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Progression of Interleukin-2 (IL-2)-Dependent Rat T Cell Lymphoma Lines to IL-2-Independent Growth Following Activation of a Gene (*Gfi-1*) Encoding a Novel Zinc Finger Protein

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During progression of Moloney murine leukemia virus (Mo-MuLV)-induced rat T cell lymphomas, growth selection results in the expansion of cell clones carrying increasing numbers of integrated proviruses. These new provirus insertions reproducibly contribute to enhanced growth, allowing the emergence of cell clones from the initially heterogeneous population of tumor cells. The Mo-MuLV-induced rat T cell lymphoma lines 2780d and 5675d, which are dependent on interleukin-2 (IL-2) for growth in culture (IL-2d), were placed in IL-2-free medium to select for IL-2-independent (IL-2i) mutants. Southern blot analysis of genomic DNA from these mutants, which was hybridized to a Mo-MuLV long terminal repeat probe, revealed that all mutants carried new provirus insertions (from one to four new proviruses per cell line). A locus of integration identified through cloning of the single new provirus detected in one of the IL-2i mutants, 2780i.5, was found to be the target of provirus insertion in 1 additional IL-2i cell line of 24 tested. A full-length cDNA of a gene (growth factor independence-1 [*Gfi-1*]) activated by promoter insertion in the 2780i.5 cells was cloned and shown to encode a novel zinc finger protein. *Gfi-1* is expressed at low levels in IL-2d cell lines cultured in IL-2-containing medium and at high levels in most IL-2i cell lines, including the two harboring a provirus at this locus. *Gfi-1* expression in adult animals is restricted to the thymus, spleen, and testis. In mitogen-stimulated splenocytes, *Gfi-1* expression begins to rise at 12 h after stimulation and reaches very high levels after 50 h, suggesting that it may be functionally involved in events occurring after the interaction of IL-2 with its receptor, perhaps during the transition from the G_1 to the S phase of the cell cycle. In agreement with this, *Gfi-1* does not induce the expression of IL-2. Expression of *Gfi-1* in 2780d cells following transfer of a *Gfi-1*/LXSN retrovirus construct contributes to the emergence of the IL-2i phenotype.

The interaction between interleukin-2 (IL-2) and the high-affinity interleukin-2 receptor (IL-2R) is a critical event in T cell activation, triggering proliferation of cells after antigen binding to the T cell receptor (1, 8, 42, 47). This clonal proliferation is obligatory for immunocompetence; mice rendered IL-2 deficient by targeted disruption of the IL-2 gene show decreased proliferative responses to mitogen and decreased T helper cell activity (40), while humans without functional IL-2 have severe combined immunodeficiency (31, 49).

The events prior to and after the interaction of IL-2 with its receptor have been explored to date by using several strategies, including the screening of T cells during activation for the expression of known genes (35), the use of subtraction cDNA libraries to clone genes whose expression is altered in activated T cells compared with in resting T cells (23), and the identification and cloning either of genes encoding proteins which interact with signalling molecules or of genes known to regulate critical activation events (21). These strategies, although informative, have limitations in that the first two may define phenotypic changes occurring during T cell activation and not regulatory events, while the third has the potential to identify only the genes involved in previously defined activation steps.

To study the regulation of T cell activation, we have chosen to use an insertional mutagenesis-based genetic strategy designed to identify genes involved in the progression of IL-2-dependent T cell lymphoma lines (IL-2d) to IL independence (IL-2i). The rationale for this strategy was based on the fact that the interaction of IL-2 with its receptor is a critical step coinciding with the commitment to later T cell activation events (1, 8, 47). We predicted, therefore, that the progression to IL-2 independence would follow the insertional activation of the IL-2 gene or of other genes regulating IL-2 gene activity directly or indirectly. Alternatively, we predicted that it would follow the insertional activation of genes involved in the transduction of IL-2-generated signals. Although these studies were designed to identify genes involved in T cell activation, we predicted that the same genes might also be involved in tumor progression. Many growth factor-independent neoplasms are thought to have arisen from cells previously under the control of autocrine or paracrine growth factors, and the ability to achieve growth factor independence is often a crucial step in malignant tumor progression (43).

The T cell lymphoma lines used in these experiments were established in our laboratory from primary thymonas induced in rats inoculated with Moloney murine leukemia virus (Mo-MuLV) (18). Maintenance of these cell lines in culture or in nude mice is associated with the acquisition of increasing numbers of integrated proviruses. Two of these proviruses, which appear spontaneously in cultured cells, have been cloned, and both activate genes which contribute to growth selection (3, 32a). These observations suggested that the process of spontaneous acquisition of new integrated proviruses, which we call spontaneous insertional mutagenesis, can be used as a genetic tool to identify genes

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expressed in association with selectable phenotypes. Three of the original twenty-nine Mo-MuLV-induced rat T cell lymphoma lines that we established in culture were dependent on IL-2 for growth. In this report, we present evidence that culture of two of these cell lines in IL-2-free medium selects for cells which contain new provirus insertions. One of these new integrated proviruses was cloned and identified a new locus of common integration (*Gfi-1*), which is targeted by provirus insertion during the transition of T cell lymphoma lines from IL-2 dependence to IL-2 independence. Provirus insertion in the *Gfi-1* locus activates a gene encoding a novel zinc finger protein whose expression is enhanced late during mitogen-induced activation.

MATERIALS AND METHODS

Tissues, cell lines, and tissue culture. The cell lines used were established from primary Mo-MuLV-induced rat T cell lymphomas and have been previously described (18). Cells were grown in RPMI 1640 medium with 10% fetal bovine serum. Of 29 cell lines, 3 were dependent on IL-2 for growth, and these were maintained in medium supplemented with 100 U of recombinant human IL-2 per ml. The isolation of IL-2-independent cell lines from two IL-2-dependent cell lines (2780d and 5675d) was achieved by employing one of the following two strategies. (i) IL-2 was withdrawn from long-term mass cultures of 10^8 tumor cells. After the number of viable cells had decreased by approximately 1,000-fold, the remaining viable cells were distributed at a concentration of 10^2 to 10^3 per ml in 0.2-ml microcultures mixed with 10^5 mouse splenocytes as feeder layers. Growth of IL-2i mutants was detected within 2 to 4 weeks. (ii) When the number of viable cells had decreased by approximately 1,000-fold following IL-2 withdrawal, the cells were placed into medium containing 1/10 the usual concentration of IL-2 (10 U/ml). Following partial recovery, the IL-2 was completely withdrawn. Selection was achieved in the absence of feeder layers.

Normal tissues were obtained from 6-month-old or younger F344 rats. Mitogen stimulation of F344 rat splenocytes was performed with concanavalin A (ConA) (2 μg/ml) at a concentration of 5×10^5 cells per ml.

The packaging-cell line PA317 (27) was grown in Dulbecco modified Eagle's minimal essential medium supplemented with HAT (hypoxanthine [100 μM], aminopterin [0.4 μM], and thymidine [16 μM]) and 10% calf serum (GIBCO). PA317 cells successfully transfected with the LXSN retroviral construct (see below) were selected in the same medium supplemented with 400 μg of G418 (Sigma) per ml.

Southern and Northern (RNA) blotting. Genomic DNA isolation and Southern blotting were carried out by using standard procedures, as described previously (44). RNA was isolated from fresh or snap-frozen cell pellets or tissue by the method of Chomczynski and Sacchi (6), and polyadenylated RNA was selected by affinity chromatography in oligo(dT) cellulose (15). Either 5 μg of poly(A)$^+$ RNA or 10 μg of total RNA was electrophoresed in 1% agarose–2.2 M formaldehyde gels and then transferred and cross-linked to nylon membranes (Hybond N, Amersham Corp.) by UV irradiation. RNA immobilized onto nylon membranes was hybridized to ^{32}P-labelled probes by using a random priming kit (Amersham Corp.) with 50% formamide–5× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate) at 42°C. Filters were washed several times in 2× SSC–0.1% sodium dodecyl sulfate (SDS) at room temperature and then washed twice for 30 min each at 65°C in 0.1× SSC–0.1% SDS.

Genomic DNA and cDNA cloning using the polymerase chain reaction (PCR). To clone genomic sequences flanking the novel provirus in the 2780i.5 cell line, 2780i.5 genomic DNA was digested with *Eco*RI, which does not cut the proviral genome, and size fractionated by agarose gel electrophoresis, and a fraction containing 9.0- to 9.5-kb DNA fragments was recovered from the gel by using the Geneclean kit (BIO-101). This DNA was digested with the restriction enzyme *Hha*I, and the resulting small DNA fragments were recircularized with T4 DNA ligase. Inverted PCR was carried our using primers 5′-GTGAATTCTCGCTTCTC GCTTCTGTTCGC-3′ and 5′-GAGAATTCTACAGGTGGG GTCTTTCA-3′, which were derived from the sequence of the proviral genome (nucleotides 8192 to 8213 and 7836 to 7817, respectively, with additional sequences corresponding to *Eco*RI restriction sites at the 5′ ends to facilitate cloning), resulting in amplification of genomic sequences flanking the 5′ long terminal repeat (LTR). PCR was carried out for 35 cycles of amplification, with each cycle consisting of 30 s each at 94, 55, and 72°C with 500 ng of template DNA, primers (1 μM), deoxynucleoside triphosphates (1 mM), reaction buffer, and Amplitaq polymerase (Perkin Elmer-Cetus). The 150-bp PCR product was digested with *Eco*RI and cloned into the *Eco*RI site of the plasmid vector in pBluescript (Stratagene).

First-strand cDNA for PCR amplification was prepared by using 2 μg of total RNA from the 2780i.5 cell line as the template, 0.1 μg random hexanucleotides (Promega), 40 U of RNasin (Promega), and 100 of Superscript Reverse Transcriptase (Bethesda Research Laboratories) in a total volume of 50 μl under the reaction conditions recommended by the supplier. Two microliters of this reaction mixture was used in an amplification PCR reaction using the conditions specified above with oligonucleotide primers from the U5 region of the LTR (5′-GGTCTCGCTGTTCCTTGGGAGG TC-3′) and from the second exon of the *Gfi-1* gene (5′-GCACGGTCTCCAGGCGCA-3′). A PCR product of approximately 270 bp was cloned into the PCR1000 vector supplied as part of the TA cloning kit (Invitrogen), according to the suppliers' recommendations.

Genomic DNA and cDNA cloning. A genomic clone was recovered from an F344 rat liver genomic DNA library in the λDash vector (Stratagene) by using standard procedures, as previously described (44). *Eco*RI fragments from the larger genomic clone were subcloned in pBluescript (Stratagene). A cDNA library was prepared with 5 μg of poly(A)$^+$ RNA from the tumor cell line 2775, using a commercially available cDNA synthesis kit (Pharmacia) and the λZapII vector (Stratagene). Following in vitro packaging, the recombinant phage particles were propagated in the *Escherichia coli* strain XL1-blue (Stratagene). The cDNA library was screened with probe A (Fig. 1). The two (of a total of five) longest (2.4-kb) cDNA clones were directly subcloned into pBluescript (SK−) by in vivo excision (41).

DNA sequence analysis. Bidirectional nested deletions of the cDNA clone C2B were generated by the exonuclease III/mung bean nuclease method (14). By using the deleted clones, both strands of the cDNA clone C2B were sequenced in their entirety. Additionally, the ends of the second clone, C1C, were sequenced. Clone C2B encompassed nucleotides 29 to 2407 of the *Gfi-1* sequence (see Fig. 6), while clone C1C contained nucleotides 1 to 2395. Sequencing reactions were carried out on alkali-denatured, double-stranded DNA by using the Sequenase version 2.0 sequencing kit (United States Biochemicals) and [^{35}S]dATP

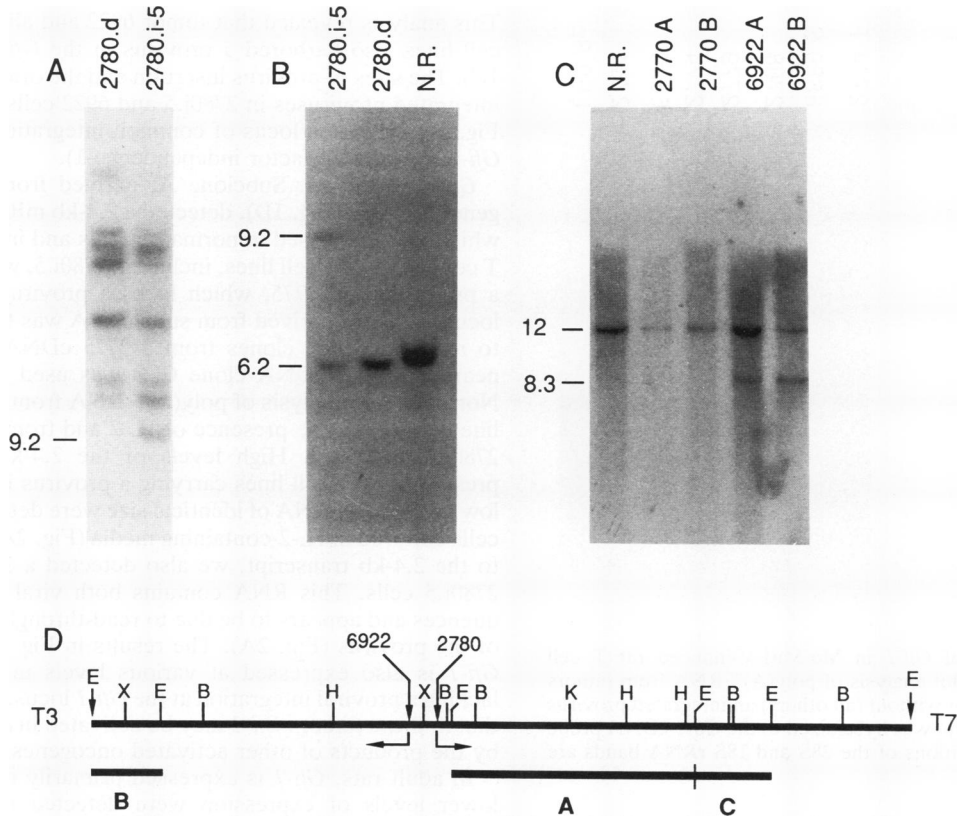

FIG. 1. Cloning of the *Gfi* locus. (A) Southern blot analysis of *Eco*RI-digested DNA from the IL-2-dependent parental cell line 2780d and from an IL-2-independent mutant, 2780i.5. The blot was hybridized to a Mo-MuLV LTR probe. The number on the left indicates the size (in kilobases) of the DNA fragment containing the novel integrated provirus. (B) Southern blot analysis of *Eco*RI-digested DNA from the IL-2d and IL-2i 2780 cell lines and from normal rat (N.R.). The probe was derived from sequences flanking the novel integrated provirus, which was cloned by inverted PCR. Numbers on the left indicate sizes (in kilobases). (C) Southern blot analysis of *Kpn*I-digested DNA from normal DNA and from multiple IL-2i T cell lymphoma lines hybridized to probe B (shown in panel D). Numbers on the left indicate sizes (in kilobases). (D) Restriction endonuclease map of a *Gfi-1* genomic clone obtained by screening an F344 rat genomic DNA library with a probe containing sequences flanking the novel integrated provirus. E, *Eco*RI; X, *Xho*I; B, *Bam*HI; H, *Hin*dIII; K, *Kpn*I. A, B, and C are subclones used as probes in the experiments described in this report. The arrows marked 6922 and 2780 indicate the site and orientation of provirus insertion in these tumors.

(Amersham). The products of the sequencing reactions were analyzed on 6 and 4% polyacrylamide–8 M urea gels.

Retroviral vector construction and infection of cell lines. The 2.4-kb *Eco*RI insert of the *Gfi-1* cDNA clone C2B, including the first ATG codon, was introduced in the sense orientation into the *Eco*RI site of the retrovirus vector LXSN (28). The resulting construct was introduced into the amphotropic packaging cell line PA317 by Ca^{2+} precipitation and selection for G418 resistance. 2780d cells were infected with the *Gfi-1*/LXSN retrovirus by cocultivation with the transfected PA317 cells and selection for resistance to G418. Infections were done in triplicate.

Nucleotide sequence accession number. The sequence of *Gfi-1* (see Fig. 4) has been submitted to the GenBank data base under accession number LO6986.

RESULTS

Selection of IL-2i mutants from IL-2d T cell lymphoma lines and identification of a new region of common integration. Our earlier studies on the progression of Mo-MuLV-induced rat T cell lymphomas suggested that mutagenesis caused by spontaneous, recurrent provirus insertions is a powerful genetic tool that can be used to identify genes whose expression is associated with selectable phenotypes. One such phenotype is IL-2-independent growth. To isolate IL-2i mutants, two IL-2d T cell lymphoma lines undergoing spontaneous insertional mutagenesis (2780d and 5675d) were selected for IL-2 independence using the strategies described in Materials and Methods. Thirty-three IL-2i cell lines were generated, three from 2780d and the remainder from 5675d. Genomic DNA from these mutants was digested with *Eco*RI (which does not cut the Mo-MuLV genome) and was hybridized after Southern blotting to a Mo-MuLV LTR probe. All contained at least one new provirus, and some carried as many as four new proviral integrations. Provirus losses were also observed in some of the mutants (data not shown).

The cell line 2780i.5 was chosen for further study, as it had acquired a single provirus, without a loss, during the transition from IL-2-dependent to IL-2-independent growth (Fig. 1A). The junction between the cellular and proviral sequences in the 9.2-kb *Eco*RI fragment containing the new provirus was cloned by inverted PCR (see Materials and Methods). The PCR-derived clone was used to probe a Southern blot of *Eco*RI-digested DNA from normal rat cells

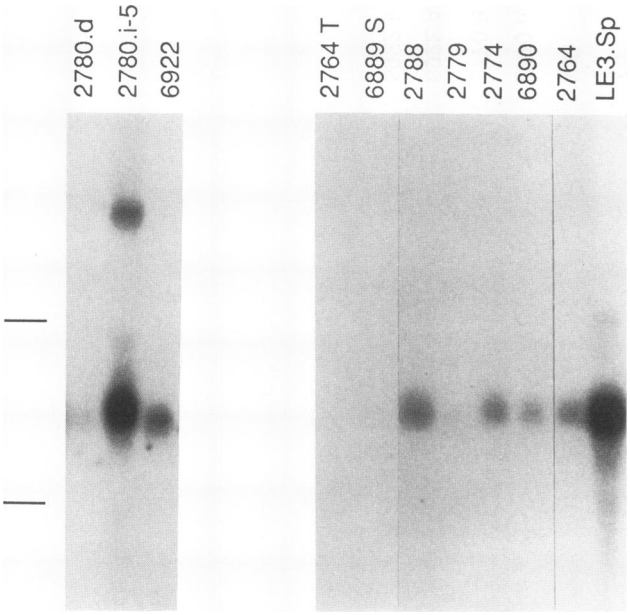

FIG. 2. Expression of *Gfi-1* in Mo-MuLV-induced rat T cell lymphomas. Northern blot analysis of poly(A)$^+$ RNA from tumors with (2780i.5 and 6922) or without (all others) an integrated provirus at the *Gfi-1* locus. The blot was hybridized to the *Gfi-1* cDNA probe C2B (see text). The positions of the 28S and 18S rRNA bands are indicated on the left.

and from the 2780d and 2780i.5 cell lines. This revealed a 6.2-kb germ line band and a 9.2-kb rearranged band which was present only in 2780i.5 cells (Fig. 1B). The PCR-derived probe was used to recover a 17.2-kb genomic clone (Fig. 1D). Subclones B and C were used to screen *Kpn*I-digested DNA from 24 independent Mo-MuLV-induced rat T cell lymphomas and/or from cell lines derived from them (18). This analysis revealed that tumor 6922 and all its derivative cell lines also harbored a provirus in the *Gfi-1* locus (Fig. 1C). The sites of provirus insertion and the orientation of the integrated proviruses in 2780i.5 and 6922 cells are shown in Fig. 1D. The new locus of common integration was named *Gfi-1* (for growth factor independence-1).

***Gfi-1* expression.** Subclone A, derived from the 17.2-kb genomic clone (Fig. 1D), detected a 2.4-kb mRNA transcript which was expressed in normal thymus and in several IL-2i T cell lymphoma cell lines, including 2780i.5, which contains a provirus, and 2775, which lacks a provirus in the *Gfi-1* locus. A probe derived from subclone A was therefore used to recover cDNA clones from a 2775 cDNA library. The near-full-length cDNA clone C2B was used as a probe in Northern blot analysis of poly(A)$^+$ RNA from the 2780d cell line cultured in the presence of IL-2 and from the cell lines 2780i.5 and 6922. High levels of the 2.4-kb RNA were present in both cell lines carrying a provirus in *Gfi-1*, while low levels of an RNA of identical size were detected in 2780d cells cultured in IL-2-containing media (Fig. 2A). In addition to the 2.4-kb transcript, we also detected a 10-kb RNA in 2780i.5 cells. This RNA contains both viral and *Gfi-1* sequences and appears to be due to read-through transcription of the provirus (Fig. 2A). The results in Fig. 2B show that *Gfi-1* is also expressed at various levels in eight tumors lacking a proviral integration at the *Gfi-1* locus. This suggests that in these tumors *Gfi-1* may be activated in *trans*, perhaps by the products of other activated oncogenes.

In adult rats, *Gfi-1* is expressed primarily in the thymus; lower levels of expression were detected in the spleen. Moreover, a short (1.7-kb) transcript was detected in testis (Fig. 3). Thus, *Gfi-1* expression in adults is restricted to lymphoid tissues and testes.

Sequence analysis and mechanism of activation of *Gfi-1*. Two 2.4-kb cDNA clones, C2B and C1C, were obtained from a cDNA library constructed from oligo(dT)-primed polyadenylated RNA from the T cell lymphoma line 2775. Sequencing the ends of these cDNAs revealed that clone

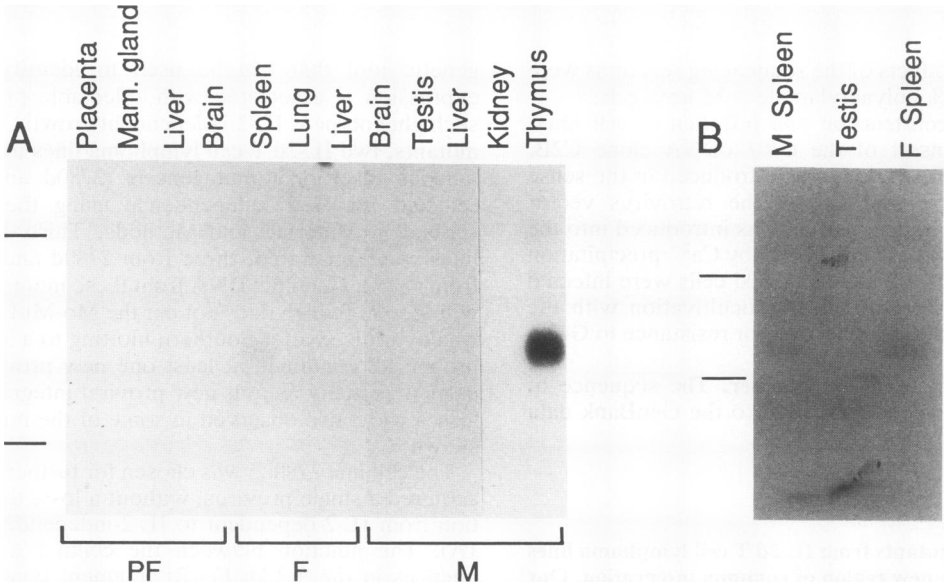

FIG. 3. *Gfi-1* expression in adult rat tissues. (A) Northern blot analysis of 5 μg of poly(A)$^+$ RNA hybridized to the *Gfi-1* cDNA probe C2B. (B) Extended exposure (14 days), revealing a smaller RNA transcript in testis RNA. PF, pregnant female; F, female; M, male. Positions of 285 and 185 rRNA bands are shown to the left.

C2B extended 12 nucleotides 3′ of C1C and lacked 28 nucleotides at the 5′ end. Both strands of clone C2B were sequenced. The C2B sequence, including the 28 nucleotides from the 5′ end of clone C1C (Fig. 4A), contains a single 423-amino-acid open reading frame, starting after an in-frame stop codon. Nucleotides −6 to +3 flanking the first AUG codon conform to the consensus for translational initiation (17). The open reading frame encodes a putative zinc finger DNA-binding protein with six zinc finger domains of the C_2H_2 type at its carboxy-terminal region. Comparison of the sequence of these domains with the consensus C_2H_2 zinc finger motif (10) revealed that the consensus is maintained, with the exception of a conservative Leu→Met substitution in the fourth domain (Fig. 4B). The seven-amino-acid linker sequence between the zinc finger motifs is also conserved (Fig. 4B).

As the provirus in 2780i.5 cells has integrated approximately 350 bp 5′ of the site of *Gfi-1* transcriptional initiation (data not shown) and the transcriptional orientation of the integrated provirus is the same as that of the *Gfi-1* gene (Fig. 1D), we hypothesized that activation of *Gfi-1* in these cells occurred via promoter insertion. Consistent with this is the detection of a 10-kb *Gfi-1* read-through transcript in 2780i.5 cells. To test this hypothesis, RNA from 2780i.5 cells was analyzed to determine whether it contained Mo-MuLV/*Gfi-1* hybrid mRNA transcripts. To this end, PCR was carried out using oligonucleotide primers from the U5 region of the LTR and from exon 2 of *Gfi-1* (the boundary between exons 1 and 2 is between nucleotides 16 and 17 of the *Gfi-1* sequence in Fig. 4A [data not shown]). The sequence of products of the amplification confirmed the synthesis of Mo-MuLV/*Gfi-1* hybrid mRNA transcripts which initiate in the proviral LTR and splice from the viral splice donor site at nucleotide position 205 to the splice acceptor site at the 5′ end of the second exon of *Gfi-1* (Fig. 5).

***Gfi-1* expression during T cell activation.** A gene activated by insertional mutagenesis could lead to IL-2 independence by one of three possible mechanisms, as suggested by the simple linear model of T cell activation shown in Fig. 6A: (i) Activation of the IL-2 gene either directly or indirectly; (ii) activation of genes expressed during T cell activation as a result of the interaction of IL-2 with its receptor; and (iii) activation of genes which induce IL-2-independent proliferation of T cells by triggering alternate pathways which bypass the need for earlier activation events.

To determine whether *Gfi-1* activity during T cell activation is required before or after the interaction of IL-2 with its receptor, RNA from 2780d and 2780i.5 cells was hybridized to a murine IL-2 probe under conditions of reduced stringency (Fig. 6B). RNA from ConA-stimulated rat splenocytes was used as a positive control. The results showed that *Gfi-1* has no effect on IL-2 expression in 2780 cells and suggested that if *Gfi-1* is normally expressed during T cell activation, its activity would be required after the interaction of IL-2 with its receptor. This was confirmed by hybridization of the *Gfi-1* probe C2B to a Northern blot of polyadenylated RNA from ConA-stimulated rat splenocytes. A dramatic increase in *Gfi-1* steady-state mRNA levels was detected. Low steady-state message levels in unstimulated splenocytes increased to maximum levels, comparable to those in 2780i.5 cells, by 50 h (Fig. 6C). Although the precise timing of events during ConA stimulation of bulk splenocytes is not possible, as the cells progress asynchronously toward mitosis, *Gfi-1* expression occurs well after the expression of the *IL-2* and the *IL-2R* genes at ~1 to 5 h (12, 20) and corresponds to the approximate timing of cells entering S phase (4). These data collectively suggest that *Gfi-1* is a transcription factor which is involved in regulating the expression of genes which are active in the S phase during cell cycle progression in T cells.

Effects of *Gfi-1* expression in 2780d cells. Three sublines of 2780d cells, which were infected with the *Gfi-1*/LXSN retroviral construct (see Materials and Methods), were obtained. Two of the three infected sublines contain multiple full-size copies of the unrearranged construct. The third subline appears to carry a provirus with a small deletion in the 3′ untranslated region (data not shown). All three express high levels of *Gfi-1*/LXSN RNA transcripts, which on the basis of their size and structure should contain the entire *Gfi-1* open reading frame. The three sublines of the 2780 cells infected with the *Gfi-1*/LXSN virus were cultured in triplicate, in parallel with the control 2780d cells, in IL-2-free media. The 2780d cells cultivated in the absence of IL-2 went into a stationary phase and finally died after a period of 1 week to 10 days. The infected sublines underwent a similar crisis. However, in ~50% of the cases (four of nine cultures), IL-2i subpopulations of these cells were selected, establishing IL-2i sublines. To confirm these results, the experiment was repeated with duplicate cultures of the three independently infected lines. After removal of IL-2, four of six infected cultures gave rise to IL-2-independent lines. These data suggest that expression of *Gfi-1* in 2780d cells via retrovirus-mediated gene transfer contributes to the emergence of the IL-2i phenotype.

DISCUSSION

Resting T lymphocytes can be triggered by a variety of stimuli to undergo a series of changes which collectively define the process of T cell activation. These changes include morphological transformation, which occurs within 12 h, progression to cell division, which occurs within 24 to 48 h, and differentiation to acquire effector function, which occurs within a week to 10 days from the initial stimulus. Like other biological processes, T cell activation is characterized by a commitment step which occurs within 2 h of the initial stimulus and irreversibly commits the cells to the late activation events. The commitment step coincides with the expression of IL-2 and its high-affinity receptor, a landmark event that divides T cell activation into early and late stages (for reviews, see references 1, 8, 42, 47).

The classical signal for T cell activation is delivered via the T cell receptor/CD3 complex and accessory molecules (9). Similar signals can be delivered by mitogenic lectins such as phytohemagglutinin and ConA or by antibodies to the T cell receptor/CD3 complex (16, 26, 39, 45, 48, 50). Stimulation of T cells via antigen plus major histocompatibility complex or lectins is sufficient for the induction of the IL-2 receptor in resting T cells. However, to induce both IL-2 and IL-2R, which is a prerequisite for cellular proliferation, additional independent signals are required. Such additional signals are delivered by accessory cells or agents that activate protein kinase C (8, 37, 51, 53). The signal requirements for T cell activation may also be influenced by the physiological state of the cell. Thus, while the activation of resting T cells requires two independent stimuli, a single stimulus is sufficient to trigger the proliferation of partially activated cells, such as $CD4^-$ $CD8^+$, $IL\text{-}2R^+$ T cells (25, 52).

Although significant progress has been made in recent years, the process of T cell activation continues to be poorly understood. To characterize this process, we have initiated studies using a novel, insertional mutagenesis-based genetic strategy. This strategy was designed on the basis of earlier

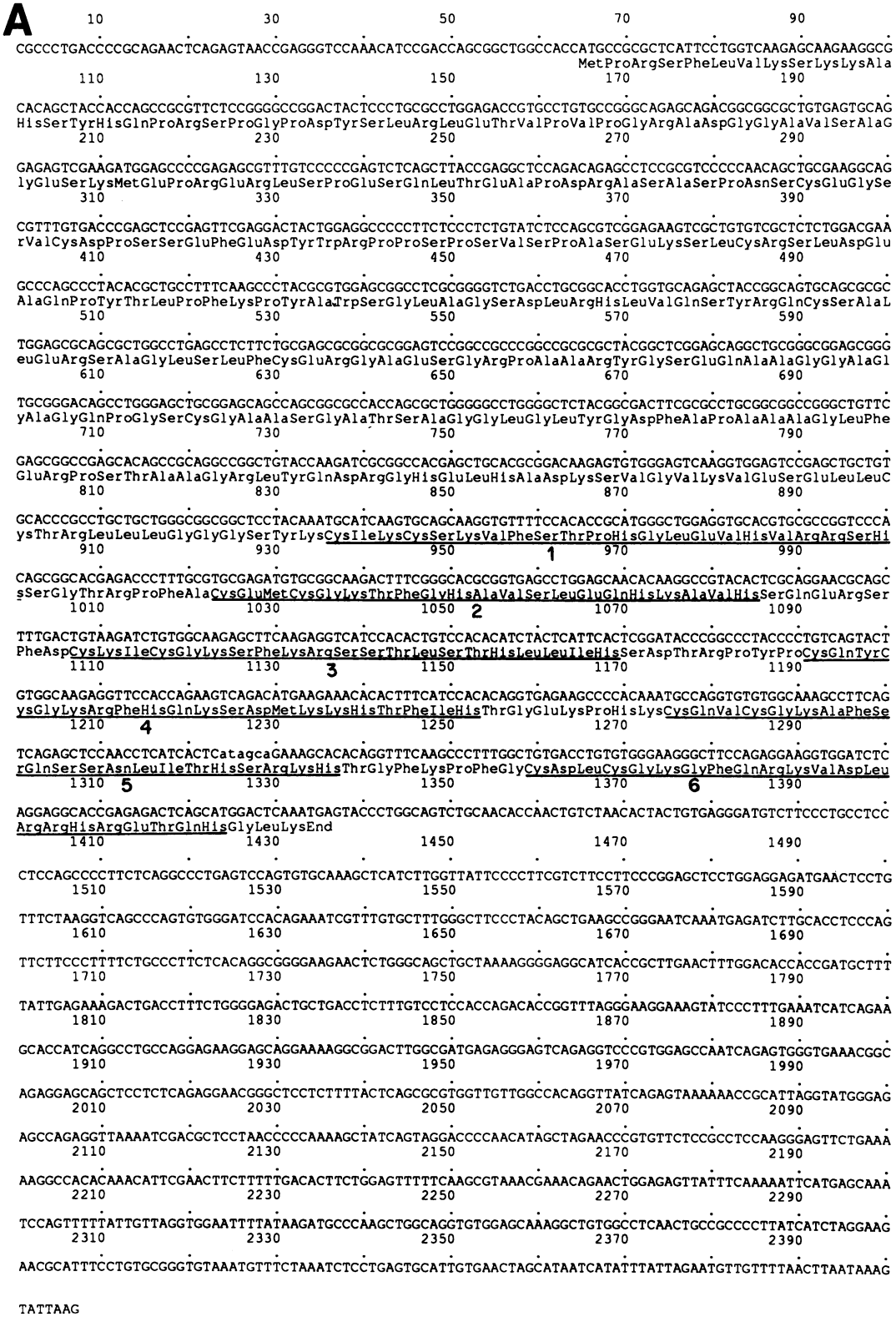

FIG. 4. Sequence of the *Gfi-1* cDNA. (A) Nucleotide and deduced amino acid sequence of the *Gfi-1* cDNA. Nucleotides 1 through 29 were derived from sequencing the 5′ end of clone C1C, while nucleotides 29 through 2407 were derived from clone C2B. The six zinc finger domains are underlined. (B) Amino acid sequence of the six zinc finger domains (left) and linker regions (right). Conserved amino acids are printed in bold letters. The C_2H_2-type zinc finger and linker consensus sequences are shown at the bottom.

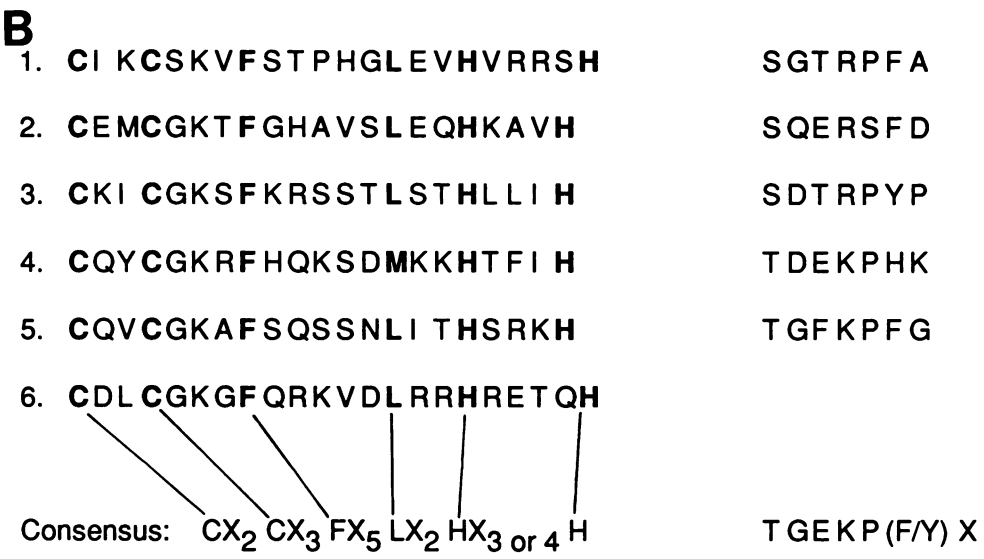

FIG. 4—*Continued.*

observations suggesting that spontaneous insertional mutagenesis can be used to identify genes associated with selectable phenotypes in Mo-MuLV-induced rat T cell lymphomas (3, 32a). The selectable phenotype in the experiments described in this report was IL-2 independence. In applying this strategy to the dissection of the process of T cell activation, we assumed that the IL-2d cell lines, although transformed, provide an accurate model of response to IL-2.

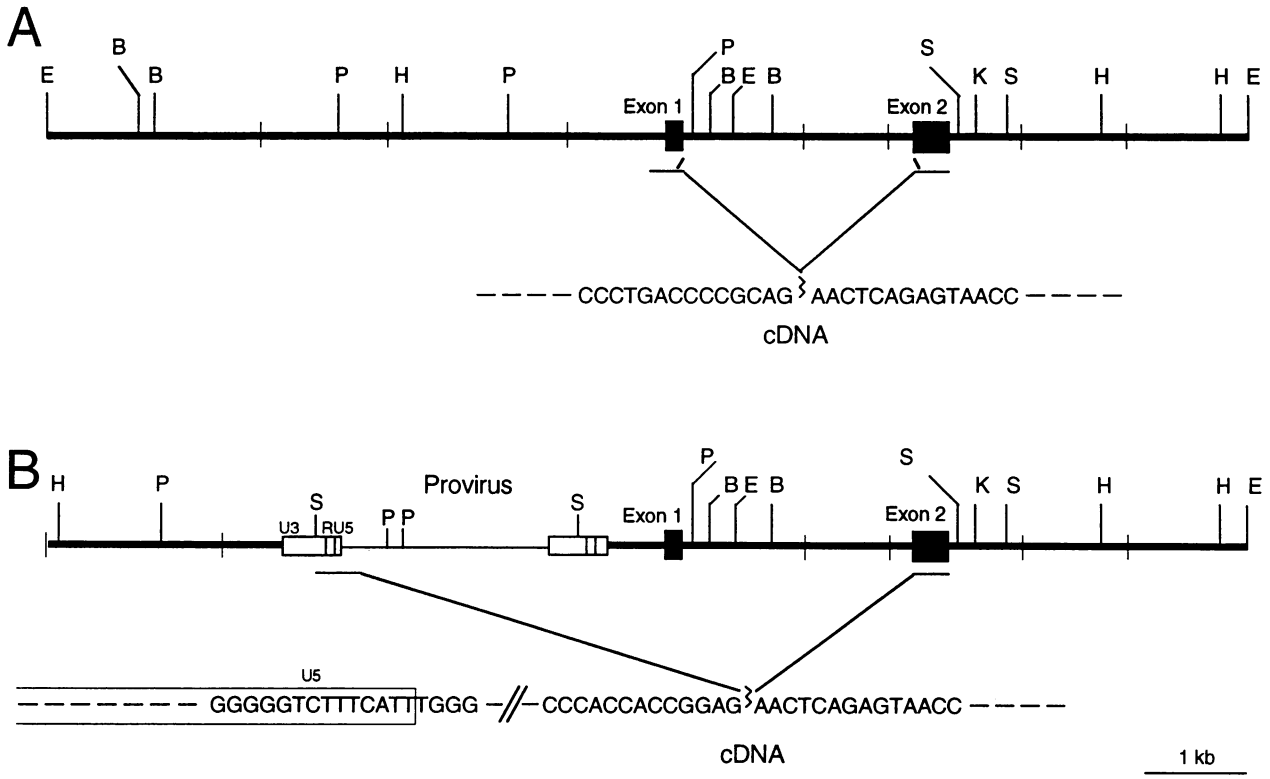

FIG. 5. Activation of *Gfi-1* by promoter insertion. (A) Restriction endonuclease map of the central portion of the *Gfi-1* genomic DNA clone. Exons 1 and 2 map within the *Eco*RI fragment A (Fig. 1D). The cDNA sequence at the splice junction between exons 1 and 2 is shown below the map. (B) In the cell line 2780i.5, provirus integration occurred 350 bp 5′ of the transcriptional start site and in the same transcriptional orientation as the *Gfi-1* gene (upper line). Transcription starts at the 5′ proviral LTR. Splicing takes place between the splice donor site 5′ of the viral *Gag* gene and the splice acceptor site of the second *Gfi-1* exon, therefore deleting exon 1 from the mature message. The bottom line shows the nucleotide sequence of the novel splice junction. E, *Eco*RI; P, *Pst*I; H, *Hin*dIII; B, *Bam*HI; K, *Kpn*I; S, *Sst*I.

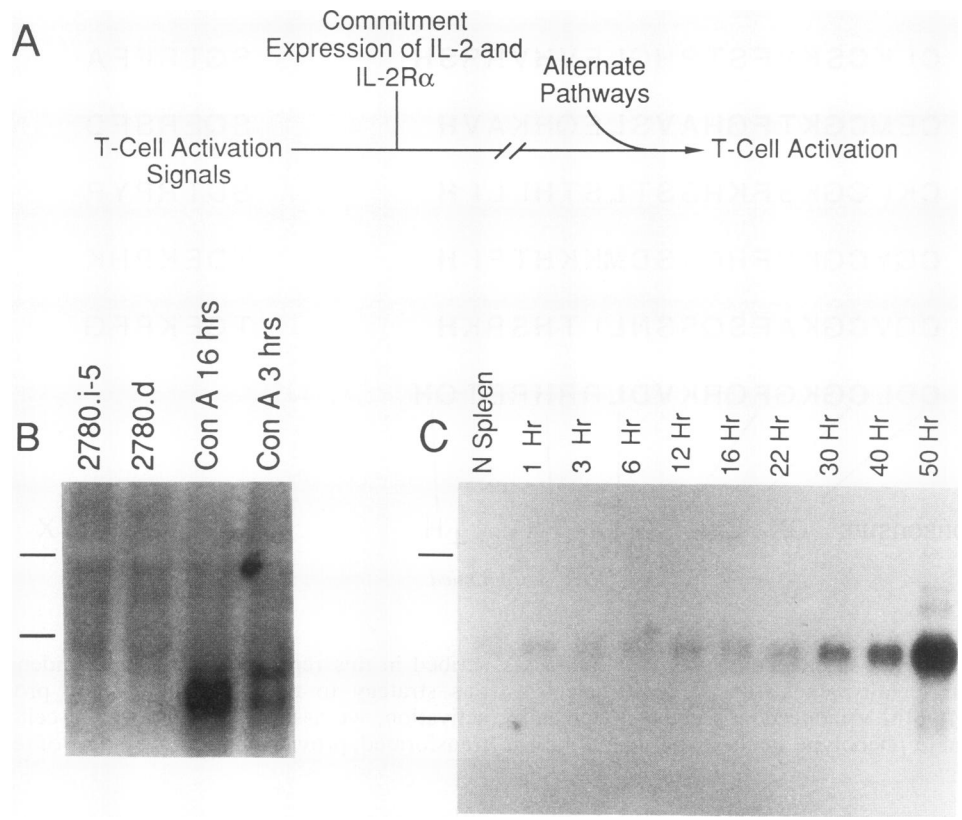

FIG. 6. *Gfi-1* expression during T cell activation. (A) Schematic diagram of events occurring during T cell activation. (B) Northern blot analysis of total RNA from the 2780 IL-2d and IL-2i cell lines and splenocytes 3 and 16 h after ConA stimulation. The blot was probed with a mouse IL-2 cDNA under conditions of reduced stringency. Under these conditions, two IL-2 transcripts were detected. The abundance of the larger transcript is markedly reduced at the 16-h time point. (C) Northern blot analysis of poly(A)$^+$ RNA from rat splenocytes before (N Spleen) and at various times after ConA stimulation. The blot was hybridized to the *Gfi-1* cDNA clone C2B. A probe for the rat ribosomal protein rpL32 was hybridized to stripped blots (panels B and C) to normalize for equal loading and transfer of RNA (data not shown). Positions of 28S and 18S rRNA are shown to the left of panels A and B.

This is a reasonable assumption given that the phenotype of the cell lines used (CD4$^-$ CD8$^+$ IL-2R$^+$) coincides with the phenotype of partially activated T cells (19, 24, 33).

The *Gfi-1* gene encodes a protein containing six zinc finger domains of the C_2H_2 type in its C-terminal region, indicating that it is a DNA-binding protein which may be involved in transcriptional regulation. The N-terminal region of this protein could function either as a transcriptional activator or as a transcriptional repressor, an issue which is currently being addressed experimentally. Interestingly, however, the region between amino acids 158 and 209 is rich in glycine and alanine residues (30 of 51 [59%]); glycine- and alanine-rich regions have been observed in the non-DNA-binding domains of transcriptional repressors (22, 29, 32), and this feature suggests that the Gfi-1 protein may down-regulate the expression of its target genes.

The activation of *Gfi-1* by promoter insertion (2780i.5 cells) results in the formation of a Mo-MuLV/*Gfi-1* hybrid transcript which lacks exon 1. The removal of exon 1 from the activated *Gfi-1* mRNA transcript may play a role in the regulation of mRNA processing and/or translational efficiency.

Northern blot analysis indicates that the expression of *Gfi-1* in adult animals is restricted to lymphoid tissues and testes. Steady-state levels of *Gfi-1* mRNA from Con-A-stimulated splenocytes begins to increase by 12 h, reaching very high levels, comparable to those seen in 2780i.5 cells, by 50 h. Precise timing of events during ConA stimulation of bulk splenocytes is not possible, as the cells progress asynchronously towards mitosis. However, the increase in *Gfi-1* steady-state message levels occurs well after IL-2 and IL-2R expression (1 to 5 h) (12, 20) and corresponds to the approximate timing of cells entering S phase (4). In agreement with this observation, the activation of *Gfi-1* does not lead to IL-2 expression in 2780i.5 cells. Collectively, these data suggest that Gfi-1 may be a transcription factor which modulates expression of genes involved in the S phase of the cell cycle in activated T cells.

Transfer of a transcriptionally active *Gfi-1* gene in 2780d cells induced a partial IL-2i phenotype. Cultivation of the 2780 cells carrying the exogenously introduced *Gfi-1* gene in medium without IL-2 allowed the rapid selection of IL-2i cells. This could be due to the selection of cells that express high levels of *Gfi-1*. Alternatively, it could be due to additional mutations affecting genes that cooperate with *Gfi-1* in the development of the IL-2i phenotype. Observations similar to the ones described here have also been made for B cells expressing *Bcl-2*. *Bcl-2* is expressed in proliferating lymphocytes (36) and in B cell lymphomas harboring the translocation t(14;18) (q32;q21) (2, 7, 46). Introduction of *Bcl-2* into early pre-B cells results in cells which are partially growth factor (IL-7) independent and which upon continu-

ous culture give rise to fully growth factor-independent cell lines (5).

Tumor progression usually proceeds in a step-wise manner (3, 30, 32a). In tumors dependent on physiological growth factors, the emergence of growth factor independence often marks the beginning of a more malignant phase of the disease (43). The release of malignant T cells from IL-2 dependence is likely to be associated with an enhancement of their malignant potential. This is suggested by the finding that high-grade T cell lymphomas may be cultured without IL-2, while low grade lymphomas require IL-2 for growth (11, 34). Similarly, human T-cell lymphotropic virus type I (HTLV-1)-infected T cells are IL-2 dependent, while high-grade T cell neoplasms developing in HTLV-1-infected individuals are IL-2 independent (13, 38). We conclude that *Gfi-1* and other genes involved in the progression of IL-2-dependent T cell lymphoma lines to IL-2-independent growth may also be involved in tumor progression.

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