene encoding the adenovirus 5 early region 1b 21,000-molecular-weight polypeptide leads to degradation of viral and host cell DNA. *J Virol* **52**, 664–671.

**Pilder, S., Moore, M., Logan, J. & Shenk, T. (1986).** The adenovirus E1B-55K transforming polypeptide modulates transport or cytoplasmic stabilization of viral and host cell mRNAs. *Mol Cell Biol* **6**, 470–476.

**Qiao, C., Li, J., Skold, A., Zhang, X. & Xiao, X. (2002).** Feasibility of generating adeno-associated virus packaging cell lines containing inducible adenovirus genes. *J Virol* **76**, 1904–1913.

**Querido, E., Marcellus, R. C., Lai, A., Charbonneau, R., Teodoro, J. G., Ketner, G. & Branton, P. E. (1997).** Regulation of p53 levels by the E1B 55-kilodalton protein and E4orf6 in adenovirus-infected cells. *J Virol* **71**, 3788–3798.

**Rabinowitz, J. E. & Samulski, J. (1998).** Adeno-associated virus expression systems for gene transfer. *Curr Opin Biotechnol* **9**, 470–475.

**Samulski, R. J. & Shenk, T. (1988).** Adenovirus E1B 55-Mr polypeptide facilitates timely cytoplasmic accumulation of adeno-associated virus mRNAs. *J Virol* **62**, 206–210.

**Schlehofer, J. R., Ehrbar, M. & zur Hausen, H. (1986).** Vaccinia virus, herpes simplex virus, and carcinogens induce DNA amplification in a human cell line and support replication of a helpervirus dependent parvovirus. *Virology* **152**, 110–117.

**Steegenga, W. T., Riteco, N., Jochemsen, A. G., Fallaux, F. J. & Bos, J. L. (1998).** The large E1B protein together with the E4orf6 protein

target p53 for active degradation in adenovirus infected cells. *Oncogene* **16**, 349–357.

**Tratschin, J. D., West, M. H., Sandbank, T. & Carter, B. J. (1984).** A human parvovirus, adeno-associated virus, as a eucaryotic vector: transient expression and encapsidation of the procaryotic gene for chloramphenicol acetyltransferase. *Mol Cell Biol* **4**, 2072–2081.

**Walz, C., Deprez, A., Dupressoir, T., Durst, M., Rabreau, M. & Schlehofer, J. R. (1997).** Interaction of human papillomavirus type 16 and adeno-associated virus type 2 co-infecting human cervical epithelium. *J Gen Virol* **78**, 1441–1452.

**Ward, P., Dean, F. B., O'Donnell, M. E. & Berns, K. I. (1998).** Role of the adenovirus DNA-binding protein in *in vitro* adeno-associated virus DNA replication. *J Virol* **72**, 420–427.

**West, M. H., Trempe, J. P., Tratschin, J. D. & Carter, B. J. (1987).** Gene expression in adeno-associated virus vectors: the effects of chimeric mRNA structure, helper virus, and adenovirus VA1 RNA. *Virology* **160**, 38–47.

**Xiao, X., Li, J. & Samulski, R. J. (1998).** Production of high-titer recombinant adeno-associated virus vectors in the absence of helper adenovirus. *J Virol* **72**, 2224–2232.

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# Roles of Bim in Apoptosis of Normal and Bcr-Abl-Expressing Hematopoietic Progenitors

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**Bcr-Abl kinase is known to reverse apoptosis of cytokine-dependent cells due to cytokine deprivation, although it has been controversial whether chronic myeloid leukemia (CML) progenitors have the potential to survive under conditions in which there are limited amounts of cytokines. Here we demonstrate that early hematopoietic progenitors (Sca-1$^+$ c-Kit$^+$ Lin$^-$) isolated from normal mice rapidly undergo apoptosis in the absence of cytokines. In these cells, the expression of Bim, a proapoptotic relative of Bcl-2 which plays a key role in the cytokine-mediated survival system, is induced. In contrast, those cells isolated from our previously established CML model mice resist apoptosis in cytokine-free medium without the induction of Bim expression, and these effects are reversed by the Abl-specific kinase inhibitor imatinib mesylate. In addition, the expression levels of Bim are uniformly low in cell lines established from patients in the blast crisis phase of CML, and imatinib induced Bim in these cells. Moreover, small interfering RNA that reduces the expression level of Bim effectively rescues CML cells from apoptosis caused by imatinib. These findings suggest that Bim plays an important role in the apoptosis of early hematopoietic progenitors and that Bcr-Abl supports cell survival in part through downregulation of this cell death activator.**

In the chronic phase, chronic myeloid leukemia (CML) is characterized by massive proliferation of granulocytes in the peripheral blood and their progenitors in the bone marrow. Abnormal hematopoietic stem cells harboring the *Bcr-Abl* chimeric gene still differentiate into mature granulocytes with apparently normal function but gradually come to occupy the hematopoietic space. They subvert the system controlling their homeostasis in the body and thus accumulate in large numbers. Because cytokines are considered to play critical roles in this homeostasis, dysregulation of cytokine-mediated cell death, cell survival, or cell division by Bcr-Abl may be responsible for leukemogenesis. Indeed, among multiple systems regulating diverse cell functions, including cell proliferation, differentiation, and apoptosis, which are dysregulated by Bcr-Abl, the reversal of apoptosis caused by cytokine deprivation is one of the most consistently observed effects (reviewed in references 15 and 23). This finding has been repeatedly demonstrated by use of different experimental systems that include murine interleukin-3 (IL-3)-dependent Baf-3 and 32D cells (8, 9, 11, 22, 28, 32, 37, 39).

We and others have investigated this cytokine-dependent cell survival system in hematopoietic progenitors by using IL-3-dependent cells and demonstrated that two distinct signaling pathways support cell survival. One pathway emanates from the membrane-proximal region of the common receptor chain (βc chain) shared by IL-3 and granulocyte-macrophage colony-stimulating factor, which activates JAK-STAT pathways and transcriptionally upregulates Bcl-$x_L$ expression (14, 45, 46). The other pathway functions via the distal portion of the βc chain and activates Ras pathways (26, 27, 30). Because experiments using Baf-3 cells expressing truncated forms of the βc chain revealed that signals from its proximal portion support cell survival only transiently, signals from its distal region, especially the activation of Ras pathways, were considered to be indispensable for long-term cell survival supported by cytokines (26; also reviewed in reference 35).

Recent progress has revealed that cell death decisions are implemented through an evolutionarily conserved mechanism (or general apoptosis program) in which members of the Bcl-2 superfamily play the central roles (reviewed in references 1 and 7). The anti- or proapoptotic family members regulate the translocation of cytochrome *c* from mitochondria to the cytosol, an event that ultimately activates the caspase cascade, while members of the BH3-only subfamily of cell death activators inhibit the function of the antiapoptotic Bcl-2 family members by binding to them. In mammals, more than three factors have been identified to be members of each subfamily. Redundancy in each category of the Bcl-2 superfamily has been explained, at least partially, by the tissue- and/or stimulus-specific response of each family member. We therefore concentrated on identifying the major Bcl-2 superfamily mem-

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TABLE 1. Primers used in this study for real-time quantitative RT-PCR

| Gene product | Product size (bp) | Forward primer | Reverse primer |
|---|---|---|---|
| A1 | 168 | GGGAAGATGGCTGAGTCTGAGCTCATG | TGACTTCAGATTCTTTTCAACTTC |
| Bad | 233 | CCACCAACAGCTATCATGGAGGCGC | GCTCTTTGGGCGAGGAAGTCCCTTG |
| Bax | 162 | AATATGGAGCTGCAGAGGATGATTG | GCACTTTAGTGCACAGGGCCTTGAG |
| Bcl-2 | 261 | GTGGTGGAGGAACTCTTCAGGGATG | GGTCTTCAGAGACAGCCAGGAGAAATC |
| Bcl-$x_L$ | 293 | GTAGTGAATGAACTCTTTCGGGATGG | ACCAGCCACAGTCATGCCCGTCAGG |
| BimEL | 324 | AGTGGGTATTTCTCTTTTGACACAG | TCAATGCCTTCTCCATACCAGACG |
| Bim(si) | 119 | AATGTCTGACTCTGACTCTCGGAC | TCTCCGCAGGCTGCAATTGTCTAC |
| Mcl-1 | 259 | GTAATGGTCCATGTTTTCAAAGATG | AAGCCAGCAGCACATTTCTGATGCC |
| DP5/Hrk | 189 | AGACCCAGCCCGGACCGAGCAA | AATAGCACTGGGGTGGCTCT |
| 28S rRNA | 324 | ACGCAGGTGTCCTAAGGCGAGCTC | CACGACGGTCTAAACCCAGCTCAC |

ber that is regulated by signals from the distal portion of the βc chain, especially via Ras pathways. We and others have found that mRNA and protein expression levels of Bim, a member of the BH3-only death activator subfamily, are downregulated by IL-3 through either the Ras/Raf/mitogen-activated protein kinase (MAPK) or the Ras/phosphatidylinositol 3-kinase (PI3-K) pathway in Baf-3 cells (13, 44). Bim was isolated independently by two groups that exploited its ability to bind Bcl-2 or Mcl1 (20, 36). Alternative splicing gives rise to three variants, BimEL, BimL, and BimS, each of which contains the BH3 domain and functions as a death inducer. It was shown that Bim was induced in Baf-3 cells by IL-3 deprivation but not by other apoptotic triggers, such as DNA damage or Fas, and that enforced expression (but not overexpression) of each form of Bim induced apoptosis in Baf-3 cells even in the presence of IL-3 (44). In addition to Bim induction in hematopoietic cells, the induction of Bim by deprivation of nerve growth factor (NGF) in primary cultures of rat sympathetic neurons, as well as in neuronally differentiated rat pheochromocytoma PC-12 cells, has been reported (5, 40, 49). These findings suggest that the level of Bim expression is a major determinant of cell fate regulated by cytokines.

In addition to its role as a key intracellular factor for cytokine-mediated cell survival, Bim was demonstrated to be an essential regulator of the total number of white blood cells by analysis of Bim-deficient mice (6). Bim-deficient mice have increased numbers of mature monocytes, granulocytes, and lymphocytes but not erythrocytes in the peripheral blood, with overgrowth of hematopoietic precursors in the bone marrow. This prompted us to investigate the roles of Bim as a possible downstream target of Bcr-Abl by using our previously established transgenic (tg) mice in addition to the conventional experimental systems for CML, such as cell lines established from patients in the blast crisis (BC) phase and cytokine-dependent cells expressing Bcr-Abl. In these tg mice, Bcr-Abl is expressed under the control of the *tec* tyrosine kinase promoter that is active in immature myeloid progenitors (16, 17, 33). Virtually all of these mice develop CML-like disease, namely, proliferation of mature myeloid precursors and megakaryocytes in the bone marrow with increased granulocytes and platelets in the peripheral blood and progressive anemia, within 8 months of birth. They generally die of the disease within 15 months (18). Moreover, when they are intercrossed with p53 haplo-deficient mice, they develop T-cell leukemia and lack functional p53 (19), indicating that this model mimics human CML in both the chronic and BC phases. Here we show that Bim plays an important role in the apoptosis of early hematopoietic progenitors and that Bcr-Abl supports cell survival in part through the downregulation of this cell death activator.

## MATERIALS AND METHODS

**Mice.** $p210^{bcr/abl}$ tg (*BCR-ABL*$^{tg/-}$) mice were previously described (18). Because the founder mice were generated by using ova derived from (C57BL × DBA)$F_2$ ($BDF_2$) mice and the tg progeny were generated by intercrossing the tg mice with $BDF_1$ mice, the genetic background of the *BCR-ABL*$^{tg/-}$ mice was a mixture of C57BL/6 and DBA. We used the normal littermates of these mice (*BCR-ABL*$^{-/-}$) as controls in this study.

**Primary culture and isolation of cytokine-dependent hematopoietic progenitors.** Mice that were 8 to 12 weeks of age were sacrificed, and bone marrow cells were harvested by a standard procedure. Cells were cultured for 5 days in serum-free medium (SF-O2; Sanko Junyaku, Tokyo, Japan) containing 10 ng of thrombopoietin (TPO) per ml and 50 ng of stem cell factor (SCF) per ml. After Ficoll gradient centrifugation to separate dead cells and mature granulocytes, cells expressing lineage-specific markers (CD4, CD8, CD11b, CD41, or Gr-1) were eliminated by using magnetic beads conjugated with specific antibodies (Miltenyi Biotec, Bergisch Gladbach, Germany). More than 90% of lineage marker-negative ($Lin^-$) cells obtained by this procedure were positive for c-Kit. These cells were further divided into Sca-1-enriched ($Sca\text{-}1^+$ $c\text{-}Kit^+$ $Lin^-$) and Sca-1-depleted ($Sca\text{-}1^-$ $c\text{-}Kit^+$ $Lin^-$) fractions by using magnetic beads conjugated with Sca-1 antibody. Viable cell counts were determined by trypan blue dye exclusion in triplicate assays. Morphology was determined by using cytospin preparations stained with May-Giemsa solution.

**TUNEL analysis.** Cells in the Sca-1-enriched fraction were cultured in cytokine-free medium for different periods. Cells were harvested and fixed with 4% paraformaldehyde for 20 min, and a terminal deoxynucleotidyltransferase-mediated nick end labeling (TUNEL) assay was performed with an apoptosis detection kit according to the manufacturer's directions (Promega, Madison, Wis.). Cells were then stained with 1 μg of propidium iodide per ml. Cytospin preparations were made, and the incorporation of dUTP was analyzed with a laser cytoscan (Olympus, Tokyo, Japan).

**Real-time quantitative RT-PCR.** Total cellular RNA was isolated with an Isogen kit according to the manufacturer's instructions (Wako Pure Chemicals, Osaka, Japan). RNA was reverse transcribed with Superscript II reverse transcriptase (Invitrogen, Carlsbad, Calif.). Real-time PCR was carried out with an ABI 7700 instrument and SYBR green PCR master mix (Applied Biosystems, Weiterstadt, Germany), which allows real-time monitoring of the increase in PCR product concentration after every cycle based on the fluorescence of the double-stranded-DNA-specific dye SYBR green. The number of cycles required to produce a product detectable above background levels was measured for each sample and used to calculate differences (*n*-fold) in starting mRNA levels for each sample. Because we had observed that levels of β-actin and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA, which are generally used for monitoring equal loading of RNA, were rapidly downregulated in the course of apoptosis by cytokine deprivation in murine IL-3-dependent cell lines (data not shown), we used 28S rRNA as an internal control. The gene primers, selected to cross introns, are listed in Table 1. The real-time reverse transcription (RT)-PCR products were resolved on a 2% agarose gel containing ethidium bromide to confirm that only single bands of the predicted size were visible.

**RNA interference.** K562 cells were cultured in medium containing 1 μM imatinib for 24 h. Cells ($2 \times 10^6$) were then transfected with 5 μg of double-stranded Cy3-labeled Bim small interfering RNA (siRNA) or control siRNA by using a hemagglutinating virus of Japan (HVJ) envelope (GenomeONE; Ishihara Sangyo Kaisha, Osaka, Japan) according to the manufacturer's directions. Cell culture was continued in the presence of imatinib for 24 h, and then cells were harvested to isolate RNA and cell lysate. Cells were also stained with annexin V-fluorescein isothiocyanate (FITC) (Promega), followed by analysis with flow cytometry. The primers used were the following, according to Reginato et al. (42): control sense, 5′-(GGCUGUAACUUACGUGUACUU)d(TT)-3′; control antisense, 5′-(AAGUACACGUAAGUUACAGCC)d(TT)-3′; Bim sense, 5′-(GACCGAGAAGGUAGACAAUUG)d(TT)-3′; Bim antisense, 5′-(CAAUUGUCUACCUUCUCGGUC)d(TT)-3′.

**Immunoblot analysis.** Cells were solubilized in Nonidet P-40 lysis buffer (150 mM NaCl, 1.0% Nonidet P-40, 50 mM Tris [pH 8.0]) containing protease inhibitor mixture (Complete; Roche Molecular Biochemicals, Mannheim, Germany); total cellular proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Cell lysates extracted from $10^5$ living cells for hematopoietic progenitors isolated from primary culture or $10^6$ living cells for Baf-3 or cell lines established from patients with leukemia were applied to each lane. After their wet electrotransfer onto polyvinylidene difluoride membranes, the proteins were detected with the appropriate antibodies by following standard procedures. The blots were then stained with primary antibodies followed by horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin secondary antibodies and subjected to chemiluminescence detection according to the manufacturer's instructions (Amersham, Little Chalfont, Buckinghamshire, United Kingdom). Bim-specific polyclonal antibodies were raised against glutathione *S*-transferase fusion proteins containing amino acids 9 to 53 of mouse BimL, as previously described (44). Bcl-2 and Bcl-x polyclonal antibodies were purchased from Transduction Laboratories (Lexington, Ky.), a monoclonal antibody against β-actin was purchased from Chemicon (Temecula, Calif.), and polyclonal antibodies against total and phosphorylated-specific Akt and MAPK were purchased from Cell Signaling Technology (Beverly, Mass.).

**Experiments using Baf-3 cells.** Murine IL-3-dependent cells were cultured in RPMI 1640 medium containing 10% fetal calf serum, 20 mM HEPES, 50 μM 2-mercaptoethanol, and 0.5% conditioned medium of 10T1/2 cells as a source of murine IL-3. To deplete IL-3, we washed the cells twice with IL-3-free growth medium. Cell lines established from patients with leukemia were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum. Cell viability was determined by trypan blue dye exclusion. For retrovirus-mediated gene expression, we constructed a control CD8-expressing vector plasmid (pMX/IRES-CD8) from the pMX retroviral vector (a gift of T. Kitamura) (38) by inserting an internal ribosomal entry site (IRES)-CD8 cassette in which the mouse CD8 cDNA was fused in frame to the IRES sequence. The *Bcr-Abl* gene was expressed by inserting the cDNA immediately after the 5′ long terminal repeat sequence. The retrovirus was made by the method described by Onishi et al. (38), using BOSC23 cells. Retroviral infection of Baf-3 cells and the selection of CD8-positive cells with a CD8 monoclonal antibody and MACS separation columns (Miltenyi Biotec) were performed according to a method described previously (27). The selection procedure was repeated until more than 95% of the cells were positive for CD8 by flow cytometry.

**Reagents and statistical analysis.** A MAPK inhibitor, PD98059 (PD), and a PI3-K inhibitor, LY294002 (LY), were purchased from Wako Pure Chemicals and Sigma-Aldrich (St. Louis, Mo.), respectively. The 2-phenylaminopyrimidine derivative imatinib mesylate was a kind gift of Elisabeth Buchdunger (Novartis, Basel, Switzerland). An analysis of variance and the post hoc method were used to compare viable cell counts in different culture conditions. Significant differences were defined as having a $P$ value of $<0.05$.

## RESULTS

**Amplification and isolation of hematopoietic progenitors from mouse bone marrow.** We initially tested the role of Bim in the regulation of cell survival by using cytokine-dependent undifferentiated hematopoietic progenitors isolated from primary cultures of bone marrow cells from normal mice. Cells from normal littermates of the Bcr-Abl tg mice (18) were cultured for 5 days in serum-free medium containing 10 ng of TPO per ml and 50 ng of SCF per ml. After the elimination of dead cells, mature granulocytes, and cells expressing lineage-specific markers (CD4, CD8, CD11b, CD41, or Gr-1), more than 90% of the cells were negative for lineage markers and positive for c-Kit (c-Kit$^+$ Lin$^-$). These cells were further divided into Sca-1-enriched fractions (Sca-1$^+$ c-Kit$^+$ Lin$^-$; typically more than 75% of cells were positive for Sca-1 immediately after separation) and Sca-1-depleted fractions (Sca-1$^-$ c-Kit$^+$ Lin$^-$; less than 5% of cells were positive for Sca-1) by using magnetic beads coated with Sca-1 antibody. Figure 1A shows the morphology of Sca-1$^+$ c-Kit$^+$ Lin$^-$ cells. Typical yields of Sca-1-enriched and Sca-1-depleted fractions were $5 \times 10^5$ and $2 \times 10^7$ cells, respectively, pooled from 10 mice.

Cells in both fractions proliferated and differentiated into mature granulocytes or monocytes when culture was continued in medium containing TPO and SCF (Fig. 1B). Cell numbers increased by around 10-fold by 5 days and then decreased, and cultures died out 10 days later. Although peak cell numbers of the progeny of Sca-1$^+$ c-Kit$^+$ Lin$^-$ cells were always greater than those of Sca-1$^-$ c-Kit$^+$ Lin$^-$ cells, the time courses were similar. When culture was continued in the absence of cytokines, Sca-1$^+$ c-Kit$^+$ Lin$^-$ cells rapidly died within 24 h without maturation (Fig. 1C, left panel). Sca-1$^-$ c-Kit$^+$ Lin$^-$ cells also died but did so more slowly than Sca-1$^+$ c-Kit$^+$ Lin$^-$ cells, and nearly half differentiated into mature granulocytes or monocytes (Fig. 1C, right panel). To confirm that the cell death observed in these experiments was apoptotic, we performed TUNEL assays (Fig. 1D). When Sca-1$^+$ c-Kit$^+$ Lin$^-$ cells were cultured in the presence of cytokines, there was a substantial number in S phase with few TUNEL-positive cells among them (Fig. 1D, left panel). In contrast, in the absence of cytokines, cells underwent $G_0/G_1$ arrest with many TUNEL-positive cells (Fig. 1D, center and right panels). These results indicated that the cell division and survival of Sca-1-positive early hematopoietic progenitors isolated by this method were cytokine dependent.

**Upregulation of Bim and downregulation of Bcl-2 in cytokine-deprived hematopoietic progenitors.** To elucidate the contribution of Bcl-2 superfamily members to cytokine-dependent cell survival in hematopoietic progenitors, expression levels of A1, Bad, Bax, Bcl-2, Bcl-$x_L$, BimEL, Mcl-1, and DP5/Hrk mRNA were assessed by using real-time quantitative RT-PCR technology. In both Sca-1$^+$ c-Kit$^+$ Lin$^-$ and Sca-1$^-$ c-Kit$^+$ Lin$^-$ cells from normal littermates, rapid downregulation of Bcl-2 and upregulation of BimEL were consistently observed in independent experiments (Fig. 2A), while mRNA expression of other Bcl-2 superfamily members did not change significantly upon cytokine deprivation (data not shown). Although downregulation of Bcl-$x_L$ following cytokine deprivation has been observed in many cytokine-dependent cell lines, including Baf-3, FL5.12, and 32D (27, 28, 39), Bcl-$x_L$ expression in hematopoietic progenitors isolated by this method was not affected by cytokine deprivation (Fig. 2A). These findings were further supported at the protein level by immunoblot analysis (Fig. 2B); simultaneous downregulation of Bcl-2 and upregulation of BimEL were observed, while Bcl-$x_L$ remained unchanged in both Sca-1$^+$ c-Kit$^+$ Lin$^-$ and Sca-1$^-$ c-Kit$^+$ Lin$^-$ cells. Importantly, the levels of the two antiapoptotic Bcl-2 family members Bcl-$x_L$ and Bcl-2 were 5- to 10-fold lower in Sca-1$^+$ c-Kit$^+$ Lin$^-$ cells than in Sca-1$^-$ c-Kit$^+$ Lin$^-$ cells, possibly explaining the rapid apoptosis observed in the former (Fig. 1C). These results suggest that the induction

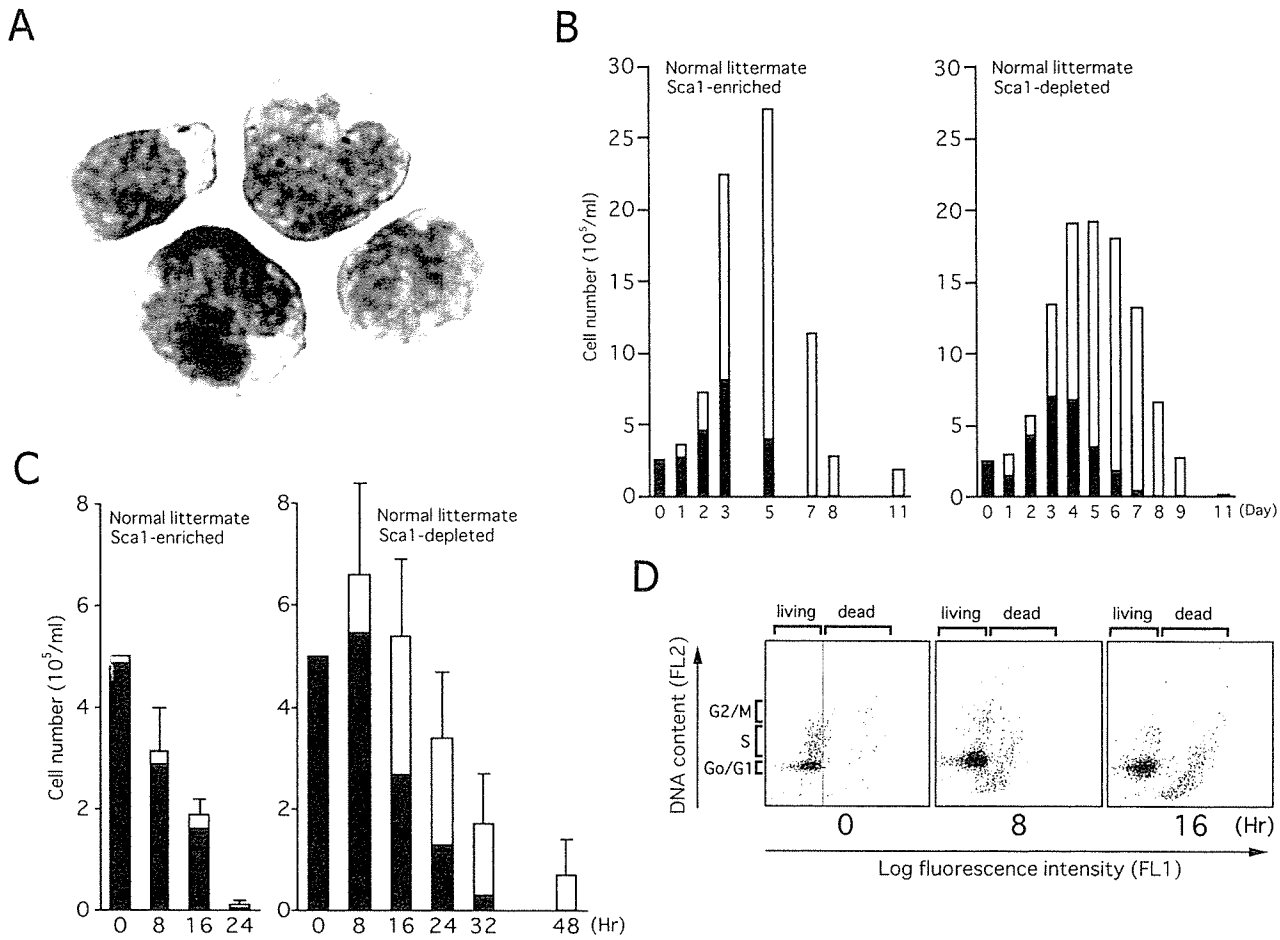


FIG. 1. Cytokine-dependent hematopoietic progenitors isolated from mouse bone marrow. (A) Cytospin preparation showing the morphology of Sca-1-positive early hematopoietic progenitors (Sca-$1^+$ c-$Kit^+$ $Lin^-$) isolated from primary cultures of mouse bone marrow cells visualized by May-Giemsa staining. (B and C) Cultures of Sca-$1^+$ c-$Kit^+$ $Lin^-$ (left panels) and Sca-$1^-$ c-$Kit^+$ $Lin^-$ cells (right panels) were continued in the presence (B) or absence (C) of SCF and TPO. The numbers of viable cells were determined by trypan blue dye exclusion. Blast cells (black bars) and terminally differentiated cells (open bars) were quantified by cytospin centrifugation. Results from one representative study (B) and the means and standard errors of results from three independent experiments (C) are shown. (D) Sca-$1^+$ c-$Kit^+$ $Lin^-$ cells were cultured in cytokine-free medium for the indicated periods. Cells were harvested, the TUNEL assay was performed with fluorescein-dUTP, and cells were stained with propidium iodide. Cytospin preparations were made and analyzed with a laser cytoscan.

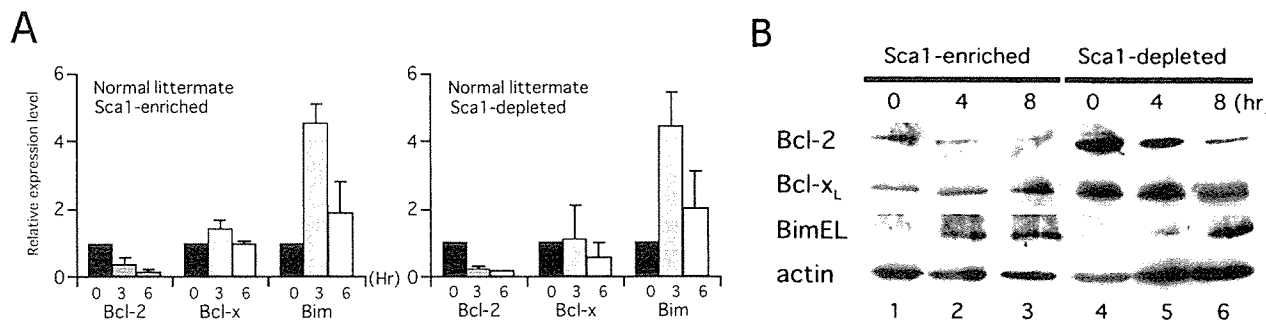


FIG. 2. Expression of Bcl-2, Bcl-$x_L$, and BimEL in Sca-$1^+$ c-$Kit^+$ $Lin^-$ and Sca-$1^-$ c-$Kit^+$ $Lin^-$ cells from normal mice. Cells were cultured in the absence of cytokines for the indicated times. (A) Real-time quantitative PCR was carried out, and the numbers of cycles required to produce a detectable product were measured and used to calculate differences (*n*-fold) in starting mRNA levels for each sample by using 28S rRNA as an internal control. mRNA expression levels in cells cultured for 0 (black bars), 3 (gray bars), and 6 (open bars) h without cytokines relative to those in cells cultured in the presence of cytokines are shown. (B) Protein expression levels of the three Bcl-2 superfamily members, as well as β-actin as a control for equal loading, were analyzed by immunoblotting with specific antibodies for each protein.

of Bim by cytokine deprivation plays an important role in regulating cell fate in Sca-1-positive early progenitors.

**Bcr-Abl reverses the upregulation of Bim by IL-3 deprivation in IL-3-dependent cells.** To test whether Bcr-Abl downregulates Bim expression, we initially used Baf-3 cells expressing Bcr-Abl. Baf-3 cells were infected with retrovirus containing Bcr-Abl and mouse CD8 cDNA as a marker (pMX-Bcr-Abl/IRES-CD8; see Materials and Methods), and infected cells were selected with magnetic beads coated with CD8 antibodies. As reported by others (11, 28), these cells proliferated in IL-3-free medium at nearly the same rate as they did in IL-3-containing medium (data not shown). As previously reported (13, 44), the simultaneous downregulation of Bcl-$x_L$ and upregulation of Bim were induced by IL-3 starvation in wild-type Baf-3 cells (Fig. 3A). In Baf-3 cells expressing Bcr-Abl, Bcl-$x_L$ expression levels were unaffected, while Bim protein was induced for 12 h after IL-3 deprivation, and then the level of Bim declined and returned to its original level within 3 days (Fig. 3B). It was also reported previously that BimEL is phosphorylated by IL-3 signaling (44), shown here by slower migrating bands (Fig. 3A, lane 1, and C, top blot). Similar slower migrating bands were observed in Baf-3 cells expressing Bcr-Abl in the absence of IL-3 [Fig. 3B and C, blots labeled Bim and Baf-3 (Bcr-Abl)], suggesting that Bcr-Abl also phosphorylates BimEL.

In wild-type Baf-3 cells, it was demonstrated previously that signals from the distal portion of the βc chain independently downregulate Bim expression through both the classical Ras/Raf/MAPK and Ras/PI3-K pathways (44). To test whether Bcr-Abl downregulates Bim expression via the same signaling pathways in this particular cell system, Baf-3 cells expressing Bcr-Abl were cultured in the absence of IL-3 for 3 days, after which they were treated with the MAPK inhibitor PD, the PI3-K inhibitor LY, or both. The effects of these inhibitors were monitored by immunoblot analysis using antibodies recognizing phosphorylated Akt (pAkt) or phosphorylated MAPK. When cells were treated with PD, phosphorylated MAPK but not pAkt decreased, and viability was slightly reduced (Fig. 3D and E). When cells were treated with LY or both PD and LY together, levels of pAkt decreased, and massive cell death occurred. Immunoblot analysis revealed a mild enhancement of Bim expression in cells treated with PD, while a marked elevation was observed in cells treated with LY or both kinase inhibitors (Fig. 3F). These results suggest that, although both Raf/MAPK and PI3-K pathways contribute to cell survival and the downregulation of Bim by Bcr-Abl kinase, PI3-K pathways are more important than Raf/MAPK pathways in this particular cell system.

**Bim expression is downregulated in cells expressing Bcr-Abl from patients with leukemia.** To gain insight into the roles of Bim in the process of human leukemogenesis, we quantified the levels of Bim and Bcl-$x_L$ proteins in cell lines established from patients in the BC phase of CML (CML/BC) and from patients with Philadelphia chromosome ($Ph^1$)-positive acute lymphoblastic leukemia (ALL) and compared them with the levels in patients with human acute myeloid leukemia (AML) and ALL cell lines that do not express the *Bcr-Abl* fusion gene. Levels of Bim in all six cell lines established from patients in CML/BC were low (Fig. 4A, lanes 1 to 6) compared with those in three control AML cell lines (Fig. 4A, lanes 7 to 9). Low levels of Bim, especially BimEL, in $Ph^1$-positive cells were also observed in five cell lines established from patients with ALL (Fig. 4B, lanes 1 to 5). In contrast, levels of Bcl-$x_L$ varied among cell lines established from patients in CML/BC and patients with AML (Fig. 4A), as expected based on results from previous studies reporting that Bcl-$x_L$ expression levels differ among AML patients (43). The levels of Bcl-$x_L$ in all ALL cell lines with or without Bcr-Abl expression seemed consistently low compared with those in AML cell lines (Fig. 4B).

To test whether the low level of Bim protein expression in $Ph^1$-positive leukemia cells was due to the potential of Bcr-Abl tyrosine kinase to downregulate it (as shown in Fig. 3B), we blocked Bcr-Abl function by using a specific inhibitor of Abl kinase, imatinib mesylate (formerly known as STI571). As previously reported (12, 24), apoptosis was induced by imatinib in five cell lines established from patients in CML/BC, and dephosphorylation of Akt and MAPK was observed in these cells (Fig. 5A and B). Increased levels of Bim proteins, especially BimEL and BimL, were induced by the addition of imatinib to KOPM30, K562, and BV173 cells (Fig. 5C). Moreover, dephosphorylation of BimEL was observed (Fig. 3C), suggesting that Bcr-Abl phosphorylates BimEL or BimL in human $Ph^1$-positive leukemia cells. Expression levels of Bcl-$x_L$ were not altered in KOPM30 and were downregulated only transiently in K562 and BV173 cells (Fig. 5C). Similar results were obtained with KOPM28 and KOPM53 (data not shown).

To examine whether the upregulation of Bim expression contributes to apoptosis induced by imatinib, K562 cells were transfected with Cy3-labeled siRNA oligonucleotides homologous to the *Bim* sequence or control siRNA (42). The induction efficiency was judged to be around 60% based on observations with fluorescence microscopy (data not shown). Real-time quantitative RT-PCR analysis using a primer set to cross the cleavage site [Bim(si)] (Table 1) revealed a 45% reduction of Bim mRNA by the Bim siRNA when whole cells were analyzed (Fig. 5D, left panel). Immunoblot analysis revealed a greater-than-60% reduction of Bim protein, while Bcl-$x_L$ analyzed as a control was reduced by less than 10% (Fig. 5D, right panel). The percentage of apoptotic cells was determined with annexin V-FITC. Cells transfected with Bim siRNA showed a significantly lower percentage of annexin V-positive cells (68.1% ± 7.3% [average ± standard deviation]) than those transfected with control siRNA (38.1% ± 9.3%) (Fig. 5E). These data suggest that Bcr-Abl supports cell survival in cell lines established from patients in CML/BC through the downregulation of Bim, although it was not clear whether the magnitude of Bim induction in these cells was sufficient to account for all of the apoptosis caused by imatinib.

**Prolonged survival of Sca-1-positive early progenitors from Bcr-Abl tg mice in cytokine-free medium.** Cell lines established with cells in CML/BC harbor additional abnormalities that develop during progression to BC and/or during adaptation to the ex vivo artificial culture environment. To test whether Bcr-Abl downregulates Bim expression in cells from patients in the chronic phase of CML, we used progenitors expressing Bcr-Abl from primary cultures of bone marrow cells obtained from Bcr-Abl tg mice that always develop CML-like myeloproliferative disease (see the introduction). Their survival and

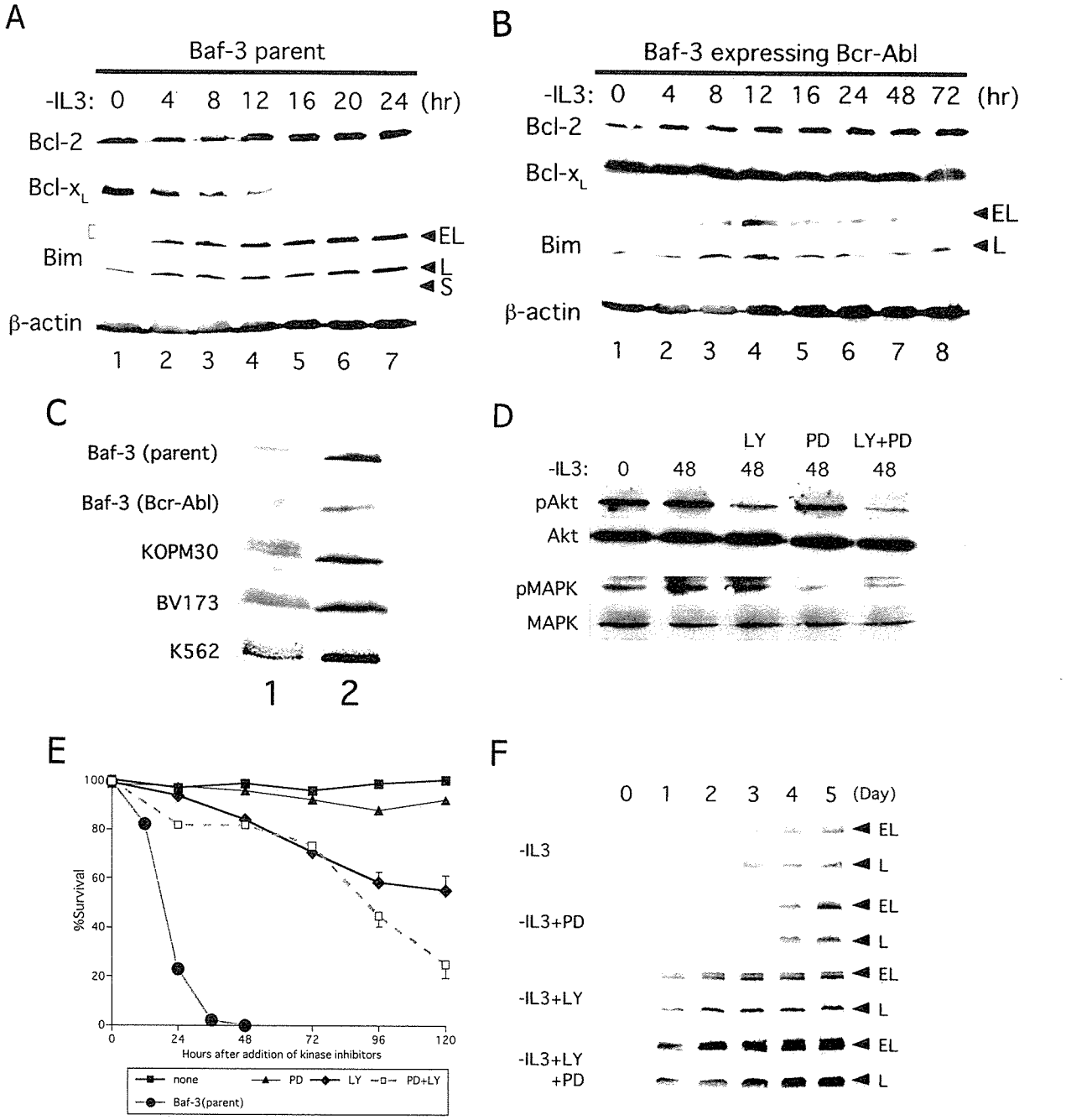


FIG. 3. Bim expression is regulated by IL-3 or Bcr-Abl through Raf/MAPK and/or PI3-K pathways in Baf-3 cells. EL, BimEL; L, BimL; S, BimS. (A and B) Expression of Bcl-2, Bcl-$x_L$, and Bim proteins in wild-type Baf-3 cells (A) and Baf-3 cells expressing Bcr-Abl after infection with a retrovirus vector (B). Cells were cultured in the absence of IL-3 for the indicated times. An immunoblot analysis using antibody specific for each protein was performed. A bracket in panel A indicates the phosphorylated forms of BimEL. (C) Phosphorylation of BimEL protein. Parental Baf-3 cells were cultured in the presence of IL-3 (lane 1) or in the absence of IL-3 (lane 2) for 4 h; IL-3-starved and Bcr-Abl expressing Baf-3, KOPM30, BV173, and K562 cells were cultured in the absence (lane 1) or presence (lane 2) of imatinib for 12 h. (D to F) Baf-3 cells expressing Bcr-Abl were cultured in IL-3-free medium for 72 h and then treated with PD, LY, or both (LY+PD) at a concentration of 50 μM for the indicated times (in hours). Immunoblot analyses using anti-phosphorylated form-specific Akt or MAPK, as well as antibodies recognizing total Akt or MAPK (D) or anti-Bim antibody (F), were performed. (E) Cell viability was determined by trypan blue dye exclusion. The survival curve of parental Baf-3 cells is shown as a control. Standard errors are shown when they were greater than 3%.

levels of Bcl-2 superfamily member expression were compared with those of their normal littermates.

Sca-$1^+$ c-$Kit^+$ $Lin^-$ and Sca-$1^-$ c-$Kit^+$ $Lin^-$ cells were isolated from primary cultures of bone marrow cells from the tg mice. When these cells were cultured in the presence of cytokines, they proliferated with kinetics similar to those of cells from the normal littermates of the tg mice (Fig. 6A), although cell numbers from the tg mice were greater than those from

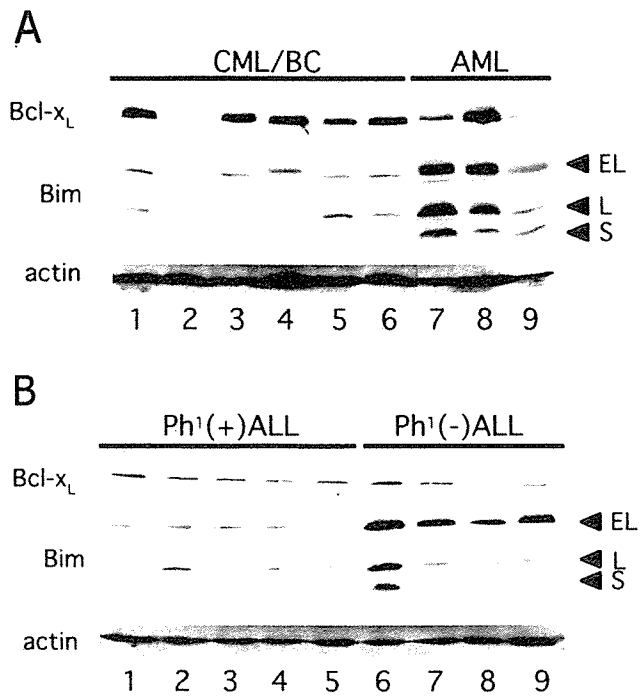


FIG. 4. Levels of Bcl-$x_L$ and Bim protein in human leukemia cell lines. Immunoblot analysis using antibody specific for each protein was performed. EL, BimEL; L, BimL; S, BimS. (A) Lanes 1 to 6, the KOPM28, KOPM30, KOPM53, K562, BV173, and KU812 cell lines, respectively, established with CML/BC cells; lanes 7 to 9, the HL60 myeloid leukemia, HEL erythroid leukemia, and U937 monocytic leukemia cell lines, respectively, lacking $Ph^1$. (B) Lanes 1 to 5, the KOPN-55bi, KOPN-57bi, KOPN-66bi, KOPN-72bi, and KOPN-30bi $Ph^1$-positive pro-B ALL cell lines, respectively; lanes 6 to 9, the 920, 697, RS4;11, and UOC-B1 pro-B ALL cell lines, respectively, lacking $Ph^1$.

their normal littermates in both fractions (Fig. 1B). When Sca-$1^+$ c-$Kit^+$ $Lin^-$ cells were cultured in cytokine-free medium, they survived for more than 3 days (Fig. 6B, left panel), much longer than those from normal littermates (Fig. 1C, left panel). Indeed, virtually no viable cells from normal littermates were observed 24 h after cytokine deprivation, while cells with immature and mature morphology from Bcr-Abl tg mice survived even after 48 h in repeated experiments. In contrast, Sca-$1^-$ c-$Kit^+$ $Lin^-$ cells underwent apoptosis with kinetics similar to those of cells from the normal littermates (Fig. 1C, right panel). To confirm that Bcr-Abl prolongs the survival of Sca-1-positive early hematopoietic progenitors in cytokine-free medium, we added 1 μM imatinib to the culture medium. Imatinib did not affect the survival of the Sca-$1^-$ c-$Kit^+$ $Lin^-$ cells (Fig. 6C, middle panel). In contrast, Sca-$1^+$ c-$Kit^+$ $Lin^-$ cells rapidly underwent apoptosis (Fig. 6C, left panel). A comparison of the numbers of living cells with those of Sca-$1^+$ c-$Kit^+$ $Lin^-$ cells from normal mice (Fig. 1C) revealed that imatinib seemed not only to reverse the antiapoptotic effects of Bcr-Abl but even to enhance apoptosis at 8 and 16 h. This finding might be explained in part by the inhibitory effects of imatinib against c-Kit function, which could persist after the removal of SCF because imatinib also induced apoptosis in Sca-$1^+$ c-$Kit^+$ $Lin^-$ cells from normal mice at 8 h (not statistically significant; $P = 0.14$) and 16 h ($P < 0.05$) after the removal of the cytokines (Fig. 6C, right panel). These data suggest that Bcr-Abl protects Sca-1-positive early progenitors, but not Sca-$1^-$ c-$Kit^+$ $Lin^-$ cells, from apoptosis caused by cytokine deprivation. Bcr-Abl mRNA expression was detected by RT-PCR in both fractions (data not shown).

**Suppression of Bim induction in cytokine-deprived progenitors by Bcr-Abl.** To clarify the mechanism through which Bcr-Abl prolongs survival of Sca-1-positive progenitors, we assessed the expression of Bcl-2 superfamily members in these cells. Real-time quantitative RT-PCR revealed that neither Bcl-2, Bcl-$x_L$, nor Bim mRNA expression was altered by cytokine deprivation in either Sca-1-positive or Sca-1-negative cells, except that Bim mRNA was induced twofold in Sca-$1^-$ c-$Kit^+$ $Lin^-$ cells (Fig. 7A). These data were confirmed at the protein level by immunoblot analysis. Bcl-2 and Bcl-$x_L$ levels were unchanged by cytokine deprivation, while Bim protein was barely detectable in either fraction (Fig. 7B), in contrast to a clear induction of Bim and downregulation of Bcl-2 in progenitors from normal littermates (Fig. 2B).

To further confirm that Bcr-Abl downregulates Bim, we analyzed the expression of the Bcl-2 superfamily members in cells cultured in cytokine-free medium in the presence of imatinib. Bim was markedly induced, while Bcl-2 was downregulated in Sca-1-positive and -negative progenitors isolated from Bcr-Abl tg mice (Fig. 7C, left and center panels). In contrast, induction levels of Bim in Sca-1-positive progenitors isolated from normal littermates were not changed by treatment with imatinib (Fig. 7C, right panel, and 2A, left panel), suggesting that the reduction of viable cells in progenitors from normal littermates by imatinib (Fig. 6C, right panel) was due to mechanisms other than Bim induction. Taken together, these data indicate that Bcr-Abl reverses the downregulation of Bcl-2 and upregulation of Bim that are observed in cytokine-starved normal hematopoietic progenitors.

## DISCUSSION

In earlier studies, it was established that the induction of Bim is an important step in Baf-3 cells undergoing apoptosis due to IL-3 deprivation (44). Here we demonstrate that Bim was induced by cytokine starvation in early hematopoietic progenitors (Sca-$1^+$ c-$Kit^+$ $Lin^-$) isolated by primary short-term culture of bone marrow cells from normal mice. In contrast, Bim was not induced by cytokine starvation in early progenitors from CML model mice that were more resistant to apoptosis than those from normal mice. We also found that Bcr-Abl downregulates Bim expression in Baf-3 cells and cell lines established with cells from patients with $Ph^1$-positive leukemia, suggesting that Bim is one of the key target factors downstream of Bcr-Abl that render CML progenitors resistant to apoptosis caused by cytokine deprivation.

The function of Bim is reported to be regulated by at least four different mechanisms. First, mRNA expression is downregulated by cytokines in mouse IL-3-dependent Baf-3, FL5.12, and 32D cells through the Ras/MAPK and PI3-K pathways, independently (13, 44). Moreover, NGF suppresses Bim mRNA expression through the inactivation of the c-jun $NH_2$-terminal kinase in NGF-dependent neuronal cells, including primary cultures of rat sympathetic neurons and neuronally differentiated PC-12 cells (5, 40, 49). In addition, serum depri-

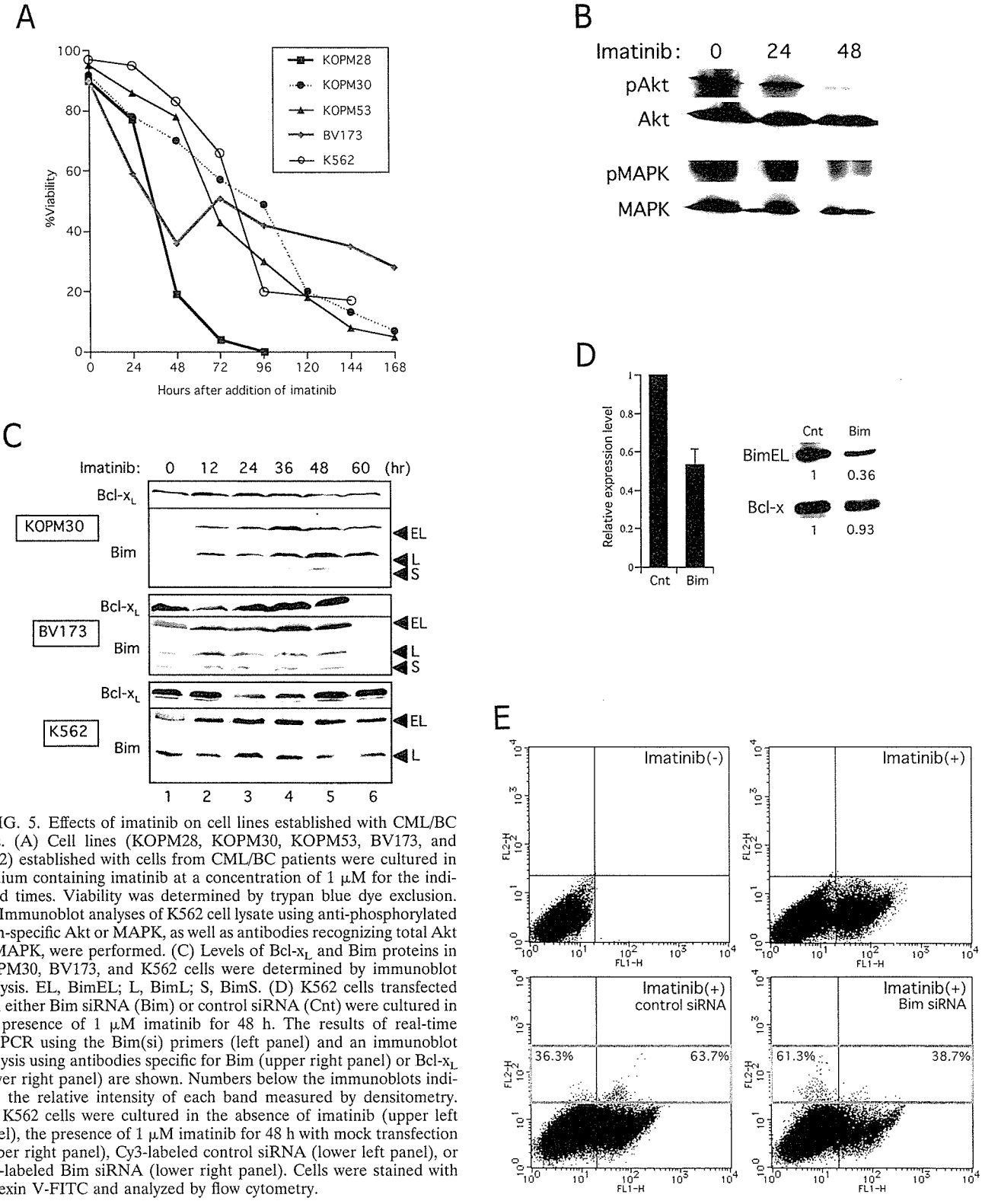


FIG. 5. Effects of imatinib on cell lines established with CML/BC cells. (A) Cell lines (KOPM28, KOPM30, KOPM53, BV173, and K562) established with cells from CML/BC patients were cultured in medium containing imatinib at a concentration of 1 μM for the indicated times. Viability was determined by trypan blue dye exclusion. (B) Immunoblot analyses of K562 cell lysate using anti-phosphorylated form-specific Akt or MAPK, as well as antibodies recognizing total Akt or MAPK, were performed. (C) Levels of Bcl-$x_L$ and Bim proteins in KOPM30, BV173, and K562 cells were determined by immunoblot analysis. EL, BimEL; L, BimL; S, BimS. (D) K562 cells transfected with either Bim siRNA (Bim) or control siRNA (Cnt) were cultured in the presence of 1 μM imatinib for 48 h. The results of real-time RT-PCR using the Bim(si) primers (left panel) and an immunoblot analysis using antibodies specific for Bim (upper right panel) or Bcl-$x_L$ (lower right panel) are shown. Numbers below the immunoblots indicate the relative intensity of each band measured by densitometry. (E) K562 cells were cultured in the absence of imatinib (upper left panel), the presence of 1 μM imatinib for 48 h with mock transfection (upper right panel), Cy3-labeled control siRNA (lower left panel), or Cy3-labeled Bim siRNA (lower right panel). Cells were stained with annexin V-FITC and analyzed by flow cytometry.

vation of CC139 fibroblasts upregulates Bim mRNA via the classical MEK/extracellular signal-regulated kinase (ERK) pathway (48). Second, subcellular localization of Bim is controlled by IL-3 in FDC-P1 cells, another mouse IL-3-dependent line, and by exposure to UV light in 293 cells (29, 41). BimEL and BimL, but not BimS, form complexes with an 8,000-molecular-weight dynein light chain, LC8 (also PIN or Dlc-1) (10, 21, 25). The Bim/LC8 complex in the presence of

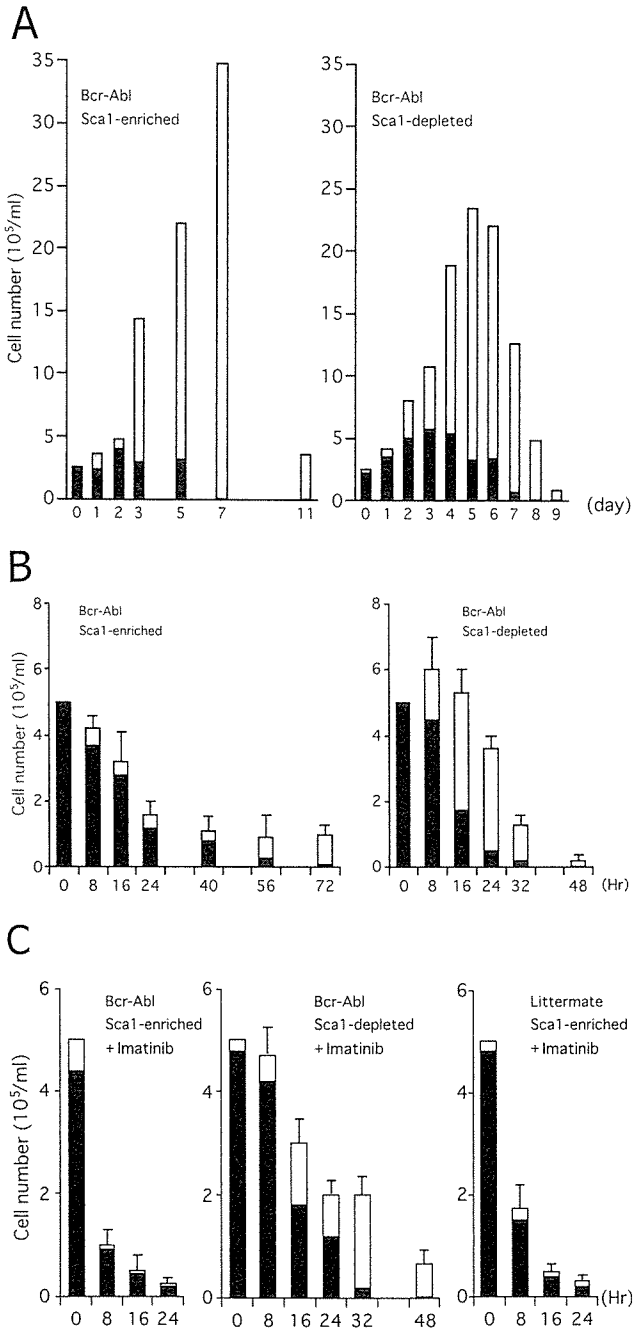


FIG. 6. (A and B) Sca-$1^+$ c-$Kit^+$ $Lin^-$ cells (left panel) and Sca-$1^-$ c-$Kit^+$ $Lin^-$ cells (right panel) amplified and isolated by primary cultures of bone marrow cells from Bcr-Abl tg mice were cultured in the presence (A) or absence (B) of SCF and TPO. (C) Sca-$1^+$ c-$Kit^+$ $Lin^-$ cells (left and right panels) or Sca-$1^-$ c-$Kit^+$ $Lin^-$ cells (middle panel) amplified and isolated by primary cultures of bone marrow cells from Bcr-Abl tg mice (left and middle panels) or their normal littermates (right panel) were cultured in the absence of SCF and TPO. Imatinib was added at a concentration of 1 μM. The number of viable cells was determined by trypan blue dye exclusion. Blast cells (black bars) and terminally differentiated cells (open bars) were determined by cytospin centrifugation. The results from one representative study (A) or the means + standard errors of results from three independent experiments (B and C) are shown.

IL-3 binds to the intermediate chain of the dynein motor complex on the microtubules. IL-3 withdrawal releases the complex from sequestration in the cytoplasm by mechanisms not yet fully understood. In the case of 293 cells exposed to UV, phosphorylation at Thr-56 of (human) BimL by activated c-jun $NH_2$-terminal kinase was reported to play an important role in this process (29). Third, NGF phosphorylates BimEL and BimL but not BimS through the MEK/MAPK pathway in neuronally differentiated PC-12 cells. Phosphorylation of (rat) BimEL at Ser-109 and Thr-110, which are adjacent to but distinct from the phosphorylation residues in 293 cells exposed to UV as mentioned above, was reported to suppress the proapoptotic function of BimEL without affecting its binding potential to LC8 or its subcellular localization (5). Fourth, proteasome-dependent degradation is involved in the regulation of Bim expression in serum-deprived fibroblasts and macrophage colony-stimulating factor-dependent osteoclasts (2, 31). These somewhat confusing results suggest that the functions of BimEL and BimL on the one hand and BimS on the other may be regulated in different ways in certain situations and that the relative importance of these four mechanisms may differ between cell types. Indeed, it has been found that the enforced expression of either BimL or BimS readily induced apoptosis in Baf-3 and 293 cells, in contrast with five glioma cell lines, in which a massive amount of BimL did not induce apoptosis, in spite of the fact that a much lower amount of BimS easily killed these cells (44, 50).

In this paper, we demonstrated that Bim is downregulated at both the mRNA and protein levels by cytokines in hematopoietic progenitors isolated from primary cultures of bone marrow cells (Fig. 2). This finding indicates that the regulation of mRNA expression is the major mechanism for controlling Bim function in early hematopoiesis. We also demonstrated that Bcr-Abl reverses the induction of Bim mRNA caused by cytokine deprivation in these progenitors (Fig. 7). Among several pathways that are reported to regulate Bim mRNA, those involved in PI3-K are most likely the major pathways for the downregulation of Bim by Bcr-Abl (Fig. 3F). In addition, phosphorylation of Bim (as with the third mechanism mentioned in the previous paragraph) might contribute to the survival of hematopoietic cells. It was previously reported that BimEL and BimL are phosphorylated in Baf-3 cells by IL-3 signaling via the same pathways that control the expression of Bim, i.e., the Ras/Raf/MAPK and Ras/PI3-K pathways (44). In this study, although the phosphorylation of BimEL in early progenitors may not be convincing (Fig. 2B), it was clearly detected in Baf-3 cells expressing Bcr-Abl cultured in IL-3-free medium and in cell lines established from patients in CML/BC cultured in the absence of imatinib (Fig. 3C). We consider it unlikely that phosphorylation plays a major role in cytokine-deprived Baf-3 cells, because cells expressing hyperphosphorylated BimEL or BimL in the presence of IL-3 still underwent apoptosis (44). However, the possibility that phosphorylation of BimEL and BimL by cytokines or Bcr-Abl contributes to cell survival to some extent in early hematopoietic progenitors or leukemic cells cannot be excluded.

There are substantial differences between early (Sca-$1^+$ c-$Kit^+$ $Lin^-$) and late (Sca-$1^-$ c-$Kit^+$ $Lin^-$) progenitors in cytokine dependence and the effects of Bcr-Abl kinase. Early progenitors undergo rapid apoptosis without maturation in the

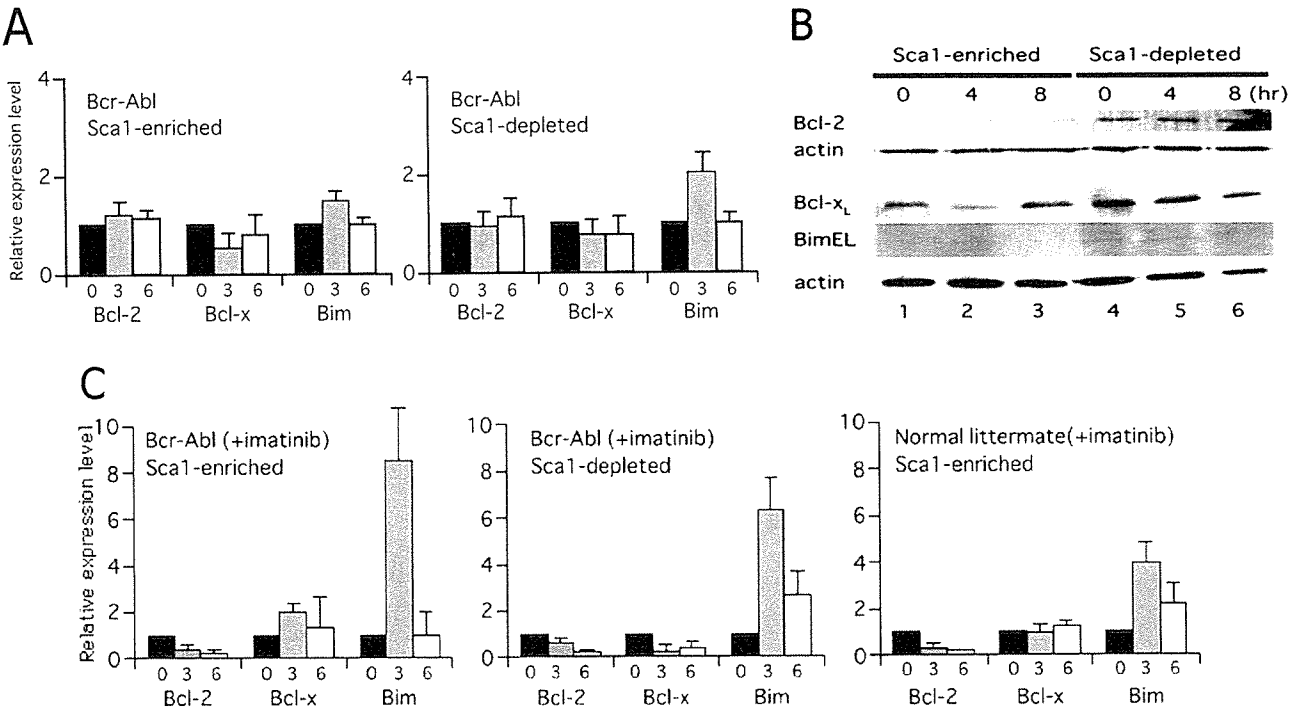


FIG. 7. Expression of Bcl-2, Bcl-$x_L$, and BimEL in Sca-1$^+$ c-Kit$^+$ Lin$^-$ and Sca-1$^-$ c-Kit$^+$ Lin$^-$ cells from Bcr-Abl tg mice and their normal littermates. Cells were cultured in cytokine-free medium in the absence (A and B) or presence (C) of imatinib at a concentration of 1 μM for the indicated times. (A and C) Real-time quantitative PCR was carried out, and the numbers of cycles required to produce a detectable product were measured and used to calculate the differences (*n*-fold) in starting mRNA levels for each sample by using 28S rRNA as an internal control. Levels of mRNA in cells cultured for 0 (black bars), 3 (gray bars), and 6 (open bars) h without cytokines relative to those in cells in the presence of cytokines are shown. (B) Levels of three Bcl-2 superfamily members, as well as β-actin proteins, as a control for equal loading were detected by specific antibodies.

absence of cytokines (Fig. 1C). This could be explained at least partially by relatively low levels of Bcl-2 and Bcl-$x_L$ expression (Fig. 2B), because it is generally accepted that cell fate is determined by the balance between pro- and antiapoptotic members of the Bcl-2 superfamily (1). Although Bcl-2 levels were downregulated by cytokine deprivation in early progenitors, this downregulation is unlikely to be the major cause of rapid apoptosis, because levels of Bcl-2 in the presence of cytokines are very low and Bcl-2-deficient mice did not show apparent abnormalities in myeloid hematopoiesis (34, 47). Thus, Bim is considered to be the major determinant of cell fate in Sca-1$^+$ c-Kit$^+$ Lin$^-$ cells, and downregulation of Bim in cytokine-deprived Sca-1-positive early progenitors isolated from Bcr-Abl tg mice may result in longer survival (Fig. 6B). On the other hand, Bim may not be the major determinant of cell fate in Sca-1$^-$ c-Kit$^+$ Lin$^-$ cells, which express Bcl-2 and Bcl-$x_L$ at high levels (Fig. 2B). Moreover, half of these cells can differentiate before undergoing apoptosis (Fig. 1C). Additionally, late progenitors from the tg mice show virtually the same time course as those from normal littermates in the absence of cytokines (Fig. 1C and 6B, right panels) in spite of the fact that there was little induction of Bim in these cells, similar to Sca-1-positive early progenitors (Fig. 7A and B).

Many reports have maintained that the growth and survival characteristics of CML progenitors in the chronic phase are similar to those in healthy bone marrow (reviewed in references 15 and 23). In spite of prominent antiapoptotic effects of Bcr-Abl in cytokine-dependent cell lines such as Baf-3, resistance of CML progenitors to cytokine deprivation is controversial. Bedi et al. reported that CD34-positive CML progenitors live longer in serum- and cytokine-free medium than CML progenitors treated with Bcr-Abl junction-specific antisense oligonucleotides and normal CD34-positive cells (4), but Amos et al. did not observe a survival advantage of CML progenitors under cytokine-free conditions (3). In the present study, we isolated early hematopoietic progenitors by using Sca-1, which is one of the most reliable markers for early progenitors, including stem cells, but is available only for the study of mouse hematopoiesis. Another advantage of our study is the use of progenitors isolated from young mice (8 to 12 weeks of age) whose peripheral blood and bone marrow are still indistinguishable from those of their normal littermates (18). Taking these advantages, we revealed a relatively small but distinct difference in apoptosis due to cytokine deprivation between normal and Bcr-Abl-expressing progenitors that correlated to the expression levels of Bim (Fig. 1, 2, 6, and 7). Moreover, Bim was induced by imatinib in CML cell lines undergoing apoptosis (Fig. 5C), and siRNA that reduced the expression level of Bim effectively rescued these cells (Fig. 5E). Taken together, these results suggest that Bim is an important downstream target that supports cell survival of Bcr-Abl-expressing hematopoietic cells. Further studies are necessary to clarify whether downregulation of Bim by Bcr-Abl contributes to the massive expansion of myeloid cells observed in CML.

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## REFERENCES

1. **Adams, J. M., and S. Cory.** 1998. The Bcl-2 protein family: arbiters of cell survival. Science **281:**1322–1326.
2. **Akiyama, T., P. Bouillet, T. Miyazaki, Y. Kadono, H. Chikuda, U. I. Chung, A. Fukuda, A. Hikita, H. Seto, T. Okada, T. Inaba, A. Sanjay, R. Baron, H. Kawaguchi, H. Oda, K. Nakamura, A. Strasser, and S. Tanaka.** 2003. Regulation of osteoclast apoptosis by ubiquitylation of proapoptotic BH3-only Bcl-2 family member Bim. EMBO J. **22:**6653–6664.
3. **Amos, T. A., J. L. Lewis, F. H. Grand, R. P. Gooding, J. M. Goldman, and M. Y. Gordon.** 1995. Apoptosis in chronic myeloid leukaemia: normal responses by progenitor cells to growth factor deprivation, X-irradiation and glucocorticoids. Br. J. Haematol. **91:**387–393.
4. **Bedi, A., B. A. Zehnbauer, J. P. Barber, S. J. Sharkis, and R. J. Jones.** 1994. Inhibition of apoptosis by BCR-ABL in chronic myeloid leukemia. Blood **83:**2038–2044.
5. **Biswas, S. C., and L. A. Greene.** 2002. Nerve growth factor (NGF) down-regulates the Bcl-2 homology 3 (BH3) domain-only protein Bim and suppresses its proapoptotic activity by phosphorylation. J. Biol. Chem. **277:** 49511–49516.
6. **Bouillet, P., D. Metcalf, D. C. S. Huang, D. M. Tarlinton, T. W. H. Kay, F. Koentgen, J. M. Adams, and A. Strasser.** 1999. Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity. Science **286:**1735–1738.
7. **Chao, D. T., and S. J. Korsmeyer.** 1998. BCL-2 family: regulators of cell death. Annu. Rev. Immunol. **16:**395–419.
8. **Cortez, D., L. Kadlec, and A. M. Pendergast.** 1995. Structural and signaling requirements for BCR-ABL-mediated transformation and inhibition of apoptosis. Mol. Cell. Biol. **15:**5531–5541.
9. **Cortez, D., G. Stoica, J. H. Pierce, and A. M. Pendergast.** 1996. The BCR-ABL tyrosine kinase inhibits apoptosis by activating a Ras-dependent signaling pathway. Oncogene **13:**2589–2594.
10. **Crépieux, P., H. Kwon, N. Leclerc, W. Spencer, S. Richard, R. Lin, and J. Hiscott.** 1997. IκBα physically interacts with a cytoskeleton-associated protein through its signal response domain. Mol. Cell. Biol. **17:**7375–7385.
11. **Daley, G. Q., and D. Baltimore.** 1988. Transformation of an interleukin 3-dependent hematopoietic cell line by the chronic myelogenous leukemia-specific P210bcr/abl protein. Proc. Natl. Acad. Sci. USA **85:**9312–9316.
12. **Deininger, M. W., J. M. Goldman, N. Lydon, and J. V. Melo.** 1997. The tyrosine kinase inhibitor CGP57148B selectively inhibits the growth of BCR-ABL-positive cells. Blood **90:**3691–3698.
13. **Dijkers, P. F., R. H. Medema, J.-W. J. Lammers, L. Koenderman, and P. J. Coffer.** 2000. Expression of the pro-apoptotic Bcl-2 family member Bim is regulated by the forkhead transcription factor FKHR-L1. Curr. Biol. **10:** 1201–1204.
14. **Dumon, S., S. C. Santos, F. Debierre-Grockiego, V. Gouilleux-Gruart, L. Cocault, C. Boucheron, P. S Mollat, S. Gisselbrecht, and F. Gouilleux.** 1999. IL-3 dependent regulation of Bcl-$x_L$ gene expression by STAT5 in a bone marrow derived cell line. Oncogene **18:**4191–4199.
15. **Holyoake, D. T.** 2001. Recent advances in the molecular and cellular biology of chronic myeloid leukaemia: lessons to be learned from the laboratory. Br. J. Haematol. **113:**11–23.
16. **Honda, H., Y. Yamashita, K. Ozawa, and H. Mano.** 1996. Cloning and characterization of mouse tec promoter. Biochem. Biophys. Res. Commun. **223:**422–426.
17. **Honda, H., K. Ozawa, Y. Yazaki, and H. Hirai.** 1997. Identification of PU.1 and Sp1 as essential transcriptional factors for the promoter activity of mouse tec gene. Biochem. Biophys. Res. Commun. **234:**376–381.
18. **Honda, H., H. Oda, T. Suzuki, T. Takahashi, O. N. Witte, K. Ozawa, T. Ishikawa, Y. Yazaki, and H. Hirai.** 1998. Development of acute lymphoblastic leukemia and myeloproliferative disorder in transgenic mice expressing p210bcr/abl: a novel transgenic model for human Ph1-positive leukemias. Blood **91:**2067–2075.
19. **Honda, H., T. Ushijima, K. Wakazono, H. Oda, Y. Tanaka, S. Aizawa, T. Ishikawa, Y. Yazaki, and H. Hirai.** 2000. Acquired loss of p53 induces blastic transformation in p210(bcr/abl)-expressing hematopoietic cells: a transgenic study for blast crisis of human CML. Blood **95:**1144–1150.
20. **Hsu, S. Y., P. Lin, and A. J. Hsueh.** 1998. BOD (Bcl-2-related ovarian death gene) is an ovarian BH3 domain-containing proapoptotic Bcl-2 protein capable of dimerization with diverse antiapoptotic Bcl-2 members. Mol. Endocrinol. **12:**1432–1440.
21. **Jaffrey, S. R., and S. H. Snyder.** 1996. PIN: an associated protein inhibitor of neuronal nitric oxide synthase. Science **274:**774–777.
22. **Kabarowski, J. H., P. B. Allen, and L. M. Wiedemann.** 1994. A temperature sensitive p210 BCR-ABL mutant defines the primary consequences of BCR-ABL tyrosine kinase expression in growth factor dependent cells. EMBO J. **13:**5887–5895.
23. **Kabarowski, J. H., and O. N. Witte.** 2000. Consequences of BCR-ABL expression within the hematopoietic stem cell in chronic myeloid leukemia. Stem Cells **18:**399–408.
24. **Kawauchi, K., T. Ogasawara, M. Yasuyama, and S. Ohkawa.** 2003. Involvement of Akt kinase in the action of STI571 on chronic myelogenous leukemia cells. Blood Cells Mol. Dis. **31:**11–17.
25. **King, S. M., E. Barbarese, J. F. Dillman III, R. S. Patel-King, H. Carson, and K. K. Pfister.** 1996. Brain cytoplasmic and flagellar outer arm dyneins share a highly conserved $M_r$ 8,000 light chain. J. Biol. Chem. **271:**19358–19366.
26. **Kinoshita, T., T. Yokota, K. Arai, and A. Miyajima.** 1995. Suppression of apoptotic death in hematopoietic cells by signaling through the IL-3/GM-CSF receptors. EMBO J. **14:**266–275.
27. **Kuribara, R., T. Kinoshita, A. Miyajima, T. Shinjyo, T. Yoshihara, T. Inukai, K. Ozawa, A. T. Look, and T. Inaba.** 1999. Two distinct interleukin-3-mediated signal pathways, Ras-NFIL3 (E4BP4) and Bcl-$x_L$, regulate the survival of murine pro-B lymphocytes. Mol. Cell. Biol. **19:**2754–2762.
28. **Laneuville, P., N. Heisterkamp, and J. Groffen.** 1991. Expression of the chronic myelogenous leukemia-associated p210bcr/abl oncoprotein in a murine IL-3 dependent myeloid cell line. Oncogene **6:**275–282.
29. **Lei, K., and R. J. Davis.** 2003. JNK phosphorylation of Bim-related members of the Bcl2 family induces Bax-dependent apoptosis. Proc. Natl. Acad. Sci. USA **100:**2432–2437.
30. **Leverrier, Y., J. Thomas, G. R. Perkins, M. Mangeney, M. K. L. Collins, and J. Marvel.** 1997. In bone marrow derived Baf-3 cells, inhibition of apoptosis by IL-3 is mediated by two independent pathways. Oncogene **14:**425–430.
31. **Ley, R., K. Balmanno, K. Hadfield, C. R. Weston, and S. J. Cook.** 2003. Activation of the ERK1/2 signaling pathway promotes phosphorylation and proteasome-dependent degradation of the BH3-only protein, Bim. J. Biol. Chem. **278:**18811–18816.
32. **Mandanas, R. A., H. S. Boswell, L. Lu, and D. Leibowitz.** 1992. BCR/ABL confers growth factor independence upon a murine myeloid cell line. Leukemia **6:**796–800.
33. **Mano, H., F. Ishikawa, J. Nishida, H. Hirai, and F. Takaku.** 1990. A novel protein-tyrosine kinase, tec, is preferentially expressed in liver. Oncogene **5:**1781–1786.
34. **Matsuzaki, Y., K. Nakayama, K. Nakayama, T. Tomita, M. Isoda, D. Y. Loh, and H. Nakauchi.** 1997. Role of bcl-2 in the development of lymphoid cells from the hematopoietic stem cell. Blood **89:**853–862.
35. **Miyajima, A., Y. Ito, and T. Kinoshita.** 1999. Cytokine signaling for proliferation, survival, and death in hematopoietic cells. Int. J. Hematol. **69:**137–146.
36. **O'Connor, L., A. Strasser, L. A. O'Reilly, G. Hausmann, J. M. Adams, S. Cory, and D. C. Huang.** 1998. Bim: a novel member of the Bcl-2 family that promotes apoptosis. EMBO J. **17:**384–395.
37. **Okabe, M., Y. Uehara, T. Miyagishima, T. Itaya, M. Tanaka, Y. Kuni-Eda, M. Kurosawa, and T. Miyazaki.** 1992. Effect of herbimycin A, an antagonist of tyrosine kinase, on bcr/abl oncoprotein-associated cell proliferations: abrogative effect on the transformation of murine hematopoietic cells by transfection of a retroviral vector expressing oncoprotein P210bcr/abl and preferential inhibition on Ph1-positive leukemia cell growth. Blood **80:**1330–1338.
38. **Onishi, M., S. Kinoshita, Y. Morikawa, A. Shibuya, J. Phillips, L. L. Lanier, D. M. Gorman, G. P. Nolan, A. Miyajima, and T. Kitamura.** 1996. Application of retrovirus-mediated expression cloning. Exp. Hematol. **24:**324–329.
39. **Packham, G., E. L. White, C. M. Eischen, H. Yang, E. Parganas, J. N. Ihle, D. A. Grillot, G. P. Zambetti, G. Nunez, and J. L. Cleveland.** 1998. Selective regulation of Bcl-XL by a Jak kinase-dependent pathway is bypassed in murine hematopoietic malignancies. Genes Dev. **12:**2475–2487.
40. **Putcha, G. V., K. L. Moulder, J. P. Golden, P. Bouillet, J. A. Adams, A. Strasser, and E. M. Johnson.** 2001. Induction of BIM, a proapoptotic BH3-only BCL-2 family member, is critical for neuronal apoptosis. Neuron **29:** 615–628.
41. **Puthalakath, H., D. C. Huang, L. A. O'Reilly, S. M. King, and A. Strasser.** 1999. The proapoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex. Mol. Cell **3:**287–296.
42. **Reginato, M. J., K. R. Mills, J. K. Paulus, D. K. Lynch, D. C. Sgroi, J. Debnath, S. K. Muthuswamy, and J. S. Brugge.** 2003. Integrins and EGFR coordinately regulate the pro-apoptotic protein Bim to prevent anoikis. Nat. Cell Biol. **5:**733–740.
43. **Schaich, M., T. Illmer, G. Seitz, B. Mohr, U. Schakel, J. F. Beck, and G. Ehninger.** 2001. The prognostic value of Bcl-$x_L$ gene expression for remission induction is influenced by cytogenetics in adult acute myeloid leukemia. Haematologica **86:**470–477.
44. **Shinjyo, T., R. Kuribara, T. Inukai, H. Hosoi, T. Kinoshita, A. Miyajima, P. J. Houghton, A. T. Look, K. Ozawa, and T. Inaba.** 2001. Downregulation of Bim, a proapoptotic relative of Bcl-2, is a pivotal step in cytokine-initiated

survival signaling in murine hematopoietic progenitors. Mol. Cell. Biol. **21:** 854–864.

45. **Silva, M., A. Benito, C. Sanz, F. Prosper, D. Ekhterae, G. Nunez, and J. L. Fernandez-Luna.** 1999. Erythropoietin can induce the expression of bcl-x(L) through Stat5 in erythropoietin-dependent progenitor cell lines. J. Biol. Chem. **274:**22165–22169.
46. **Socolovsky, M., A. E. Fallon, S. Wang, C. Brugnara, and H. F. Lodish.** 1999. Fetal anemia and apoptosis of red cell progenitors in Stat5a$^{-/-}$5b$^{-/-}$ mice: a direct role for Stat5 in Bcl-X(L) induction. Cell **98:**181–191.
47. **Veis, D. J., C. M. Sorenson, J. R. Shutter, and S. J. Korsmeyer.** 1993. Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. Cell **75:**229–240.
48. **Weston, C. R., K. Balmanno, C. Chalmers, K. Hadfield, S. A. Molton, R. Ley, E. F. Wagner, and S. J. Cook.** 2003. Activation of ERK1/2 by deltaRaf-1:ER* represses Bim expression independently of the JNK or PI3K pathways. Oncogene **22:**1281–1293.
49. **Whitfield, J., S. J. Neame, L. Paquet, O. Bernard, and J. Ham.** 2001. Dominant-negative c-Jun promotes neuronal survival by reducing BIM expression and inhibiting mitochondrial cytochrome c release. Neuron **29:**629–643.
50. **Yamaguchi, T., T. Okada, T. Takeuchi, T. Tonda, M. Ohtaki, S. Shinoda, T. Masuzawa, K. Ozawa, and T. Inaba.** 2003. Enhancement of thymidine kinase-mediated killing of malignant glioma by BimS, a BH3-only cell death activator. Gene Ther. **10:**375–385.