c-*fos* Is Not Essential for v-*abl*-induced Lymphomagenesis[1]

Enrique Saez, Heather Oppenheim, Jennifer Smoluk, Janet W. Andersen, Richard A. Van Etten, and Bruce M. Spiegelman[2]

Dana-Farber Cancer Institute, Department of Cell Biology [E. S., H. O., J. S., J. W. A., B. M. S.], and Department of Genetics, Center for Blood Research [R. A. V.], Harvard Medical School, Boston, Massachusetts 02115

ABSTRACT

Recent studies have suggested that cellular transformation by abl on-coproteins may be mediated by the *ras* signaling pathway. One of the main nuclear targets of this signal transduction cascade is the Fos and Jun family of transcription factors. To test the relevance of the c-*fos* proto-oncogene for v-*abl*-induced cancer development, we inoculated c-*fos*-deficient mice with the Abelson murine leukemia virus. Neonatal c-*fos*-deficient mice infected with the Abelson complex are able to develop the pre-B-cell lymphoma that characterizes Abelson disease. c-*fos*-deficient animals succumb to the disease with similar kinetics as their wild-type and heterozygous littermates. Moreover, the transformed cell that brings about the malignancy in mutant mice is the same pre-B-cell lymphoblast that is seen in control animals. These results demonstrate that c-*fos* is not required for *in vivo* transformation by v-*abl*.

INTRODUCTION

The Ab-MuLV[3] was isolated from a mouse infected with Mo-MuLV that developed an aggressive lymphoma spontaneously (1). In contrast to the slow thymic lymphoma typically induced by Mo-MuLV, neonatal mice inoculated i.p. with Ab-MuLV develop a non-thymic lymphoma with a short latency period (reviewed in Ref. 2). The transformed cell is a primitive lymphocyte that expresses the B-cell marker B220 and has a rearranged immunoglobin heavy chain. Although Abelson virus infection *in vivo* results in other hematopoietic malignancies (*e.g.*, erythroleukemia and myeloid leukemias) rarely, many cell types, including fibroblasts, can be transformed by Ab-MuLV *in vitro* (3–5).

Cloning and analysis of the Ab-MuLV genome revealed that this retrovirus arose from a recombination between Mo-MuLV and the cellular gene c-*abl* (reviewed in Ref. 6). In Ab-MuLV, the *gag* gene of Mo-MuLV has fused to a large portion of the coding sequence of c-*abl*, giving rise to a protein of M_r 160,000 known as p160^{v-abl}. A variety of other oncogenic *abl* fusions have been isolated. Of particular significance is a class of *abl* fusions in which this gene is fused to the *bcr* gene, because these aberrant proteins have been implicated in the pathogenesis of human chronic myelogenous leukemia and acute lymphoblastic leukemia.

Little is known regarding the mechanism of transformation of mutant abl proteins. c-*abl* encodes a cytoplasmic tyrosine kinase with homology to Src. Molecular analysis has shown that an active tyrosine kinase domain is required for *abl*-derived proteins to retain their transforming properties. Nevertheless, few cellular substrates have been identified thus far. Some studies have suggested that the Philadelphia chromosome bcr/abl fusion protein that induces chronic myelogenous leukemia (p210) uses the *ras* signaling pathway (7–9). In

these experiments, bcr/abl-induced p21 Ras activation led to constitutive c-*fos* and c-*jun* expression that correlated with transformation.

More substantial evidence that the AP-1 family may be a downstream target of oncogenic abl proteins came from experiments using temperature-sensitive kinase mutants of v-*abl* (10). These studies demonstrated that activation of the v-Abl kinase activity in stably transfected NIH3T3 cells resulted in the appearance of two distinct cell populations. The large majority of cells ceased growing and were arrested in G_1. However, a small minority became serum and anchorage independent. The transformed (positive) phenotype was dominant to the arrested (negative) one. Activation of the v-Abl kinase activity in the positive cells resulted in a strong, sustained induction of c-*fos* and c-*jun*. These AP-1 genes were induced in both quiescent and exponentially growing positive cells. Furthermore, v-Abl activation resulted in the transactivation of the c-*fos* promoter exclusively in positive cells. Taken together, these data suggested that constitutive AP-1 activity could be important for v-*abl* transformation. We have taken advantage of the existence of c-*fos*-deficient mice to determine the importance of this proto-oncogene for transformation *in vivo* by v-*abl*.

MATERIALS AND METHODS

Viral Stocks. The Abelson complex was prepared by harvesting media from plates of the cell line N54. This cell line, a gift from Dr. David Baltimore (Whitehead Institute for Biomedical Research), is a 3T3 fibroblast clone that produces high titers of Ab-MuLV pseudotyped with Mo-MuLV. N54 generates the p160 form of v-*abl*. To prepare the infectious cocktail, supernatant was harvested from confluent N54 cells and passed through a 0.45-μm filter. Viral stocks were titered by a focus formation assay in NIH3T3 fibroblasts (5).

Animals. c-*fos*-deficient animals were generated as described previously (11). Animals were housed under BL2 containment conditions in a viral antibody-free environment. Breeding pairs of mice heterozygous for the c-*fos* null mutation were monitored regularly for the appearance of litters. Neonatal mice (48 h or less postpartum), were injected i.p. with 100 μl Abelson complex at a titer of 2–5 $\times$ 10^6 focus-forming units/ml. Infected mice were inspected daily for the development of symptoms of Abelson disease. Afflicted mice were observed until the disease became near terminal. At the time of sacrifice, mice were genotyped by PCR.

Pathological Analysis. Diseased animals were sacrificed by CO_2 asphyxiation. Immediately afterward, cardiac puncture was performed to obtain a blood sample. Automated total blood cell counts and differential WBC counts were performed by the hematology laboratory at the Dana-Farber Cancer Institute. All lymph nodes were taken and either fixed in Optifix (American Histology Labs), or flash frozen in an ethanol and dry ice bath. The liver, spleen, and thymus were processed similarly. Additional tumor masses and abnormal organs were also preserved for pathology and RNA isolation.

FACS and Immunohistochemical Staining of Cells. Cells were isolated by grinding pieces of freshly excised tissue loosely in PBS. The primary antibodies used in this analysis were 6B2 (α-B220; Becton-Dickinson), α-Thy-1.2 (Becton-Dickinson), M1-70 (α-Mac-1; Becton Dickinson), and 8C5 (α-Gr-1; Pharmingen). Antibodies were used according to the manufacturers' recommendations. A no-primary antibody control was run in parallel. FACS analysis and immunohistochemical staining were performed as described by Ambrosino *et al.* (12).

Statistical Analysis. The association between c-*fos* genotype and survival was analyzed with a log rank test. $P < 0.05$ was considered significant. Plots

of the survival distributions were generated by the method of Kaplan and Meier.

RESULTS

c-fos-deficient Mice Are Susceptible to Infection with the Abelson Complex. To evaluate whether the development of Abelson disease was affected by the absence of c-fos, mating pairs of mice heterozygous for the c-fos null mutation were set up to generate mice of all three desired genotypes. Neonatal mice derived from these crosses were injected i.p. with 2-5 × 10^5 focus-forming units of the Abelson complex within 48 h of birth. At the time of inoculation, mice of different c-fos genotypes were indistinguishable. Infected mice were monitored daily for the appearance of disease symptoms; those clearly sick were separated from their littermates. When the disease became terminal, mice were sacrificed, and blood and other organs were examined to establish a diagnosis. Abelson disease was recognized by a combination of the following criteria: (a) the presence of external symptoms (bulging of the skull and hind-limb paralysis); (b) the absence of grossly elevated WBC counts or other hematological abnormalities in peripheral blood; (c) gross morphology of tumor cells as evaluated by a peripheral blood smear and lymph node pathology; (d) enlargement of the spleen and lymph nodes and histology to confirm invasion by lymphoblasts; (e) infiltration of the meninges and displacement of skull bones by tumor cells; and (f) lack of significant thymus involvement.

Fig. 1 shows a Kaplan-Meier plot comparing the survival rates of mice of all three genotypes infected with the Abelson complex and diagnosed with Abelson disease. Statistical analysis of these numbers demonstrates that no significant differences in the time to terminal disease development were observed between mice of varying c-fos genotypes. Specifically, when the survival rate of wild-type (n = 39) and c-fos-deficient mice (n = 13) was compared in log rank tests, P was 0.41; when heterozygous mice (n = 69) were compared with c-fos null mutants, P was 0.12; when wild-type and heterozygous mice were compared, the P was 0.24. Furthermore, the median survival time was very similar among all mice: 52 days for wild-type; 48 days for heterozygous; and 52 days for c-fos-

deficient animals. These figures are within the previously described range for the C57BL/6J strain (1).

The rate of disease progression was similar in all infected animals; once symptoms were detected, the afflicted animal invariably needed to be sacrificed within 5-16 days, irregardless of c-fos genotype. Some differences were noticed in how Abelson disease manifested itself in individual mice. In some mice, a particular lymph node became very prominent (as large as 2 cm in diameter), with tumor cells infiltrating surrounding muscle tissue. In others, large paraspinous tumor masses were detected. A few mice with facial deposits of tumor cells were also observed. Nevertheless, none of these extreme symptoms could be associated with a specific c-fos genotype.

Neonatal infection with Ab-MuLV sometimes results in hematological malignancies distinct from Abelson pre-B-cell lymphoma (reviewed in Ref. 6). A few infected mice in each group developed some of these other disorders, usually erythroleukemias. The incidence of erythroleukemias, however, was not high enough to draw any conclusions regarding the influence of c-fos in their pathogenesis, except to say that c-fos is not absolutely required for erythroleukemias to arise. A small minority of mice (~5%) in all genotypes proved resistant to Abelson complex infection. Given the technical difficulty of performing i.p. injections in fragile neonatal mice, this resistance is likely to be a reflection of unsuccessful infections.

The Target Cell of Abelson Virus Is the Same in Wild-Type and c-fos-deficient Mice. To examine in detail whether Abelson disease developed in a similar manner in a c-fos-deficient background, tumor cells were isolated from infiltrated lymph nodes, and their identity was established by immunohistochemistry and FACS analysis. The thymuses of those animals from which tumor tissue was obtained were also studied. Cells isolated from tumor-infiltrated lymph nodes (>99% tumor cells) or thymuses were stained with antibodies specific for a range of hematopoietic lineage markers. Immunohistochemistry was used to monitor the quality and specificity of the staining. FACS analysis was used to quantitate the percentage of cells staining with each antibody. Fig. 2 shows the results of FACS analysis of cells
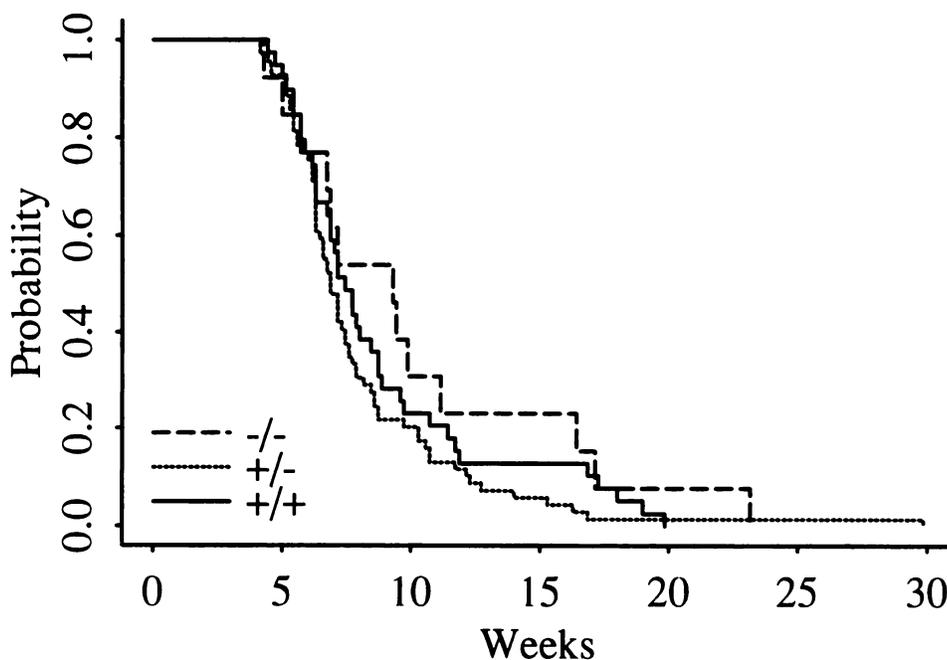
Fig. 1. Survival after infection with the Abelson virus. Kaplan-Meier plot showing the probability of survival as a function of time (weeks) after infection. Wild-type (———, n = 39), heterozygous (·····, n = 69), and c-fos-deficient mice (– – – –, n = 13) are represented.
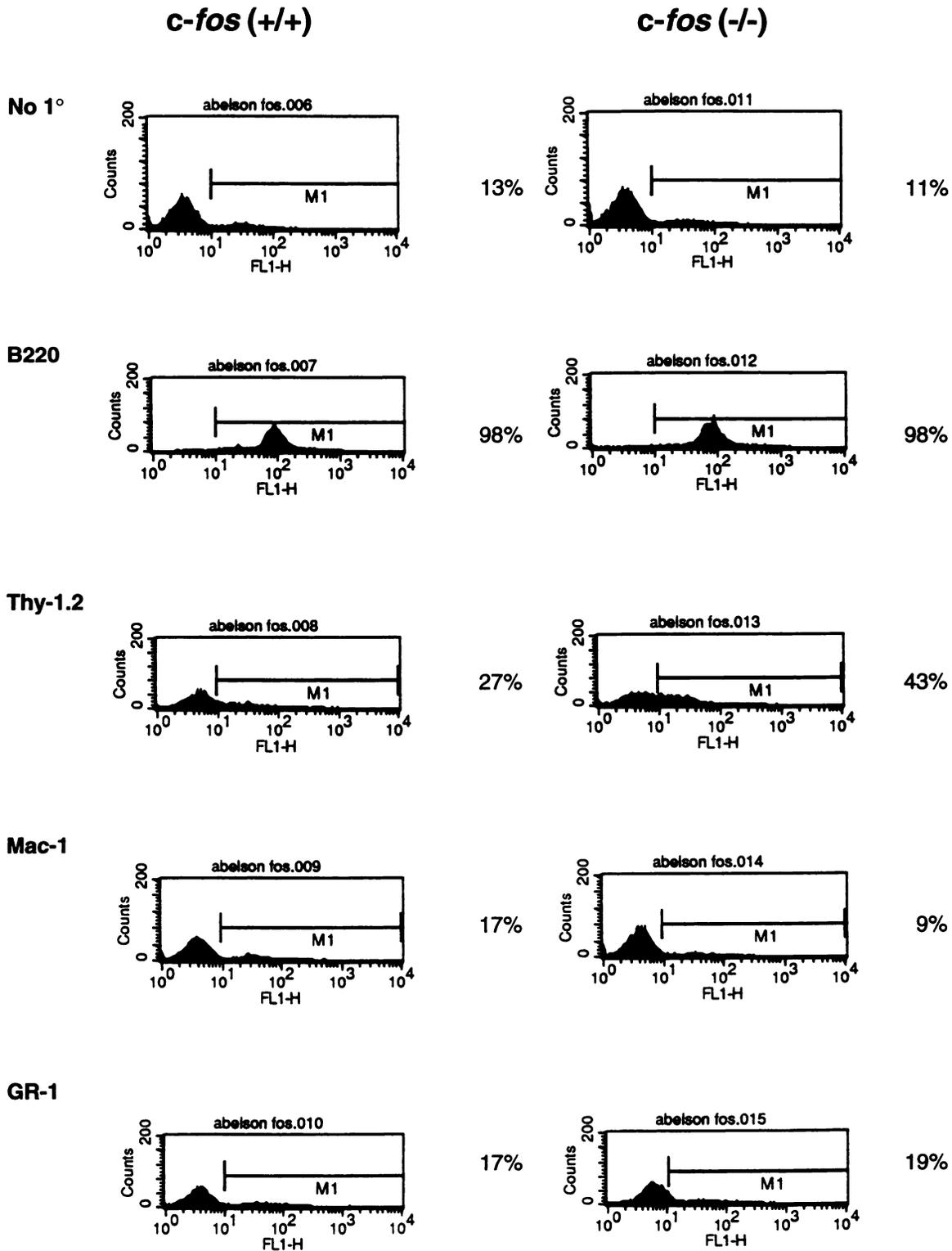
Fig. 2. FACS analysis of tumor cells. Cells isolated from tumor-infiltrated lymph nodes were stained with primary antibodies against hematopoietic markers, incubated with a fluorescence-conjugated secondary antibody, and counted using an automated FACS analyzer. The number of positive cells (substracting background) is shown under the *bar* labeled *M1*. The percentage of positive cells in the sample is shown next to the graph. The *left column* corresponds to cells from a wild-type tumor; the *right column* displays the analysis of c-fos-deficient tumor cells. Primary antibodies are as indicated next to the graphs. Note that B220-positive cells are the predominant cell type.

isolated from wild-type and mutant tumors and stained with anti-bodies against four antigens: B220 (a B-cell marker); Thy-1.2 (specific for T cells); Mac-1 (a monocyte-macrophage antigen); and Gr-1 (expressed in cells of the granulocyte lineage). As expected for Abelson disease, tumor cells of both genotypes stained with B220 and were essentially negative for all other markers. Evaluation of the immunohistochemical staining pattern of tumor cells (data not shown), combined with the FACS analysis data, allowed us to conclude that the transformed cell was the same in both genotypes: an immature B lymphocyte.
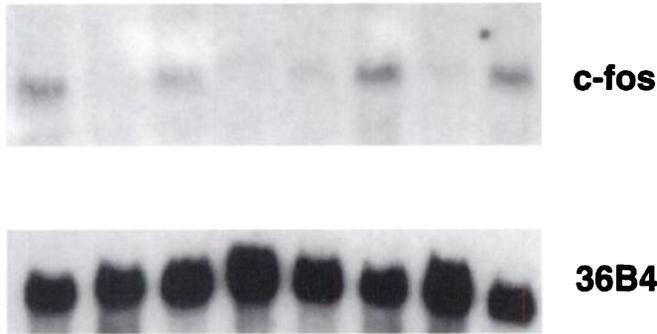
Fig. 3. c-fos expression in wild-type, Abelson virus-induced tumors. Total RNA was prepared from freshly isolated tumor cells and analyzed by Northern blot. Each lane contains 20 μg RNA from a diseased wild-type mouse (eight different animals are shown). To control for loading and RNA quality, the blot was stripped and reprobed with ribosomal associated protein 36B4.

c-fos Expression Is Variable in Ab-MuLV-induced Lymphomas. Given that c-fos-deficient mice developed Abelson disease in a standard manner, we explored whether in wild-type mice, transformation by v-abl led to the induction of c-fos expression. To establish whether this was the case, RNA was isolated from fresh tumors and analyzed by Northern blot. Significant levels of c-fos expression could be detected in only about one-half of all wild-type tumors examined (four of eight). The level of c-fos mRNA expression was quite variable even within expressing tumors (Fig. 3). These results (i.e., the absence of significant c-fos expression in several wild-type Abelson tumors) illustrate that transformation by v-abl does not always lead to constitutive c-fos expression.

DISCUSSION

Neonatal infection with the Abelson murine leukemia virus leads to the development of an aggressive pre-B-cell lymphoma. Some studies had suggested that the AP-1 family of transcription factors might be one of the nuclear mediators of Abelson transformation. In vitro experiments using temperature-sensitive mutants of the oncogene carried by Ab-MuLV had shown that v-abl could either inhibit or stimulate cell growth, depending on the cell context (10). High-levels of c-fos and c-jun expression were detected in cellular environments permissive to v-abl transformation. These AP-1 family proto-oncogenes were dramatically induced upon v-Abl activation, even in actively proliferating cells. In this study, we have tested directly whether c-fos is required for in vivo transformation by v-abl. Our results demonstrate that c-fos is not necessary for the development of Abelson disease.

In the absence of c-fos, the Abelson lymphoma arose and progressed normally. The survival time of mutant mice infected with the Abelson complex was similar to that of their wild-type littermates. c-fos-deficient mice succumbed to the disease with standard kinetics and incidence rates. Moreover, the same immature pre-B cell targeted by Ab-MuLV in wild-type mice was responsible for inducing the malignancy in mutant mice. We were unable to detect uniform c-fos expression in wild-type, Abelson virus-induced tumors. This result illustrates that constitutive c-fos expression is not always associated with the transformed phenotype in Abelson disease. Taken together, these observations allow us to conclusively rule out a significant role for c-fos in the pathogenesis of Abelson virus infection. Interestingly, because the bone marrow cavity of c-fos null mice is very ossified and does not allow much cell proliferation to occur there (11, 13), the observation that no differences in survival time (indicative of a slower-growing tumor) were found between genotypes suggests that the bone marrow might not be an important site of residence and proliferation for the Abelson lymphoma.

Our findings in this study contrast with previous work, in which we showed that c-fos is required for malignant progression of certain solid tumors (14). Several reasons can explain why c-fos-mediated, elevated AP-1 activity is not necessary for the development of Abelson pre-B-cell lymphoma. Although c-fos is known to regulate the expression of several AP-1-dependent genes that are important for tumor development (e.g., tumor metalloproteases and angiogenic factors), it is unclear what the contribution of these particular genes could be to the development of a lymphoma (14–16). Because lymphoblasts are independent cells, they do not require proteases to emancipate themselves from binding, neighboring cell interactions. Moreover, in contrast to epithelial cells, lymphoblasts are not arranged in an encapsulated tissue. Therefore, to become invasive, malignant lymphoblasts are not compelled to degrade the basement membrane surrounding the cellular entity to which they belong. Because tumor cells are mobile and do not form part of an organized, multicellular arrangement, the development of Abelson lymphoma does not require the formation of new blood vessels either. Given that the tumor metalloproteases, their inhibitors, the angiogenic vascular endothelial growth factor, and some genes important for invasion and metastasis are the only AP-1-regulated genes that have been associated with cancer development thus far, if these proteins are not important for the pathogenesis of the neoplasm under study, the absence of c-fos may have little impact on the progression of the disease.

ACKNOWLEDGMENTS

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