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Tyrosine Kinase Oncogenes Abrogate Interleukin-3 Dependence of Murine Myeloid Cells through Signaling Pathways Involving c-*myc*: Conditional Regulation of c-*myc* Transcription by Temperature-Sensitive v-*abl*

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Retroviral expression vectors carrying the tyrosine kinase oncogenes *abl*, *fms*, *src*, and *trk* abrogate the requirements of murine myeloid FDC-P1 cells for interleukin-3 (IL-3). Factor-independent clones constitutively express c-*myc* in the absence of IL-3, whereas in parental cultures c-*myc* transcription requires the presence of the ligand. To directly test the effect of a tyrosine kinase oncogene on c-*myc* expression, retroviral constructs containing three different temperature-sensitive mutants of v-*abl* were introduced into myeloid IL-3-dependent FDC-P1 and 32D cells. At the permissive temperature, clones expressing temperature-sensitive *abl* behaved like wild-type *abl*-containing cells in their growth properties and expressed c-*myc* constitutively. Temperature shift experiments demonstrated that both IL-3 abrogation and the regulation of c-*myc* expression correlated with the presence of functional v-*abl*. Induction of c-*myc* expression by reactivation of temperature-sensitive v-*abl* mimicked c-*myc* induction by IL-3 in that it did not require protein synthesis and occurred at the level of transcription, with effects on both initiation and a transcription elongation block. However, v-*abl*-regulated FDC-P1 cell growth differed from IL-3-regulated growth in that c-*fos* and *junB*, which are normally induced by IL-3, were not induced by activation of v-*abl*.

An important achievement of molecular oncology is the finding that oncogenes are activated versions of components of growth factor signal transduction pathways. This connection became apparent through the molecular cloning of cellular homologs that function at various points in signal transduction from the cell membrane to the nucleus, including oncogenes representing extracellular ligands (*sis* and *int*-2), cell membrane-associated growth factor receptors (*fms* and *erbB*), tyrosine kinase (*src*, *abl*, and *ros*) and GTP-binding transducers (*ras* oncogenes), cytosolic serine-threonine kinase transmitters (*raf*-family oncogenes), and nuclear transcription factors (c-*jun*, c-*fos*, and c-*myc*) (reviewed in reference 29). After binding of the ligand to its cognate receptor, a complex set of second messengers is generated (1, 2), some of which appear to activate a protein kinase cascade (17, 26, 30). These signals are then transmitted to the nucleus, presumably via modification of preexisting transcription factors (30a, 40), which then induce the expression of a specific set of genes, including the proto-oncogenes c-*fos*, c-*jun*, and c-*myc* (3, 7, 9, 13, 18, 32).

A major obstacle to the study of growth factor signal transduction has been the complexity of components involved and the difficulty in identifying the intermediates between specific transducers and their targets. This is especially true in deciphering signaling pathways that control expression of c-*myc*, since this gene is induced by a diverse assortment of growth factors and is repressed by numerous growth inhibitors.

To examine signal transduction mechanisms regulating the expression of c-*myc*, we have chosen the interleukin-3 (IL-3)-dependent murine myeloid FDC-P1 cell line, which requires only this ligand for growth and viability (9, 10, 14). Stimulation of these cells with purified IL-3 leads to the induction of c-*fos* and c-*myc* expression yet differs from fibroblast cell systems in that both genes are coordinately induced and the induction of c-*myc* is not transient but remains high as long as the ligand is provided (9). In this study, we tested the possible connection between the induction of tyrosine phosphorylation and c-*myc* expression by introducing constitutive and conditional derivatives of tyrosine kinase oncogenes and examining their ability to regulate c-*myc* transcription.

MATERIALS AND METHODS

Cell lines. Growth and culturing of the FDC-P1 myeloid cell line in purified IL-3 have been previously described (10, 14). A subclone of this line, designated FD-1, was selected for use because of its low rate of generating spontaneous IL-3-independent clones. The 32D cell line is a multipotent myeloid stem cell line that also absolutely requires IL-3 for growth and viability (34). In this study, we used a subclone of 32D cells, termed 32DC3 (kindly provided by Prem Reddy, Wistar Institute); in agar cloning experiments and in bulk culture, we have been unable to isolate spontaneous IL-3-independent cells. Both FD-1 and 32DC3 cells have a normal diploid karyotype (10, 34) and are nontumorigenic.

Recombinant retroviruses and infection of FD-1 and 32DC3 cells. Several different recombinant retroviruses carrying tyrosine kinase oncogenes, all having a Moloney murine leukemia virus (MuLV) backbone and controlled by the

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Moloney MuLV long terminal repeat, were used. Two constructs, *fms* MuLV (37) and *trk* MuLV (17a), also contain the selectable *neo* gene conferring resistance to the antibiotic geneticin (G418). The v-*src* MuLV construct has been previously described (36). Five different retroviruses carrying versions of v-*abl* were tested. Two contain wild-type v-*abl*; Ab-MuLV (22, 38) expresses a $p120^{gag-v\text{-}abl}$ fusion protein (here designated MO), and Ab-MuLV-P70 (12) expresses a truncated form containing only the v-*abl* kinase domain (here referred to as WT). To conditionally express functional v-*abl*, retroviruses carrying three different temperature-sensitive (*ts*) mutations in v-*abl* were used. Ab-MuLV RK and Ab-MuLV DP *ts* viruses (here designated RK and DP) contain point mutations at $P160^{v\text{-}abl}$ amino acids 449 (insertion of valine and proline) and 461 (insertion of a methionine), respectively (19). Ab-MuLV P70/H590 (here referred to as TS) was created by site-directed mutagenesis of the tyrosine codon at position 590 to histidine (12). All *ts abl* constructs display conditional kinase activity and morphological transforming potential on NIH 3T3 cells when shifted to a nonpermissive temperature (37 to 39°C [12, 19]). FD-1 and 32DC3 cells were infected with comparable titers of the recombinant retroviruses for 24 h as previously described (9) by using Leuk-MuLV, a leukemogenic strain of Moloney MuLV, as a helper virus (6). Infected cells were then plated into 0.4% agar without IL-3 and containing 200 to 400 μg of G418 per ml when appropriate. With infections involving wild-type and *ts* v-*abl* constructs, agar plates were kept at the permissive temperature (32°C), and IL-3-independent clones were picked and expanded at this temperature. All IL-3-independent clones were positive for transforming virus, as judged by their ability to induce focus formation of NIH 3T3 cells by infectious cell center assays at the appropriate temperature.

Biological assays. Viability of control cells and of cultures containing wild-type and *ts abl* viruses was assessed at permissive and nonpermissive temperatures as previously described (9).

Isolation and analysis of RNA and DNA. Cells were harvested by centrifugation, and DNA and total RNA were isolated by the guanidine thiocyanate lysis procedure, followed by pelleting of the RNA by CsCl gradient centrifugation (5). High-molecular-weight DNA and total and poly(A)$^+$ RNA were purified as previously described (6, 7) and analyzed by using standard Southern and Northern (RNA) blot hybridization protocols. The DNA probes used for analyses of Southern and Northern blots were as follows: c-*fos*, a 1.2-kilobase-pair (kbp) *Eco*RI-*Cla*I fragment (exons 2 to 4) of mouse c-fos; c-*myc*, a 900-bp *Xba*I-*Sac*I fragment (exon 2) of mouse c-*myc*; v-*abl*, a 1.6-kbp *Bgl*II fragment; actin, a 760-bp *Bal*I fragment of chicken β-actin; and *junB*, a 1.8-kbp *Eco*RI cDNA insert.

S1 nuclease and RNase protection assays. S1 nuclease analyses using 2 μg of poly(A)$^+$ RNA and 250,000 cpm of 5′-end-labeled DNA probe were carried out as previously described (6). S1-resistant hybrids were separated by electrophoresis on 6% denaturing polyacrylamide gels and visualized by autoradiography. The murine c-*myc* exon 1 *Xba*I-*Sac*I probe of clone pBS-*myc*-1 was specifically labeled at the *Sac*I site with T4 polynucleotide kinase (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) to 10^7 cpm/pmol, using conditions required for labeling recessed 5′ends (24).

RNase mapping of c-*myc* transcripts was carried out as described previously (23). Hybrids were digested with 20 U of RNase A per ml and 35 U of RNase T_1 per ml (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) for 30 min at ambient temperature, and RNase-resistant species were separated by electrophoresis on 6% denaturing polyacrylamide gels and visualized by autoradiography.

Immunoblotting. Cell extracts were made as previously described (33), and the protein concentration was determined by using the Bio-Rad Protein Assay Kit (Bio-Rad, Inc., Richmond, Calif.). Equivalent amounts of protein were immunoprecipitated with monoclonal Moloney MuLV $p12^{gag}$ antibody H548 (4), rabbit anti-mouse immunoglobulin G (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa.), and protein A-Sepharose (Bio-Rad) overnight at 4°C. Immunoprecipitates were washed three times and then loaded onto an 8.5% sodium dodecyl sulfate–polyacrylamide gel. Gels were transferred to diazobenzyloxymethyl paper and reacted with monoclonal antibody H548 and anti-mouse immunoglobulin G as described previously (33).

Nuclear run-on analyses. Nuclei were isolated and nascent transcripts were elongated as described by Eick and Bornkamm (11). After elongation in vitro for 15 min at 28°C, the reaction was stopped by the addition of guanidine thiocyanate solution, and total nuclear RNA was prepared by pelleting through CsCl gradients (5). RNA pellets were dissolved, degraded to a 200-nucleotide size range by boiling, ethanol precipitated, suspended in hybridization buffer, and hybridized to Southern blots (5×10^6 cpm of run-on probe per ml). Nascent c-*myc* transcription was analyzed by using blots having 2 μg of an M13 clone containing a 350-base antisense *Bam*HI-*Xho*I fragment of mouse c-*myc* exon 1 (6) and 4 μg of clone pSVc-*myc* (21; containing mouse c-*myc* exons 2 and 3) restricted with *Bgl*I. Blots were prehybridized, hybridized, and washed as described elsewhere (33a).

RESULTS

FDC-P1 cells abrogated of IL-3 dependence by tyrosine kinase oncogenes constitutively express c-*myc*. To test whether the ability to abrogate IL-3 dependence is a general property of tyrosine kinase oncogenes and whether abrogation is associated with changes in c-*myc* regulation, we first introduced activated forms of four different tyrosine kinase oncogenes into these cells. FD-1 cells, a subclone of FDC-P1, were infected overnight with Ab-MuLV (22), *src* MuLV (36), *fms-neo* MuLV (37), or *trk-neo* MuLV (17a) and then directly plated in soft agar without IL-3. All tyrosine kinase-containing retrovirus constructs generated factor-independent clones with frequencies at least 5 orders of magnitude higher than that seen with control *neo* virus infections (data not shown).

To determine the status of c-*myc* expression, several IL-3-independent clones were expanded in the absence of IL-3 and examined by Northern hybridization analyses. With the use of probes specific for the various tyrosine kinase oncogenes, all infected lines expressed high levels of the predicted viral transcripts (data not shown). c-*myc* expression in these cells was compared with that of FD-1 cells grown in IL-3 and of FD-1 cells deprived of this ligand for 24 h. Levels of c-*myc* transcripts were tightly controlled by the presence of IL-3 in parental FD-1 cells and dropped to almost undetectable levels when the ligand was removed (Fig. 1A). In contrast, this dependence was alleviated in all tyrosine kinase oncogene factor-independent clones, since c-*myc* RNA levels were as high as in control FD-1 cells grown in IL-3. To examine whether constitutive expression of tyrosine kinase genes had effects on the expression of

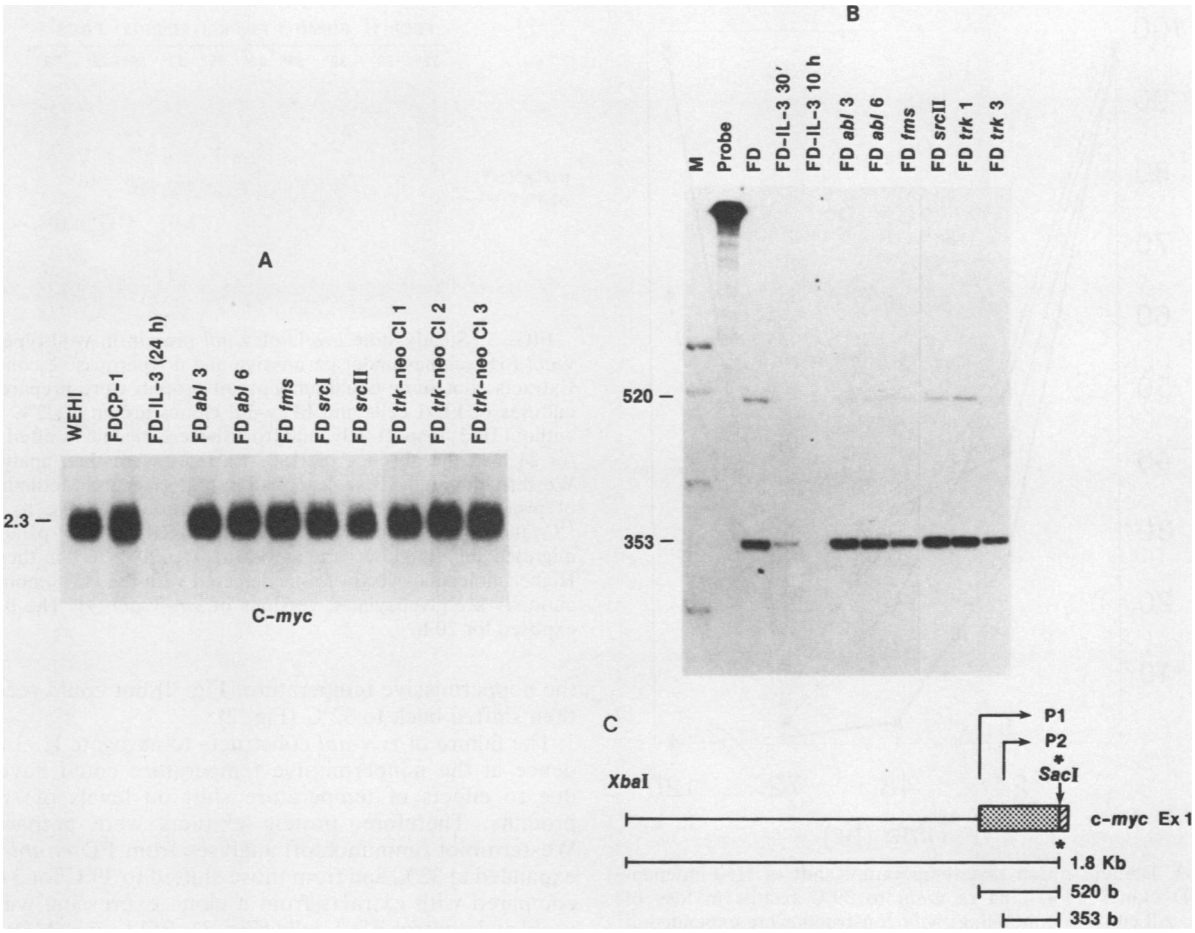

FIG. 1. Expression and promoter usage of the c-*myc* gene in tyrosine kinase IL-3-independent lines. (A) Expression analyses of factor-dependent FDC-P1 cells and IL-3-independent clones derived by infection with *abl*, *fms*, *src*, and *trk* tyrosine kinase retroviruses. Total poly(A)$^+$ RNA was isolated from the independent cell lines grown in the absence of IL-3 and from control FD-1 cultures grown in the presence of IL-3 or deprived of this ligand for 24 h. A 5-μg sample of each RNA preparation was analyzed by Northern blotting with a mouse c-*myc* exon 2 probe. Sizes of mRNAs are shown in kilobases and were determined from migration relative to that of size standards. The blot was exposed for 2 days. (B) S1 nuclease analysis of c-*myc* RNA cap sites. A 2-μg sample of poly(A)$^+$ RNA from the factor-independent clones and from control FD-1 cells grown in IL-3 or deprived of the ligand for 30 min or 10 h was hybridized with a 1.9-kbp *Xba*I-*Sac*I probe 5′ end labeled at the *Sac*I site (shown in panel C) and digested with S1 nuclease; the resistant hybrids were analyzed on a sequencing gel. M, ^{32}P-end-labeled restriction fragments of pBR322 cut with *Msp*I. The gel was exposed for 20 h.

other nuclear oncogenes, these blots were also hybridized with c-*fos*, c-*jun*, and *junB* probes. Expression of c-*fos* and *junB*, which are maximally induced 30 min after stimulation of FDC-P1 cells with IL-3 (9; J. L. Cleveland, unpublished results), and c-*jun*, which is not (Cleveland, unpublished results), was undetectable in all of the IL-3-independent clones (data not shown).

Since c-*myc* expression in the factor-independent cell lines appeared to be deregulated, it was important to examine whether the c-*myc* locus had been amplified or rearranged. Southern blot analysis of *Hin*dIII-digested high-molecular-weight DNA from these cells did not show any detectable alterations or amplification of c-*myc* in any of the clones (data not shown). Alternatively, constitutive c-*myc* expression could have been a consequence of altered promoter usage in these cells. Therefore, S1 nuclease mapping experiments were performed by using a 1.8-kbp mouse *Xba*I-*Sac*I probe 5′ end labeled at the *Sac*I site of mouse c-*myc* exon 1 (nucleotide 520). Promoter 2 (P_2) was used in preference to P_1 in c-*myc* transcripts from control FD-1 cells grown in IL-3, and levels of both decreased dramatically when IL-3 was removed from these cells (Fig. 1B). Similar ratios of P_2- versus P_1-derived transcripts were also observed in the IL-3-independent clones, and no novel RNA start sites were detected (Fig. 1B). In addition, S1 nuclease mapping experiments with probes specific for mouse c-*myc* exons 2 and 3 confirmed that c-*myc* transcripts from these cells were not altered relative to normal splicing patterns (data not shown).

Conditional abrogation of IL-3 dependence and regulation of c-*myc* expression by *ts* v-*abl*. The constitutive expression of c-*myc* in the factor-independent clones could have been due to primary effects of these tyrosine kinases on c-*myc* regulation or alternatively to secondary events associated with abrogation of IL-3 dependence. To discriminate between these possibilities, several retroviral constructs bearing wild-type virus (Ab-MuLV [MO] and Ab-MuLV strain p70 [WT]) or *ts* mutants (RK-MuLV, DP-MuLV, and Ab-MuLV p70/H590 [TS]) of v-*abl* were introduced into FD-1 cells and also into another IL-3-dependent murine myeloid cell line, 32DC3 (34). Cells were infected overnight and then immediately plated in soft agar without IL-3 at the permissive temperature (32°C). All v-*abl* retroviruses generated IL-

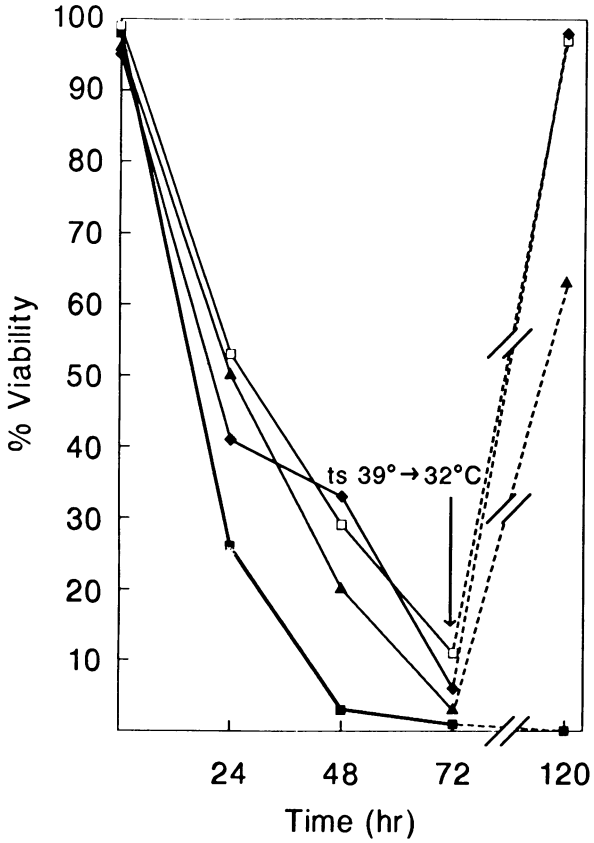

FIG. 2. Demonstration that temperature shift of IL-3-independent 32D clones containing *ts* v-*abl* to 39°C results in loss of viability. All cultures containing v-*abl* constructs were expanded at 32°C in the absence of IL-3 and then shifted to 39°C. For comparison, 32D cells were also expanded at 32°C but in medium containing IL-3, washed twice to remove the ligand, and then put in medium alone and shifted to 39°C. At the indicated times, samples of cells were removed and the percentage of viable cells was determined by their ability to exclude trypan blue. Viability profiles are shown for 32D cells (■) and RK *ts* v-*abl* clones 1 (▲), 2 (□), and 3 (◆) after the shift to the nonpermissive temperature for 72 h in the absence of IL-3 and then shiftdown to 32°C for an additional 120 h.

3-independent clones. In contrast, no IL-3-independent clones were obtained by infection with control *neo* virus at 32°C (data not shown).

It was previously demonstrated that infection of a different subclone of FDC-P1 cells with the RK and DP constructs abrogated IL-3 dependence at permissive but not nonpermissive temperatures (20). To test whether IL-3 independence of FD-1 and 32DC3 cells infected with *ts* v-*abl* viruses was similarly dependent on functional v-*abl* protein, several clones from each of the infected cultures were picked and expanded at 32°C in medium without IL-3, and their viability was examined after a shift to the nonpermissive temperature. As a control, uninfected FD-1 and 32DC3 cells were expanded at 32°C in IL-3, washed to remove the ligand, and then shifted to 39°C. Both FD-1 and 32DC3 cells died rapidly at 39°C when IL-3 was removed (Fig. 2 and data not shown). In agreement with previous results (20), FD-1 clones infected with *ts* v-*abl* viruses died rapidly when shifted to 39°C, whereas clones infected with wild-type viruses remained viable at this temperature (data not shown). Similarly, 32D clones infected with *ts abl* constructs also lost their IL-3 independence when these cultures were shifted to

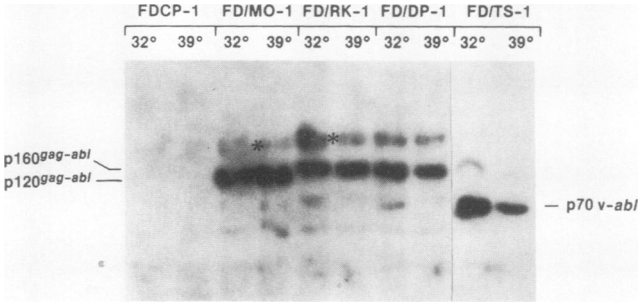

FIG. 3. Steady-state levels of v-*abl* protein in wild-type and *ts* v-*abl* FD-1 clones under permissive and nonpermissive conditions. Extracts containing equal amounts of protein were prepared from cultures of FD-1 cells and FD/v-*abl* clones grown at 32°C with or without IL-3, respectively, and from these same lines shifted to 39°C for 24 h in the absence of IL-3. Extracts were then analyzed by Western blot analysis as described in Materials and Methods. Sizes of proteins were determined from migration relative to that of ^{14}C-labeled molecular weight standards. RK and DP p160$^{gag\text{-}v\text{-}abl}$ migrated slightly slower than Ab-MuLV p120$^{gag\text{-}v\text{-}abl}$ in these gels. Higher-molecular-weight forms detected with the H548 monoclonal antibody are glycosylated versions of *gag*-v-*abl* (*). The blot was exposed for 20 h.

the nonpermissive temperature (Fig. 2) but could recover if then shifted back to 32°C (Fig. 2).

The failure of *ts* v-*abl* constructs to abrogate IL-3 dependence at the nonpermissive temperature could have been due to effects of temperature shift on levels of *ts* v-*abl* proteins. Therefore, protein extracts were prepared for Western blot (immunoblot) analyses from FD *ts abl* clones expanded at 32°C and from those shifted to 39°C for 24 h and compared with extracts from a clone expressing wild-type v-*abl* and control FD-1 cells (Fig. 3). Wild-type (MO) and *ts* (RK, DP, and TS) v-*abl* virus clones expressed high levels of the predicted-size v-*abl* proteins (Fig. 3) at both temperatures. Therefore, loss of IL-3 independence of *ts* v-*abl*-containing cells likely reflects an effect of the specific *ts* mutations on the kinase activity in these proteins.

The inability of the three different *ts* v-*abl* constructs to abrogate IL-3 dependence at the nonpermissive temperature may have been due to a failure of *ts* v-*abl* to induce c-*myc* expression at 39°C. To test whether v-*abl* tyrosine kinase regulates c-*myc* expression, we next examined whether a shift of FD-1 and 32D *ts abl* cultures to the nonpermissive temperature and subsequent temperature shiftdown would influence c-*myc* expression (Fig. 4 and 5, respectively). Wild type (MO) and *ts* (RK, DP, and TS) v-*abl* clones and control FD-1 and 32D cells were shifted to 39°C, followed by a shift to 32°C, and total RNA was prepared at various time intervals. In addition, 32D cells deprived of IL-3 were treated with IL-3. In both control IL-3-depleted FD-1 and 32D cells and in all *ts* v-*abl* clones, c-*myc* RNA levels dropped dramatically when the cultures were shifted to 39°C, whereas clones expressing wild-type v-*abl* did not show significant changes in c-*myc* transcript levels (Fig. 4 and 5). When the *ts* v-*abl* cultures were shifted back to the permissive temperature, a rapid (within 30 min) induction of c-*myc* RNA levels occurred that was similar to the kinetics of c-*myc* induction by IL-3 in FDC-P1 (9) and 32D (Fig. 5) cells. In contrast, there was virtually no effect of temperature shiftdown on induction of c-*myc* transcripts in control FD-1 and 32D cells (Fig. 4 and 5) or in wild-type *abl* clones (Fig. 4). All *ts* v-*abl* clones tested to date show a similar potential to rapidly induce c-*myc* with temperature shift-

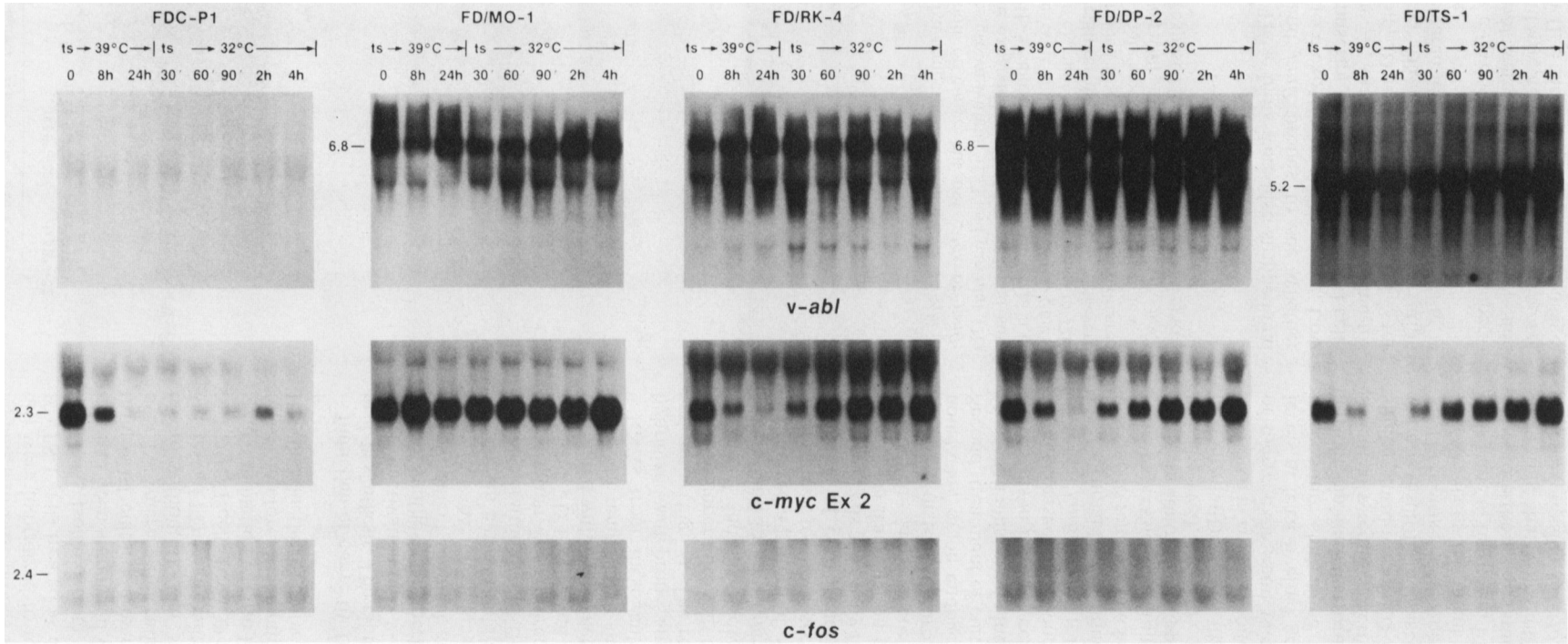

FIG. 4. Regulation of c-*myc* expression by *ts* v-*abl* in FD-1 cells. Cultures of FD/v-*abl* clones grown without IL-3 and FD-1 cells grown in IL-3 were expanded at 32°C, washed twice with medium, suspended in RPMI 1640, and shifted to 39°C. After 24 h in the absence of IL-3 at 39°C, the cultures were shifted to 32°C; at various times, total RNA was prepared. A 20-μg sample of each RNA sample was analyzed by Northern blot hybridization with probes specific for v-*abl*, mouse c-*myc*, and mouse c-*fos*. Blots were exposed for 20 h (v-*abl*) to 2 days (c-*myc* and c-*fos*).

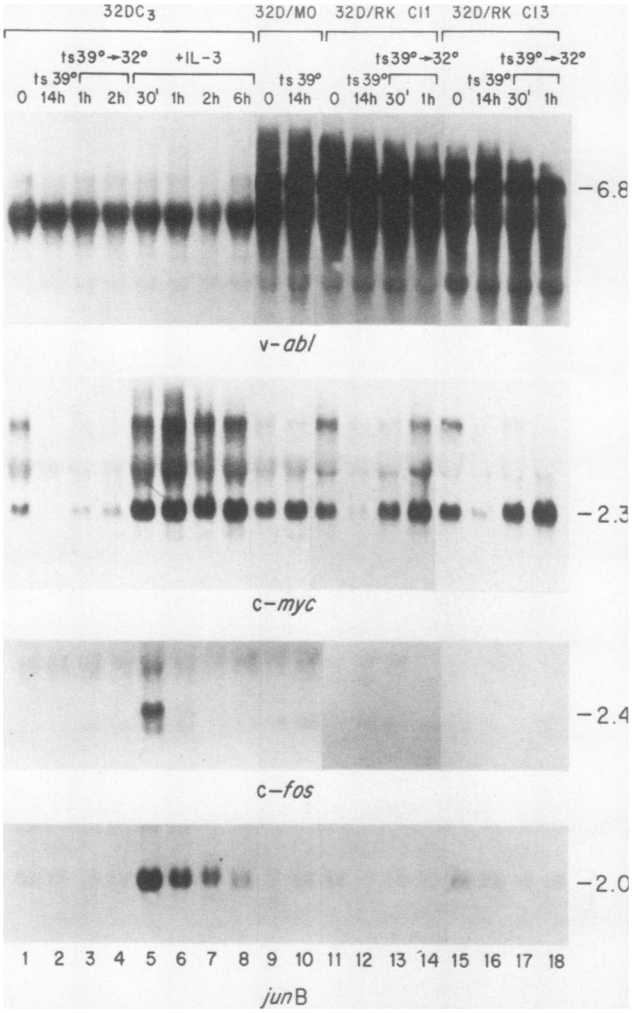

FIG. 5. Demonstration that temperature activation of *ts* v-*abl* in 32D cells induces expression of c-*myc* but not c-*fos* or *junB*. Cultures of 32D/v-*abl* clones grown without IL-3 and parental 32D cells grown with IL-3 were expanded at 32°C, washed and suspended in medium lacking IL-3, and shifted to 39°C. After 14 h at this temperature, the cultures were shifted to 32°C; at various times, total RNA was prepared. To examine the induction of genes in 32D cells by IL-3, parallel 32D cultures deprived of IL-3 for 14 h were also treated with 50 U of purified IL-3 per ml, and at the indicated times RNA was isolated. A 20-μg amount of each RNA sample was analyzed by blot hybridization with probes specific for v-*abl*, c-*myc*, c-*fos*, and *junB*. Blots were exposed for 20 h (v-*abl* and c-*myc*) to 3 days (c-*fos* and *junB*).

down (data not shown). These blots were also stripped and hybridized with c-*fos* and *junB* probes to see whether reactivation of v-*abl* induced expression of either gene. Low levels of a 2.4-kb c-*fos* transcript were detected in FD-1 cells grown in IL-3 at 32°C (Fig. 4), and higher levels of both c-*fos* and *junB* were observed in 32D cells stimulated with IL-3 for 30 min (Fig. 5), yet neither wild-type v-*abl* nor reactivation of *ts* v-*abl* induced detectable levels of either gene (Fig. 4 and 5).

To determine whether c-*myc* RNAs induced by temperature activation of *ts* v-*abl* were bona fide c-*myc* P_1- and P_2-derived transcripts and whether v-*abl* activation of c-*myc* expression differed from IL-3 induction in promoter usage, RNA protection experiments were performed. Three overlapping antisense probes spanning mouse c-*myc* exon 1 (Fig. 6B) were hybridized to total RNA isolated from FD-1 and FD/TS-1 cells treated as described above. In addition, total RNA was isolated from control IL-3-deprived FD-1 cells that had been stimulated with purified IL-3. Promoter usage from FD-1 cultures grown at 32°C in IL-3 again favored P_2 over P_1, whereas this ratio was almost 1:1 in FD/TS-1 cells grown without IL-3 at 32°C (Fig. 6A). Treatment of IL-3-deprived FD-1 cells with purified IL-3 for 1 h or shiftdown of FD/TS-1 cells from 39 to 32°C resulted in a dramatic and similar induction of both P_1- and P_2-derived transcripts (Fig. 6A). Therefore, reactivation of *ts abl* kinase was similar to IL-3 in induction of c-*myc* expression from promoters P_1 and P_2.

Induction of c-*myc* by IL-3 or by *ts* v-*abl* does not require protein synthesis. The rapid induction of c-*myc* either by IL-3 or by reactivation of *ts* v-*abl* protein suggested that de novo protein synthesis was perhaps not required. To test for such a requirement, control and *ts* v-*abl* cells were shifted to nonpermissive conditions for 24 h and then treated with anisomycin before readdition of IL-3 to FD-1 cells or temperature shiftdown of FD/TS-1 cells. To determine whether transcription was required to induce c-*myc* by IL-3, factor-deprived FD-1 cells and FD/TS-1 cells at 39°C were also pretreated with 2 μg of actinomycin D per ml before addition of IL-3 or temperature shiftdown.

RNAs from these cultures was isolated, blotted, and hybridized with probes specific for v-*abl*, c-*myc*, c-*fos*, *junB*, and actin (Fig. 7). The results are summarized as follows. First, a temperature shift to 39°C in the absence of IL-3 (Fig. 7, lanes 2 and 10) followed by treatment with either IL-3 (lanes 4 and 15) or a temperature shift to 32°C (lanes 3 and 11) showed a pattern of c-*myc* RNA depletion and reinduction similar to those seen in Fig. 4 and 5. Notably, the induction of c-*myc* and the weaker induction of *junB* in FD/TS-1 cells at 39°C by IL-3 (lane 15) demonstrated that the IL-3 signal transduction pathway inducing these genes still functioned. Again, there was no apparent effect of factor depletion or temperature shift on the expression of exogenous v-*abl* or on endogenous c-*fos*, *junB*, or actin levels. Second, treatment of cultures at the nonpermissive temperature with anisomycin increased steady-state levels of v-*abl* transcripts in FD/TS-1 cells about twofold (lane 12), moderately increased expression of c-*myc* transcripts, and induced c-*fos* and *junB* RNAs in FD and FD/TS-1 cells (lanes 5 and 12). Presumably, the induction of these genes by anisomycin was due to removal of a labile repressor that down-regulates their expression at transcriptional or posttranscriptional levels. In contrast, no appreciable effect on actin RNA levels was observed. Third, a combination of anisomycin and IL-3 in control FD-1 cells and of anisomycin and temperature shiftdown in *ts* v-*abl* clone TS-1 resulted in a superinduction of c-*myc* expression (lanes 6 and 13, respectively), with levels at least fivefold greater than that seen with IL-3 or temperature shift alone. The superinduction of c-*myc* by anisomycin and temperature shiftdown in FD/TS-1 cells specifically required activation of *ts* v-*abl* protein, since a similar shiftdown of FD-1 cells pretreated with anisomycin did not result in increased levels of c-*myc* expression (lane 7). There was also a synergism in the induction of c-*fos* and *junB* levels by treatment with anisomycin and IL-3 in FD-1 cells (lane 6) but not in FD/TS-1 cells treated with anisomycin before the temperature shiftdown (lane 13), again demonstrating that v-*abl* differed from IL-3 in its inability to affect expression of c-*fos* and *junB*. Finally, pretreatment with actinomycin D abolished induction of c-*myc* RNA

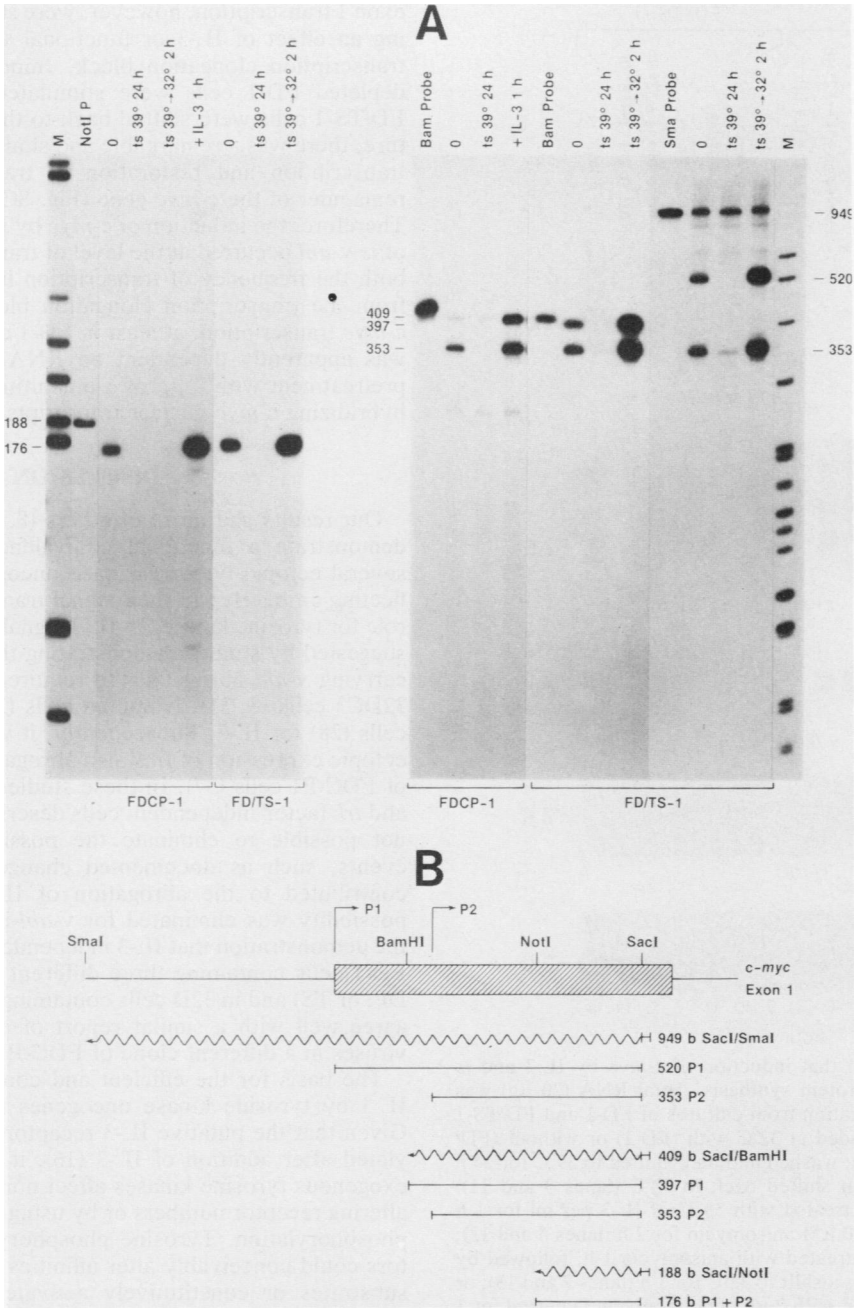

FIG. 6. Demonstration that induction of c-*myc* by IL-3 or by *ts* v-*abl* is similar in promoter usage. Cultures of FD-1 cells and the *ts* v-*abl* clone FD/TS-1 were expanded at 32°C with and without IL-3, respectively, washed twice, shifted to 39°C in the absence of IL-3 for 24 h, and shifted back to 32°C; at various times, total RNA was prepared. For comparison of c-*myc* induction by IL-3 versus *ts* v-*abl*, FD-1 factor-deprived cultures were also treated with purified IL-3 (at 50 U/ml) for 1 h, and total RNA was isolated. A 20-μg sample of total RNA was hybridized to 500,000 cpm of ^{32}P-labeled run-off transcripts generated from *Sma*I-, *Bam*HI-, and *Not*I-linearized pBS-*myc*-1 templates (see panel B), the hybrids were digested with RNases A and T_1, and the resistant hybrids were analyzed on sequencing gels. (A) Gel profile of RNase-resistant species. The dried gels were exposed for 20 h. (B) Probes used and protected species observed. All RNA antisense probes contained 12 nucleotides derived from the T7 promoter, in addition to the c-*myc* exon 1 sequence, which were digested by the RNases.

levels by IL-3 in FD-1 cells (lane 8) and by temperature shiftdown in FD/TS-1 cells (lane 14). Levels of viral v-*abl* RNA also dropped dramatically (lane 14), whereas actin levels were not appreciably affected (lanes 8 and 14). Therefore, the induction of c-*myc* by IL-3 or by reactivation of v-*abl* was independent of protein synthesis and appeared to require new transcription.

Induction of c-*myc* by IL-3 or reactivation of *ts* v-*abl* occurs at the level of transcription. To demonstrate that induction of c-*myc* P_1 and P_2 transcripts by IL-3 and *ts* v-*abl* occurred at the level of transcription and to see whether this regulation involved changes in transcription initiation frequency or in the transcription elongation block present in mouse c-*myc* exon 1 (27, 39), nuclear run-on assays were performed. Nuclei were isolated from FD-1 and FD/TS-1 cells grown at permissive and nonpermissive temperatures with or without

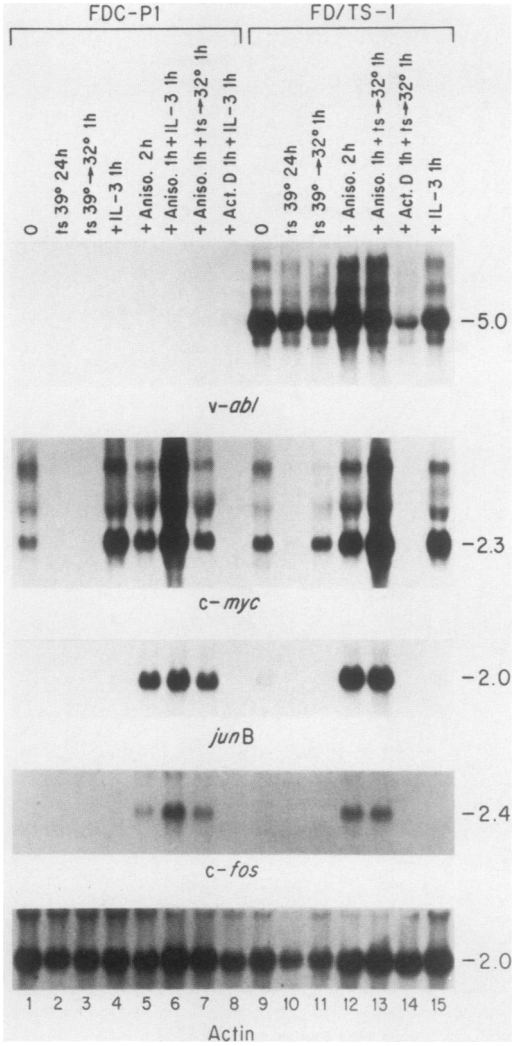

FIG. 7. Demonstration that induction of c-*myc* by IL-3 and *ts* v-*abl* does not require protein synthesis. Total RNA (20 μg) was analyzed by blot hybridization from cultures of FD-1 and FD/TS-1 treated as follows: expanded at 32°C with (FD-1) or without (FD/TS-1) IL-3 (lanes 1 and 9); washed and then shifted to 39°C for 24 h (lanes 2 and 10) and then shifted back to 32°C (lanes 3 and 11); shifted to 39°C and then treated with 50 U of IL-3 per ml for 1 h (lanes 4 and 15) or with 10 μM anisomycin for 2 h (lanes 5 and 12); shifted to 39°C and then treated with anisomycin 1 h, followed by addition of IL-3 (lane 6) or a shift to 32°C for 1 h (lanes 7 and 13); or shifted to 39°C and treated with 2 μg of actinomycin D per ml for 1 h before addition of IL-3 (lane 8) or a shift back to 32°C (lane 14). Duplicate gels were blotted and sequentially hybridized with probes specific for v-*abl*, c-*myc* exon 2, *junB*, c-*fos*, and β-actin. Blots were exposed from 20 h (v-*abl*, c-*myc*, and actin) to 3 days (*junB* and c-*fos*).

IL-3 supplement, and nascent transcripts were elongated and hybridized to Southern blots (Fig. 8).

In both control FD-1 cells expanded at 32°C in IL-3 and FD/TS-1 cells grown at 32°C without IL-3, transcription of c-*myc* was discontinuous, with higher levels of exon 1 and intron 2 transcripts than of exon 2 and 3 transcripts (Fig. 8A and E). When both IL-3-deprived FD-1 and FD/TS-1 cells were shifted to 39°C, there was a dramatic decrease in c-*myc* transcription (Fig. 8B and F), since no hybridization was seen with exon 2 and 3 and intron 2 fragments. Levels of exon 1 transcription, however, were still detectable, suggesting an effect of IL-3 or functional v-*abl* depletion on the transcription elongation block. Importantly, when factor-depleted FD-1 cells were stimulated with IL-3 or when FD/TS-1 cells were shifted back to the permissive temperature, there was a remarkable and similar induction of exon 1 transcription and restoration of transcription across the remainder of the c-*myc* gene (Fig. 8C and G, respectively). Therefore, the induction of c-*myc* by IL-3 or by reactivation of *ts* v-*abl* occurred at the level of transcription and affected both the frequency of transcription initiation and a release from the transcription elongation block. The induction of c-*myc* transcription, at least in FD-1 cells treated with IL-3, was apparently dependent on RNA polymerase II, since pretreatment with 2 μg of α-amanitin per ml abolished any hybridizing c-*myc* nuclear transcripts (Fig. 8D).

DISCUSSION

Our results and those of others (8, 20, 25, 28, 31, 36, 37) demonstrate a functional equivalence between IL-3 and several ectopic tyrosine kinase oncogenes, presumably reflecting an overlap in their signal transduction pathways. A role for tyrosine kinases in IL-3 signal transduction was first suggested by studies demonstrating that expression vectors carrying v-*abl* abrogated the requirements of FDC-P1 and 32DC3 cells (8, 31), lymphoid cells (25), and primary mast cells (28) for IL-3. Subsequently, it was demonstrated that ectopic expression of *fms* also abrogated factor dependence of FDC-P1 cells (37). In these studies, and in the *src*, *fms*, and *trk* factor-independent cells described in this report, it is not possible to eliminate the possibility that secondary events, such as documented changes in karyotype (35), contributed to the abrogation of IL-3 dependence. This possibility was eliminated for v-*abl*-induced abrogation by the demonstration that IL-3 independence was conditional in FD-1 cells containing three different *ts* v-*abl* viruses (RK, DP, or TS) and in 32D cells containing RK virus. These data agree well with a similar report of effects of RK and DP viruses in a different clone of FDC-P1 cells (20).

The basis for the efficient and conditional abrogation of IL-3 by tyrosine kinase oncogenes is poorly understood. Given that the putative IL-3 receptor is tyrosine phosphorylated after addition of IL-3 (16), it is possible that these exogenous tyrosine kinases affect normal IL-3 receptors by altering receptor numbers or by using them as substrates for phosphorylation. Tyrosine phosphorylation of IL-3 receptors could conceivably alter affinities of these receptors for substrates or constitutively activate any intrinsic kinase activity. However, binding experiments with iodinated IL-3 and analyses with antiphosphotyrosine antibody have failed to detect any changes in IL-3 receptor number, affinity, or tyrosine phosphorylation state in these abrogated cells (15). Abrogation of IL-3 dependence by tyrosine kinases could also involve the induction of growth factors that function in autocrine loops. We have tested conditioned medium from these cells and found that the cells do not contain any factors that have mitogenic activity for themselves or for the parental cells (J. L. Cleveland and U. R. Rapp, unpublished data). In addition, hybridization analyses have failed to detect transcripts for IL-3, IL-4, or granulocyte-macrophage colony-stimulating factor in any of the cell lines (data not shown).

Another mechanism by which tyrosine kinases could relieve IL-3 requirements, which is supported by the data presented in this report, is a bypass mechanism whereby

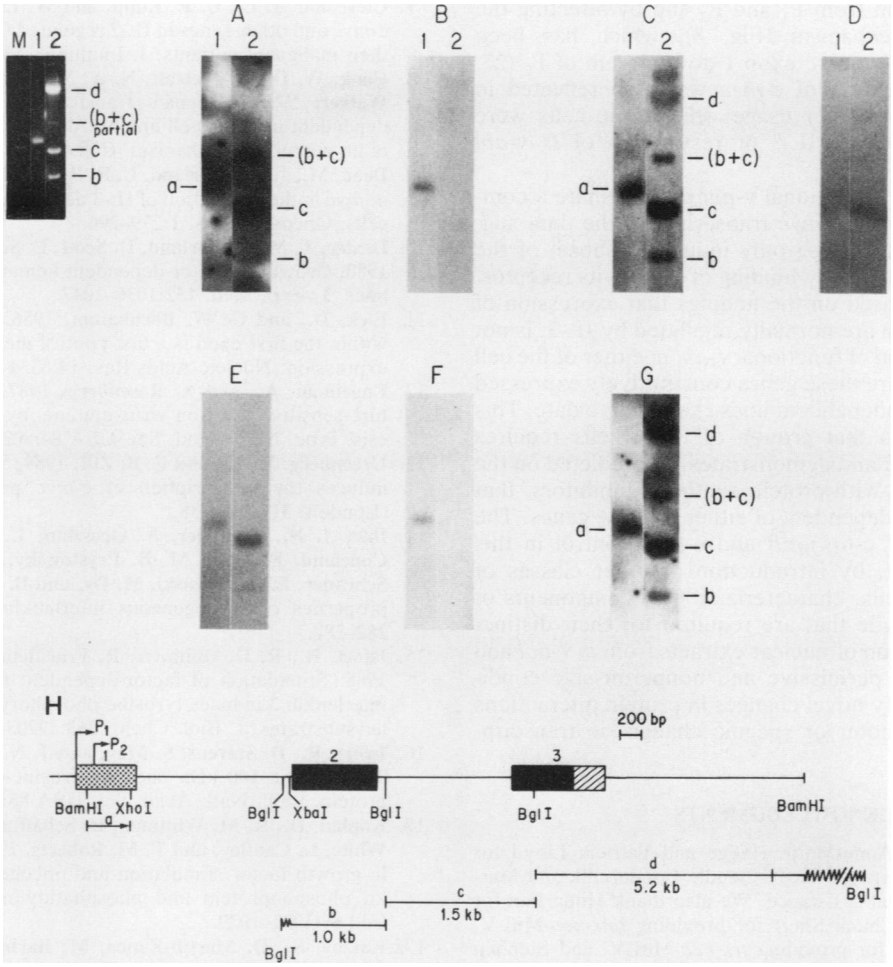

FIG. 8. Transcriptional activity of the c-*myc* gene under permissive, nonpermissive, and inducible conditions. Nuclei were isolated from cultures expanded at 32°C with (FD-1 [A]) or without (FD/TS-1 [E]) IL-3, from cultures shifted to 39°C or 24 h (B and F), from FD/TS-1 cells shifted to 39°C and then shifted back to 32°C for 1 h (G), and from factor-deprived FD-1 cells that had been treated with IL-3 (C). To determine whether transcription was dependent on RNA polymerase II, nuclei from FD-1 cultures stimulated with IL-3 were pretreated with 2 μg of α-amanitin per ml for 5 min at 0°C before addition of [^{32}P]UTP (D). ^{32}P-labeled transcripts were hybridized to nylon filters containing 2 μg of single-stranded M13p*myc*1-BamHI/XhoI (mouse c-*myc* exon 1 antisense fragment; lane 1) and 4 μg of plasmid pSV-c-*myc* restricted with *BglI* (lane 3). Blots with control M13 DNA revealed no hybridization to any of the nuclear run-on transcript preparations (data not shown). Blots were exposed for 4 days. (H) Positions of relevant restriction fragments within c-*myc*. ∿∿∿, Position of *BglI* site within the pSV c-*myc* plasmid.

these proteins induce a signal cascade similar to that induced by IL-3 which would alter the activities of genes regulating cellular proliferation. The downstream target genes whose expression might be regulated by signal cascades generated by these tyrosine kinases would logically include those that are rapidly induced by IL-3, which in these two cell lines include c-*fos*, c-*myc*, and *junB*. Of this set of genes, several lines of evidence indicate that c-*myc* is a key target in IL-3 signal transduction and that this induction is necessary for IL-3-mediated growth and viability. First, c-*myc* RNA (9; this report) and transcription (this report) levels are tightly regulated by IL-3 in these cells, and in contrast to the transient induction of c-*fos* (9) and *junB* RNA, c-*myc* RNA levels remain high and constitutive as long as the ligand is provided. Second, the introduction of vectors expressing near-normal levels of mouse c-*myc* has a pronounced effect on FDC-P1 cell viability, since infected clones remain viable in the absence of IL-3 yet are not fully factor independent, as their ability to proliferate is restricted. With time in culture, all of these partially abrogated clones became fully factor independent and notably constitutively expressed endogenous c-*myc* or N-*myc* (9) but not c-*fos* or *junB* (unpublished data). Third, spontaneous factor-independent FDC-P1 cells constitutively express c-*myc* but again not c-*fos* or *junB* (Cleveland, unpublished data).

Our results demonstrating a similar regulation of c-*myc* expression by IL-3 and *ts* v-*abl* protein suggests that other tyrosine kinases, including those responsible for tyrosine phosphorylation seen after binding of IL-3, might function similarly in their ability to regulate c-*myc*. Although effects of conditional *abl* and IL-3 upon c-*myc* mRNA stability were not directly tested, the results indicate that the control of c-*myc* RNA levels is quite comparable and, on the basis of effects of actinomycin D and nuclear run-on analyses, occurs predominately at the level of transcription. Similar to regulation of c-*myc* in other cell types, transcriptional control by IL-3 and *ts* v-*abl* does not require protein synthesis (Fig. 7) and appears to occur at least at two levels, by changing the

frequency of initiation from P_1 and P_2 and by affecting the elongation block mechanism (Fig. 8), which has been mapped within mouse c-*myc* exon 1 downstream of P_2 (27, 39). Analogous regulation of c-*myc* was also reflected in similar changes in promoter usage when these cells were stimulated by addition of IL-3 or restoration of *ts* v-*abl* protein (Fig. 6).

Although IL-3 and conditional v-*abl* protein share a common target in regulating c-*myc* transcription, the data suggest that this tyrosine kinase only induces a subset of the cascade normally induced by binding of IL-3 to its receptor. This conclusion is based on the findings that expression of c-*fos* and *junB*, which are normally regulated by IL-3, is not induced by restoration of functional v-*abl* in either of the cell lines examined, nor are these genes constitutively expressed in any of the factor-independent lines examined to date. This observation indicates that growth of these cells requires neither c-*fos* nor *junB* and demonstrates, as predicted on the basis of experiments with protein synthesis inhibitors, that c-*myc* induction is independent of either of these genes. The unique separation of c-*fos*/*junB* and c-*myc* control in this system should allow, by introduction of other classes of kinases into these cells, characterization of components of the IL-3 signal cascade that are required for their distinct induction. Examination of nuclear extracts from *ts* v-*abl* and parental cells under permissive and nonpermissive conditions may also identify novel changes in protein interactions with the c-*myc* promoter or specific changes in transcriptional complexes.

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