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Replacement of Interleukin-2 (IL-2)-Generated Mitogenic Signals by a Mink Cell Focus-Forming (MCF) or Xenotropic Virus-Induced IL-9-Dependent Autocrine Loop: Implications for MCF Virus-Induced Leukemogenesis

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In earlier studies, we have shown that superinfection of an interleukin-2 (IL-2)-dependent, Moloney murine leukemia virus (MoMuLV)-induced rat T-cell lymphoma line (4437A) with mink cell focus-forming (also called polytropic) murine retroviruses induces rapid progression to IL-2-independent growth. In this report, we present evidence that the vast majority (>90%) of the IL-2-independent lines established from polytropic or xenotropic virus-infected 4437A cells carry provirus insertions in the 3′ untranslated region of the IL-9 receptor gene (*Gfi-2* [for *growth factor independence-2*]/IL-9R). Prior to superinfection, the cells express neither *IL-9* nor *IL-9R*. Following superinfection and provirus insertion in the *Gfi-2/IL-9R* locus, the cells express high levels of mRNA transcripts with a truncated 3′ untranslated region which are predicted to encode the normal IL-9R protein product. The same IL-2-independent cells also express IL-9 which is induced by an insertional mutagenesis-independent mechanism. The establishment of an IL-9-dependent autocrine loop was sufficient to render the cells IL-2 independent, as suggested by the finding that 4437A cells, expressing a stably transfected *Gfi-2/IL-9R* construct, do not require IL-2 when maintained in IL-9-containing media. Additional experiments designed on the basis of these results showed that *IL-9* gene expression is induced rapidly following the infection of 4437A cells by polytropic or xenotropic viruses and occurs in the absence of selection for IL-2-independent growth. Taken together, these data suggest that infection of 4437A cells by mink cell focus-forming or xenotropic viruses induces the expression of IL-9, which in turn rapidly selects the cells expressing the IL-9 receptor through an insertional mutagenesis-dependent mechanism. Given that both the polytropic and xenotropic viruses can induce the IL-9-dependent autocrine loop, the reduced ability of the xenotropic viruses to rapidly induce IL-2 independence in culture and tumors in animals is likely to be the result of their lower growth rates.

Early studies of viral gene expression in highly leukemia-prone mouse strains led to the isolation of a class of recombinant retroviruses which were thymotropic and had an extended host range. These viruses, which arise by recombination between replicating ecotropic viruses and endogenous murine retroviral loci, were named either mink cell focus-forming viruses (MCF) because they form cytopathic foci in mink lung cells (17) or polytropic because they infect mouse as well as heterologous cells in culture (18). The MCF viruses and the ecotropic viruses from which they are derived differ mainly within the envelop (*env*) gene and the long terminal repeat (LTR) (12, 13, 17, 18). The *env* and LTR both contribute to the leukemogenic potential of the MCF viruses as confirmed by testing natural polytropic virus isolates and recombinants constructed in vitro (7, 19, 20).

Several mechanisms have been proposed to explain the role of the MCF envelope glycoprotein in leukemogenesis (34). (i) A virus with a novel *env* gene may circumvent superinfection resistance in ecotropic virus-infected cells. Such cells, representing the putative progenitors of the tumor cells, are proliferating actively in the spleen in the early stages of virus infection. Their efficient infection by MCF viruses arising in the thymus may be the critical step responsible for cellular transformation via insertional mutagenesis (10, 11). (ii) Simultaneous infection by ecotropic and MCF viruses may suppress the growth of bone marrow stromal cells, which in turn inhibits hematopoiesis. According to this hypothesis, the virus-induced proliferation of hematopoietic cells in the spleen, a required manifestation of the early stages of oncogenesis, is the result of compensatory extramedullary hematopoiesis (4). (iii) Interaction of the MCF envelope gene product with membrane receptors may stimulate cell growth (36).

The last of these hypotheses received strong support recently when it was shown that the defective envelope glycoprotein (gp55) of the spleen focus-forming virus, which displays significant sequence similarity to the envelope glycoprotein of the MCF viruses, binds to and activates the erythropoietin (Epo) receptor (24). This observation led to experiments which showed that (i) superinfection of an interleukin-2 (IL-2)-dependent rat T-cell lymphoma line with polytropic murine retroviruses rapidly confers IL-2 independence (33) and (ii) expression of the polytropic virus envelope glycoprotein in IL-3-dependent T-cell lymphoma lines, transfected with an IL-2R expression construct, confers IL-3 independence (25).

In this report, we present evidence that infection of 4437A cells with MCF and xenotropic viruses leads to the rapid induction of *IL-9* gene expression. This, combined with the withdrawal of IL-2, rapidly selects for cells in which the IL-9 receptor is upregulated through an insertional mutagenesis-dependent mechanism. The autocrine loop IL-9/IL-9R can replace IL-2 in maintaining T-cell growth.

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MATERIALS AND METHODS

Cells and viruses. 4437A is an IL-2-dependent T-cell lymphoma line established from a Moloney murine leukemia virus (MoMuLV)-induced rat thymoma (22, 33). The uninfected and virus-infected *Mus dunni* cells were kindly provided by J. Coffin (Tufts University School of Medicine) and have been described previously (33). COS-1 cells were kindly provided by J. Chernoff (Fox Chase Cancer Center). The 4437A cells were maintained in RPMI medium supplemented with horse serum (10%), penicillin (50 U/ml), streptomycin (50 μg/ml), kanamycin (100 μg/ml), and IL-2 (50 U/ml). The cocultivation of 4437A cells with *M. dunni* cells was carried out in the same media (33). *M. dunni* and COS-1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin (50 U/ml), streptomycin (50 μg/ml), and kanamycin (100 μg/ml).

Southern and Northern (RNA) blotting. Cellular genomic DNA and RNA were prepared as previously described (14, 29, 33). Southern and Northern blots were prepared and analyzed by standard procedures as previously described (14, 29, 33).

Construction and screening of genomic and cDNA libraries. Partial genomic DNA libraries were constructed and screened as previously described (14, 29). Briefly, *Sal*I-digested genomic DNA from the MCF virus-infected, IL-2-independent 4437A subline mM9 was fractionated by agarose gel electrophoresis. An approximately 12-kb fraction was subsequently ligated into the *Sal*I site of the bacteriophage lambda vector λKT3, previously described (3). Following in vitro packaging and amplification in *Escherichia coli* LE392, the recombinant phage were screened with a 625-bp *Bam*HI-*Eco*RI MCF *env*-specific probe (33). This yielded a 12.1-kb *Sal*I genomic clone containing proviral and flanking cellular DNA (Fig. 1B).

cDNA libraries were constructed from poly(A)$^+$ RNA from normal Fisher 344 rat thymus and the MCF virus-infected IL-2-independent 4437A subline M9 with a cDNA cloning kit (Stratagene). The cDNA was synthesized with an oligo(dT) primer and was ligated into the bacteriophage lambda vector lambda uni-ZapXR (Stratagene). Both cDNA libraries were screened with the IL-9R genomic probe A (Fig. 1B). Initial characterization of the clones obtained from the screening of the normal thymus cDNA library identified at least one nearly full-length clone (clone 5a). Moreover, initial screening of the M9 library showed that almost all the isolated clones represented promoter insertion-induced transcripts containing only sequences derived from the 3′ untranslated region of the gene. This suggested that provirus insertion occurred in the 3′ untranslated region of the *IL-9R* gene, and therefore, it would be likely to give rise to 3′ truncated mRNA transcripts. To obtain cDNA clones of these transcripts, the M9 cDNA library was rescreened with a 1.3-kb cDNA probe derived from the 5′ end of clone 5a. The M9 library was also screened with a mouse IL-9 probe kindly provided by D. Cossman (Immunex). In vivo excision of individual clones was carried out as previously described (14, 19).

Cloning virus-host junctions by PCR. Virus-host junctions were amplified from poly(A)$^+$ RNA (2) isolated from individual MCF virus-infected 4437A sublines (33). PCR primers were derived from the *IL-9R* cDNA sequence 5′CTGCAGC TAGCTTGCTAAGCC3′) and the MCF virus LTR (3′CATC CTCTCTACAGTGTG5′). Amplification was carried out for 30 cycles (denaturing for 0.5 min at 94°C, annealing for 1 min at 50°C, and extension for 2.5 min at 72°C). Following cloning into the plasmid vector pBluescript SK$^-$, individual clones were sequenced.

DNA sequencing. DNA sequencing was carried out on alkali-denatured double-stranded DNA by the dideoxy chain termination method and by employing Sequenase version 2 (U.S. Biochemicals) and [α-^{35}S]dATP (NEN Dupont) (14, 29). The 3,692-bp *Gfi-2* cDNA clone 5a was sequenced in full with nested oligonucleotide primers.

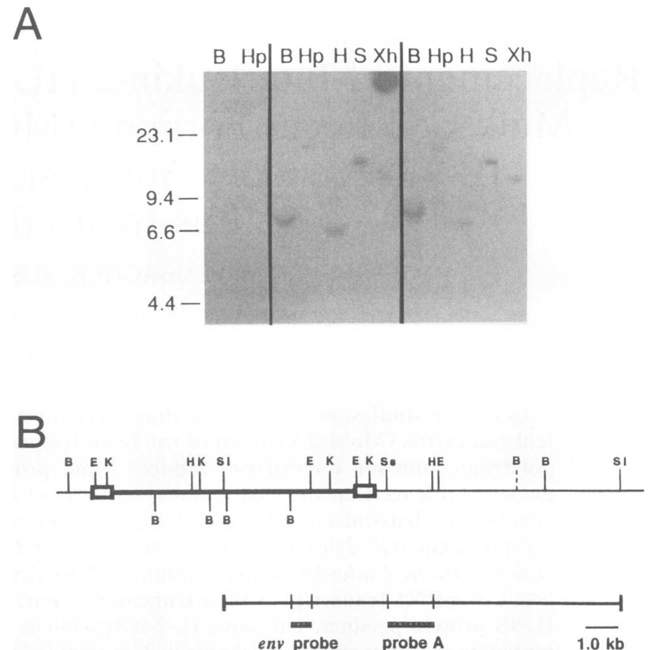

FIG. 1. Identification and cloning of the *Gfi-2* locus. (A) Genomic DNA from nonsuperinfected 4437A cells and two MCF virus-infected, IL-2-independent 4437A sublines (mM9 and M6) was digested with *Bam*HI (B), *Hpa*I (Hp), *Hin*dIII (H), *Sal*I (S), and *Xho*I (Xh), and it was hybridized to a 625-bp *Bam*HI-*Eco*RI MCF *env* probe (33). The numbers on the left are marker lengths in kilobases. (Left panel) Nonsuperinfected 4437A cells. (Middle panel) mM9 cells. (Right panel) M6 cells. (B) Restriction endonuclease map of the integrated mMCF-V33 provirus and the flanking cellular DNA cloned from the IL-2-independent 4437A subline mM9. The dashed line identifies a polymorphic *Bam*HI site (also see Fig. 2). Alignment of the *Sal*I genomic clone with the virus-*Gfi-2* restriction endonuclease map is shown in the bottom diagram. The solid bars under the diagram of the *Sal*I clone define the MCF *env*-specific probe and probe A. B, *Bam*HI; E, *Eco*RI; K, *Kpn*I; H, *Hin*dIII; SI, *Sal*I; Ss, *Sst*I.

Expression constructs and transfection. To express the *IL-9R* gene in mammalian cells, a 2.2-kb *Eco*RI cDNA clone derived from the M9 cDNA library was introduced into the *Eco*RI site of the retrovirus vector pBABE puro (27). The 5′ end of this cDNA clone starts at nucleotide position −22 relative to the translation initiation (ATG) codon (see Fig. 5). The 3′ end is truncated by comparison with the normal thymus-derived cDNA clones and contains MCF virus LTR sequences. The *Gfi-2* expression construct was transferred into 4437A cells by a modified calcium phosphate-mediated transfection protocol (5, 6). To express IL-9 in mammalian cells, a 0.6-kb full-length *Eco*RI-*Sal*I, IL-9 cDNA clone was subcloned in the respective sites of the mammalian expression vector pCMV5 (pIL9CMV5) (1). This construct was transferred into COS-1 cells by the DEAE-dextran-chloroquine method (26). Briefly, 24 h prior to transfection COS-1 cells were transferred into 60-mm-diameter petri dishes at a density of 7.5×10^5 cells per dish. Immediately before transfection, the cells were washed twice with phosphate-buffered saline and overlaid with 2 ml of Opti-MEM medium (Gibco-BRL) containing DEAE-

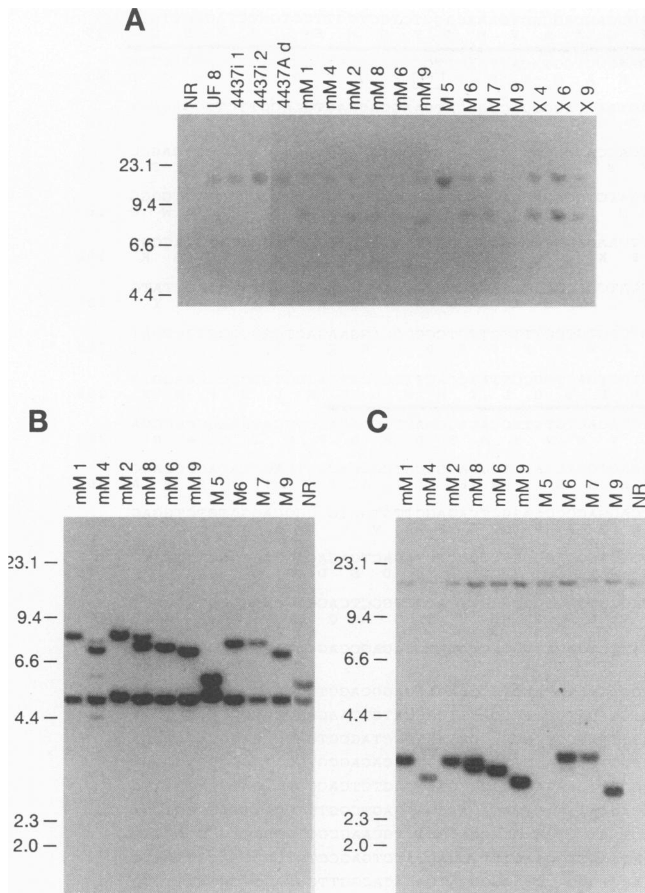

FIG. 2. *Gfi-2* rearrangements in 11 MCF virus-infected, IL-2-independent 4437A sublines. (A) Genomic DNA was digested with *Kpn*I and hybridized to probe A (Fig. 1). (B) Genomic DNA digested with *Bam*HI. (C) Genomic DNA digested with *Eco*RI. All the cell lines with one exception (M5) carry rearrangements in the *Gfi-2* locus. Three cell lines (mM4, mM8, and M6) appear to contain multiple subclones with independent provirus insertions in the *Gfi-2* locus. Note that normal Long Evans rats (NR) and 4437A cells (data not shown) exhibit a *Gfi-2* restriction fragment length polymorphism and that all *Gfi-2* provirus insertions occur in one of the two *Gfi-2* alleles, suggesting that the other one may be inactive (B). NR, normal rat; UF8, uninfected IL-2-independent 4437A cells number 8. 4437i-1 and 4437i-2 define two IL-2-independent 4437A sublines which were isolated spontaneously from nonsuperinfected cells. 4437Ad is the parental IL-2-dependent cell line; mM1, mM4, mM2, mM8, mM6, and mM9 are six IL-2-independent cell lines isolated from cultures infected with the modified MCF virus V33; M5, M7, M6, and M9 are four IL-2-independent MCF-247-infected sublines; while X4, X6, and X9 are three xenotropic virus-infected sublines. The numbers on the left of each panel are marker lengths in kilobases.

dextran (Pharmacia) (400 μg/ml) and chloroquine (Sigma) (0.1 mM). Transfections were carried out with 5 μg of DNA per dish. To supplement 4437A cell cultures with IL-9, we cultivated the 4437A cells in media supplemented with supernatants of COS-1 cells transfected with the IL-9 expression construct pIL9CMV5 (0.1%, vol/vol). Supernatants of COS-1 cells transfected with the pCMV5 vector were used as controls.

Nucleotide sequence accession numbers. The sequence of the *Gfi-2* cDNA was submitted to GenBank under accession number L36459. The rat IL-9 cDNA was submitted under accession number L36460. The sequences of the virus *Gfi-2*

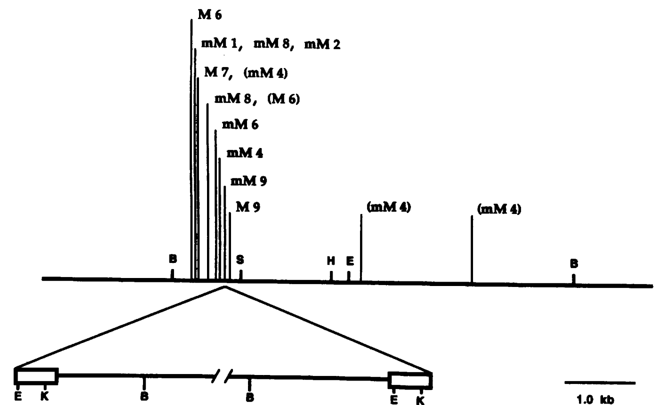

FIG. 3. Sites of provirus insertion in the *Gfi-2* locus in individual IL-2-independent 4437A sublines. Sublines mM4, mM8, and M6 contain more than one population of cells with different provirus insertions in the *Gfi-2* locus. mM8 is composed of two equally represented cell populations. mM4 and M6 are composed of one dominant and several minor cell populations. The minor populations are shown in parentheses. B, *Bam*HI; E, *Eco*RI; K, *Kpn*I; H, *Hin*dIII; S, *Sst*I.

junctions in 4437A sublines M6, mM1, mM6, and M9 were also submitted to GenBank (accession numbers L36600, L36598, L36597, and L36599, respectively).

RESULTS

Earlier studies have shown that MCF viruses play an important role in the induction of T-cell lymphomas in mice (7, 12, 13, 17–20, 34). To define the role of MCF viruses in leukemogenesis, we have previously examined whether infection of an IL-2-dependent rat T-cell lymphoma line (4437A) with MCF or xenotropic viruses affected its requirement for IL-2. Infection of the 4437A cells with the polytropic viruses MCF-247 and mMCF-V33 or the xenotropic virus NZB-X was achieved by cocultivation with virus-infected *M. dunni* embryo fibroblasts. 4437A cells, cocultivated with uninfected *M. dunni* embryo fibroblasts, were used as controls. Following 5 days of cocultivation, the IL-2 was withdrawn from the medium. Upon IL-2 withdrawal, the uninfected and NZB-X virus-infected cells went through a crisis period characterized by massive death, while all the independently maintained cultures of polytropic virus-infected cells became IL-2 independent with a minimal crisis. These data showed that the polytropic viruses can alter the growth requirements of T-cell lymphomas (33).

Southern blot analysis of genomic DNA from the MCF virus-infected IL-2-independent cells, 2 weeks after the selection was applied, revealed the outgrowth of clonal cell populations (33). Two MCF virus-infected IL-2-independent lines, derived from independent infections (mM9 and M6), were shown to be clonally derived, in that they carried a single integrated polytropic provirus. Digestion of genomic DNA from these cell lines with five restriction endonucleases revealed that the provirus had targeted the same locus (*Gfi-2*) in both (Fig. 1A).

The single integrated polytropic provirus from one of the IL-2-independent lines (mM9) was cloned by using the 625-bp *Bam*HI-*Eco*RI MCF-247 *env*-specific probe. Figure 1B shows a restriction map of the integrated provirus and the flanking cellular DNA. Hybridization of the cellular DNA probe A derived from this clone to *Kpn*I-digested genomic DNA from all the xenotropic and polytropic virus-infected IL-2-indepen-

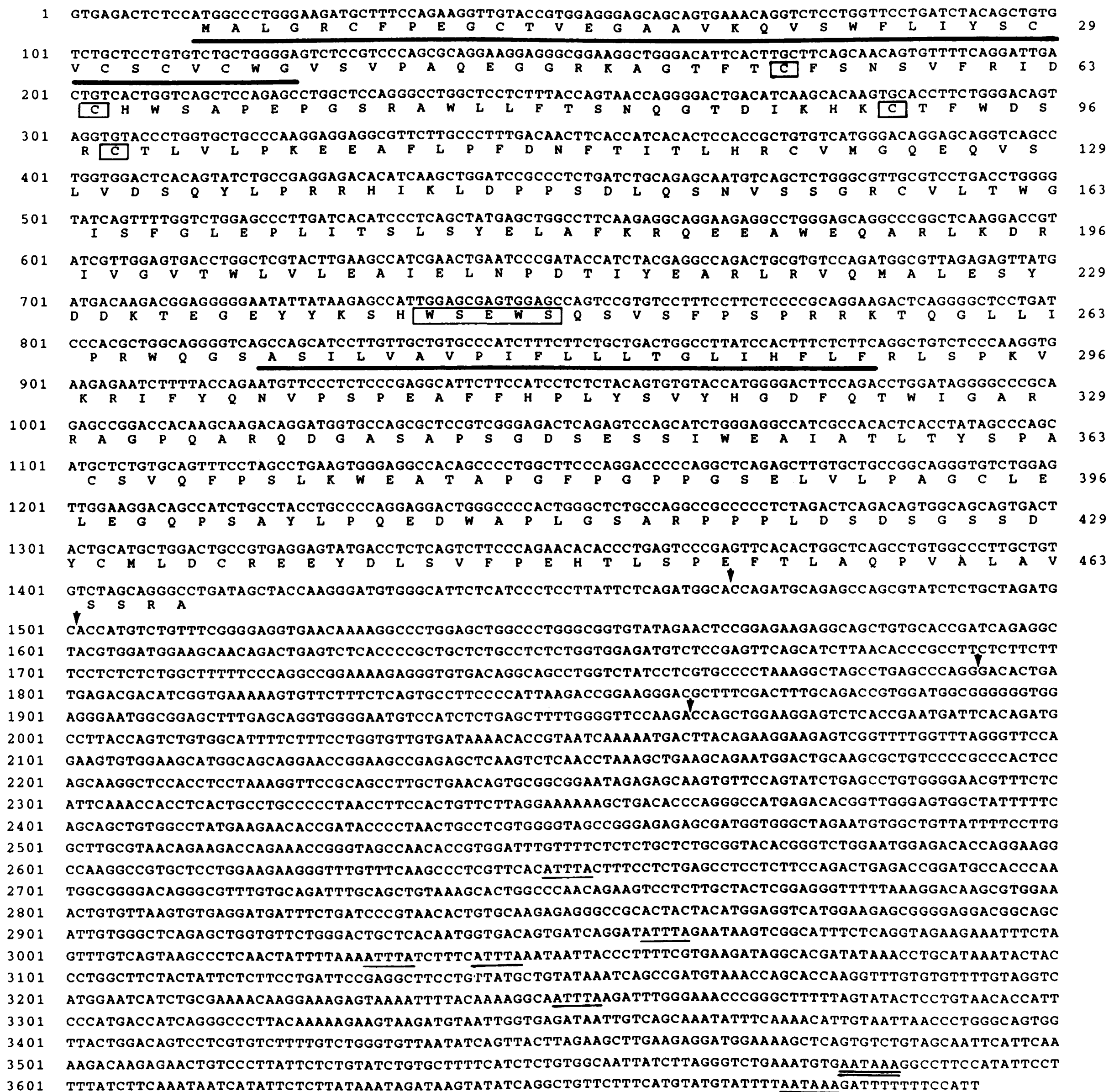

FIG. 4. *Gfi-2* encodes the rat homolog of the IL-9 receptor. Sequences characteristic of all the hematopoietic receptors are boxed. The leader sequence and the transmembrane domain are underlined with bold lines. Instability sequence motifs are underlined with thin lines, and polyadenylation signals are underlined with double lines. The sites of provirus integration in four MCF virus-infected IL-2 independent sublines are shown by arrows. Probe A corresponds to sequences from nucleotide positions 2142 to 3453 in the 3′ untranslated region of the gene. The 1.3-kb 5′ probe extends from nucleotide positions 1 to 1272.

dent cell lines revealed rearrangements in all but one (Fig. 2A). Further analysis showed that at least three of the lines progressing to IL-2 independence were composed of multiple populations of cells, each carrying independent provirus insertions in the *Gfi-2* locus (Fig. 2B and C). The transcriptional orientations of all the integrated proviruses were the same. A summary of the results, with the relative map position of the integration sites within the *Gfi-2* locus, is shown in Fig. 3. This analysis also showed that the 4437A cells were heterozygous for a *Gfi-2* locus restriction fragment length polymorphism (Fig. 2B). Interestingly, all the *Gfi-2* provirus insertions occurred in the same allele. Given that provirus insertion results in a gain of function mutation, this result suggests that the nonfavored allele may be inactive. The significance of this finding is currently under further investigation.

Northern blot analysis of RNA from normal rat tissues using the genomic *Gfi-2* probe A revealed that *Gfi-2* expression is restricted to thymus and spleen (data not shown).

A cDNA library was constructed from oligo(dT)-primed poly$(A)^+$ RNA from normal rat thymus and the MCF-247 virus-infected, IL-2-independent 4437A subline M9. Both libraries were screened with the *Gfi-2* genomic probe A. Sequence analysis of the normal thymus cDNA clone 5a revealed that it encodes the rat IL-9 receptor (IL-9R) (Fig. 4). Following the initial characterization of the resulting cDNA clones, the M9 library was rescreened with a 1.3-bp probe derived

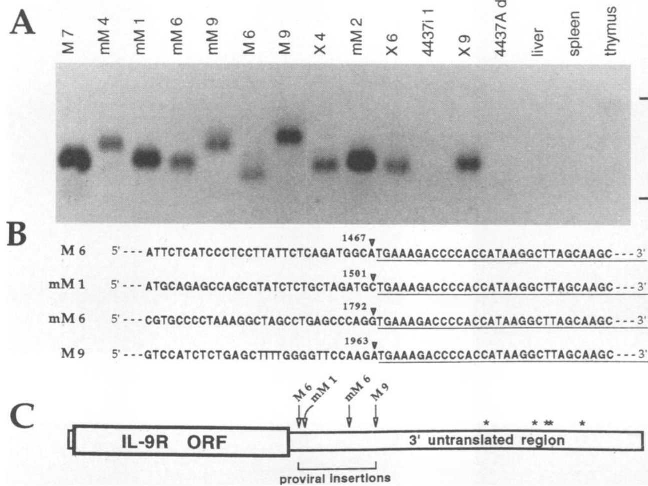

FIG. 5. Expression of *Gfi-2/IL-9R* in the IL-2-dependent (4437Ad), IL-2-independent 4437A cells and normal liver, spleen, and thymus. (A) IL-2-dependent 4437A cells (4437Ad) and nonsuperinfected IL-2-independent cells (4437i-1) do not express the *IL-9R* gene. The size of the *Gfi-2/IL-9R* mRNA detected with the 1.3-kb probe from the 5' end of the normal IL-9R clone 5a varies between sublines, depending on the site of the provirus insertion. The positions of rRNA are indicated (–). (B) Nucleotide sequence of the IL-9R/MuLV junction in four MCF or mMCF virus-infected IL-2-independent lines, including the ones with the largest and smallest IL-9R mRNA transcripts. The provirus insertion sites are indicated (arrowheads). (C) Diagram of the IL-9R cDNA clone showing the domain of the 3' untranslated region of the gene where all provirus integrations were detected. The asterisks show the map positions of all the instability motifs (AUUUA) detected in the 3' untranslated region of the gene.

from the 5' end of the normal *IL-9R* cDNA clone 5a. Sequence analysis of the tumor-specific cDNA clone 20, obtained from the second screening of the cDNA library, revealed that the mRNA from which it was derived terminated in the proviral LTR and was truncated in its 3' untranslated region.

These findings suggested that the activation of the *IL-9R* gene in the M9 cells was due to the provirus insertion-mediated truncation of the 3' untranslated region of the gene. To determine whether this mechanism of activation was common among the MCF virus-infected IL-2-independent lines, a Northern blot of poly(A)$^+$ RNA from 11 such lines, as well as from uninfected IL-2-dependent 4437A cells, was hybridized to a 1.3-kb cDNA probed from the 5' region of the *IL-9R* gene. The results showed that the MCF virus-infected IL-2-independent sublines express high levels of *IL-9R*, while the IL-2-dependent and IL-2-independent cells that are not infected with MCF viruses do not. The size of the *IL-9R* mRNA transcripts varies among sublines (Fig. 5A). It is interesting that high levels of the *IL-9R* mRNA were detected in all the MCF virus-infected 4437A sublines, including M5 which does not carry a provirus in the *Gfi-2* locus (data not shown). These findings provided support to the hypothesis that the activation of the *IL-9R* gene in the great majority of the MCF-virus infected lines was due to the provirus insertion mediated truncation of the 3' untranslated region of the gene. To map precisely the sites of provirus insertion, we carried out reverse transcriptase-PCRs using *IL-9R* and MCF virus LTR oligonucleotide primers (see Materials and Methods) and template cDNAs synthesized by oligo(dT) priming of poly(A)$^+$ RNA from representative sublines, including the ones expressing the largest and smallest RNA transcripts. The sequence of the resulting PCR products, at the junction between the virus and the cell, revealed that, in all cases, provirus insertion occurred in the 3' untranslated region of the gene (Fig. 5B and C).

The preceding experiments showed that all but one of the MCF virus-infected, IL-2-independent 4437A sublines carried a rearranged *IL-9R* gene. Moreover, at least 3 of 10 sublines were composed of multiple populations of cells, each containing an independent *IL-9R* provirus. To determine whether provirus insertion in the *IL-9R* gene occurred in any of the MoMuLV-induced rat T-cell lymphomas, under study in this laboratory, genomic DNA from 120 tumors including 40 IL-2-independent cell lines was digested with *Kpn*I and was hybridized to the *Gfi-2* probe A. This analysis revealed no rearrangements of the *IL-9R* gene in any of the MoMuLV-induced tumors and cell lines (data not shown).

The targeting of the *IL-9R* locus only in MCF (or NZB-X) virus-infected cells suggested that cells overexpressing the IL-9R have a growth advantage over cells that do not only when superinfected with MCF or xenotropic viruses. This suggested two possibilities. (i) A protein, perhaps the envelope glycoprotein, encoded by the MCF or xenotropic viruses, may interact with the IL-9 receptor, generating mitogenic signals similar to those generated through the interaction of IL-9 with its receptor. (ii) MCF and xenotropic virus infection, induced by cocultivation with virus-infected *M. dunni* cells, may lead to the expression of IL-9. The parallel activation of the IL-9 receptor through insertional mutagenesis may thus establish an IL-9-dependent autocrine loop. A prerequisite of both models is that mitogenic signals originating in the IL-9 receptor can substitute for the mitogenic signals originating in the IL-2 receptor. To address this question, a pBABE puro construct of the *IL-9R* cDNA clone, isolated from the M9 cDNA library, was introduced stably by transfection in the IL-2-dependent 4437A cells. The growth of the transfected cells was subsequently examined in media not supplemented with growth factors or in media supplemented with IL-2 or IL-9. The rat IL-9 (Fig. 6) was harvested from culture supernatants of COS-1 cells transfected with rat IL-9 expression constructs generated as described in Materials and Methods. The results shown in Fig. 7 confirmed that the IL-9R-transfected 4437A

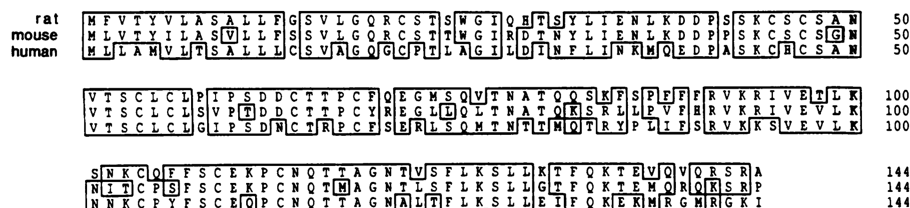

FIG. 6. Amino acid sequence alignment of rat, human, and mouse IL-9. The rat IL-9 (top line) is 58% identical to the human IL-9 (bottom line) and 74% identical to the mouse IL-9 (middle line). Conserved sequences are boxed. Sequence position numbers are shown on the right.

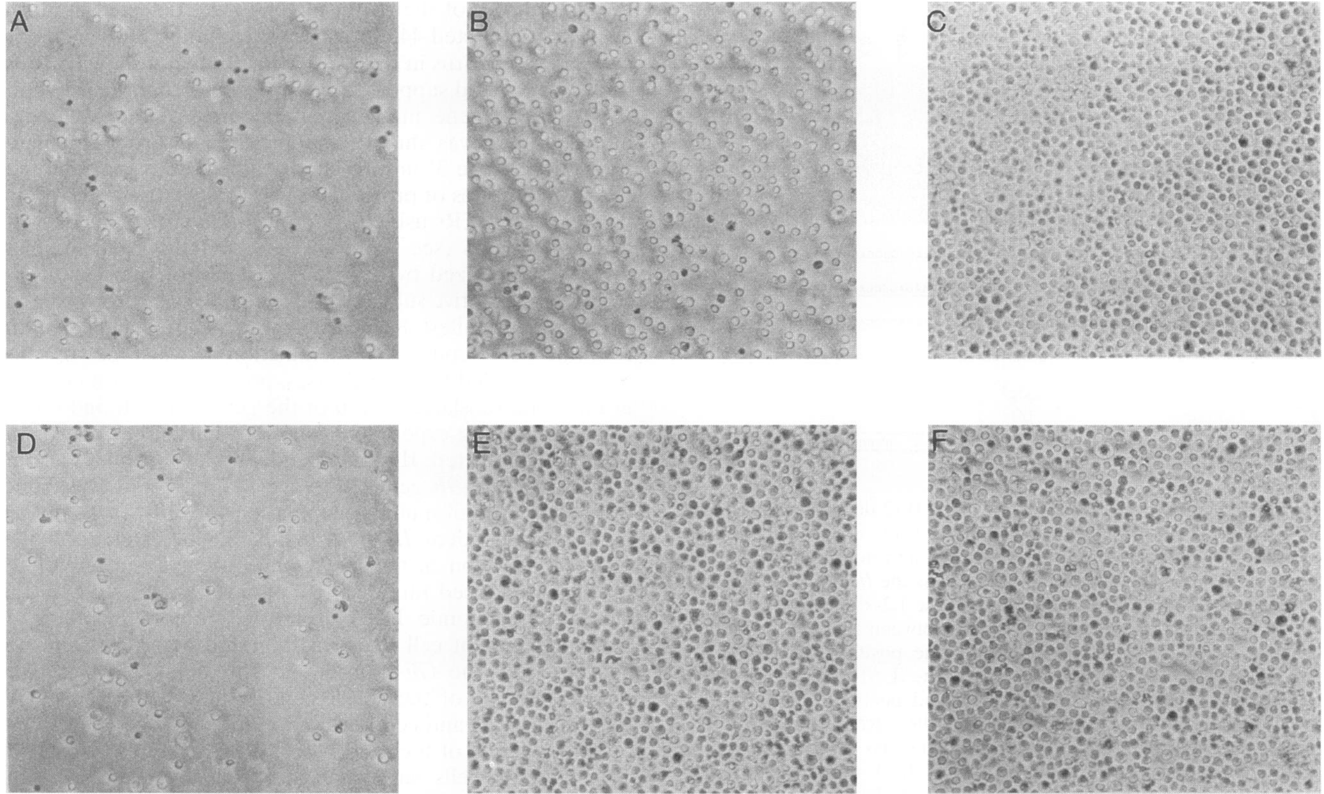

FIG. 7. IL-9 can replace IL-2 in 4437A cells transfected with a *Gfi-2/IL-9R* expression construct. (A) 4437A cells cultured in the absence of either IL-2 or IL-9. (B and C) The same cells maintained in IL-9- or IL-2-containing medium, respectively. (D) 4437A cells transfected with an IL-9R expression construct, cultured in the absence of IL-2 or IL-9. (E and F) The same cells maintained in IL-9- or IL-2-containing medium, respectively.

cells continue to be growth factor dependent but that the mitogenic signal generated through the triggering of the IL-2 receptor can be replaced by signals originating in the IL-9 receptor. It is interesting that the nontransfected 4437A cells also responded to IL-9, although minimally (Fig. 7B). Since these cells do not express detectable levels of IL-9R, their minimal response may depend on the interaction of IL-9 with unknown low-affinity IL-9 receptors. Alternatively, it may be due to the interaction of IL-9 with the IL-9R expressed at very low levels.

The expression of IL-9 in IL-2-dependent and IL-2-independent 4437A cells with or without rearrangements in the *Gfi-2* locus was subsequently examined by Northern blotting and hybridization to the rat IL-9 probe. The results revealed that while the IL-2-dependent cells do not express IL-9, all of the MCF and xenotropic virus-infected IL-2-independent cells, including subline M5 which carries an unrearranged *Gfi-2* locus, express high levels of this cytokine (Fig. 8). Hybridization of Southern blots of genomic DNA isolated from the same cell lines to an IL-9 probe revealed no rearrangements of this gene (data not shown). These findings suggested that MCF virus infection may induce the expression of IL-9 directly, by an insertional mutagenesis-independent mechanism. Alternatively, they suggested that the expression of IL-9 may be induced by signals originating in the *M. dunni* cells. In support of the alternative hypothesis, IL-9 was shown to be expressed by an IL-2-independent 4437A subline established by cocultivation with uninfected *M. dunni* cells (UF8) but not by an IL-2-independent subline established from noncocultivated cells (4437i-1) (Fig. 9A). To distinguish between these possibilities, IL-2-dependent 4437A cells were cocultivated with uninfected *M. dunni* cells or with *M. dunni* cells infected with MCF-247, mMCF-V33 or NZB-X. Eleven days later, total RNA from superinfected and nonsuperinfected cells, maintained in IL-2-containing media, was analyzed by Northern blotting for the expression of the *IL-9* gene. The results showed that indeed superinfection of the 4437A cells with MCF or xenotropic viruses rapidly induces the expression of IL-9 (Fig. 9B). In parallel, 4437A cells transfected with a *Gfi-2* expression construct were infected with MCF-247 in the presence of IL-2. Following IL-2 withdrawal, 1 week later, the cells

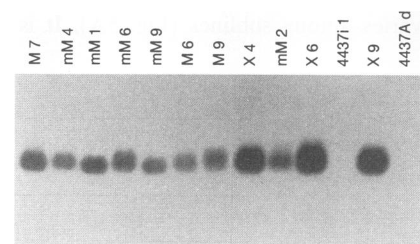

FIG. 8. Expression of IL-9 in IL-2-dependent (4437Ad) and IL-2-independent (4437A) cells. IL-9 is expressed in all the MCF, mMCF, and xenotropic virus-infected IL-2-independent cells but not in the IL-2-dependent (4437Ad) and the nonsuperinfected IL-2-independent (4437i-1) cells.

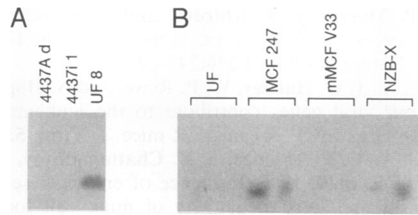

FIG. 9. Expression of IL-9 in 4437A cells following cocultivation with uninfected and MCF-247, mMCF-V33, or NZB-X virus-infected *M. dunni* fibroblasts. (A) Northern blot hybridization of a rat IL-9 cDNA probe to poly(A)$^+$ RNA from IL-2-dependent 4437A cells (4437Ad) and two IL-2-independent 4437A cell lines not superinfected. Of the two IL-2-independent lines, one (4437i-1) was not cocultivated while the other (UF8) was cocultivated with *M. dunni* embryo fibroblasts. (B) Northern blot hybridization of an IL-9 cDNA probe to total cell RNA from 4437A cells cocultivated with uninfected (UF), MCF-247-infected (MCF247), mMCF-V33-infected (mMCFV 33), and NZB-X-infected (NZB-X) *M. dunni* fibroblasts. Although the level of *IL-9* gene expression in mMCF-V33-infected cells in this experiment was low, it was higher than the level of *IL-9* expression in uninfected cells. The variability observed between experiments is likely to result from variability in the efficiency of infection.

continued to grow, while cell death occurred in uninfected control flasks of *Gfi-2*-transfected 4437A cells (data not shown). This result suggested that the virus-infected cells not only express IL-9 RNA but they also produce biologically active IL-9.

DISCUSSION

Our earlier studies had shown that the selection of IL-2-independent lines from the MCF virus-infected 4437A cultures occurred rapidly without a major crisis. The data presented in this report provide an explanation of these earlier findings. These data suggest that the MCF or xenotropic virus infection of 4437A cells initiates a series of events that ultimately lead to the expression of IL-9. Given that signals generated by the triggering of the IL-9 receptor can replace functionally the mitogenic signals originating in the IL-2 receptor, the expression of IL-9 establishes conditions that, following IL-2 withdrawal, select the cells expressing the IL-9 receptor. In the great majority of cases the expression of IL-9R is induced by an insertional mutagenesis-dependent mechanism. Since 4437A cells not expressing IL-9R also respond, although poorly, to IL-9, expression of IL-9 following MCF or xenotropic virus infection may enhance the overall survival of the cultured cells. This could facilitate the emergence and selection of cell clones expressing IL-9R, thus explaining the apparent lack of a major crisis during the selection of MCF virus-infected cells for IL-2-independent growth.

One of the main questions raised by these findings is whether the induction of the *IL-9* gene, following MCF virus infection, contributes to MCF-induced leukemogenesis in vivo. Work from other laboratories showing that a nononcogenic mouse T-cell lymphoma line becomes oncogenic following transfection of an IL-9 expression construct supports this hypothesis (35). To address this issue, we are currently examining a series of MCF virus-induced mouse lymphomas for *IL-9* and *IL-9R* gene expression, and for rearrangements of the *IL-9R* gene. Moreover, we are in the process of characterizing the oncogenic potential of the nononcogenic lymphoma line 4437A in a nude mouse transplantation assay following transfection of the IL-9 and IL-9R expression constructs.

The mechanism by which MCF viruses contribute to the induction of T-cell lymphomas in rodents has been under study for many years (34). Recently, it was suggested that the MCF envelope glycoprotein, which is responsible at least in part for the oncogenic potential of these viruses (17, 34), may initiate mitogenic signals in T cells, perhaps by interacting with cellular receptors, such as the IL-2 receptor (25, 33). The interaction between viral and cellular proteins and its consequences is an issue that has received considerable attention in recent years. Thus, it has been shown that the defective MCF-like envelope glycoprotein of the spleen focus-forming virus interacts with the erythropoietin receptor and stimulates the growth of erythroid precursors in vivo and in culture (24). Moreover, the peptide encoded by the E5 gene of the bovine papillomavirus induces transformation by interacting with the platelet-derived growth factor receptor (15, 30, 31) and the polyomavirus middle T antigen may induce transformation by binding to and activating Src (21). Finally, a series of other proteins exert direct effects on transcription, mRNA trafficking, and translation (reviewed in references 8, 9, and 32) or interact with proteins that play important roles in cell cycle regulation (23). Although the expression of IL-9 in MCF or xenotropic virus-infected 4437A cells may be the direct result of the interaction between the MCF or xenotropic envelope glycoprotein and a given cytokine receptor, other possibilities cannot be excluded. The system we have established allows us to address questions regarding the interaction of the MCF envelope glycoprotein with the signal transduction machinery in T cells.

The data presented in this report do not explain why the xenotropic virus-infected 4437A cells did not progress to become IL-2 independent with efficiency equal to that of the MCF virus-infected cells. One possible explanation is that xenotropic viruses grow much less efficiently than the MCF viruses. To address this hypothesis, we examined the titer of the virus produced by the MCF-247- and NZB-X-infected *M. dunni* cells. To this end, virus dilutions were used to infect mink lung cells, and the presence of virus in the culture supernatants of the infected cells was monitored by a reverse transcriptase assay. The results showed that the titer of the xenotropic virus produced by the *M. dunni* cells was approximately 5% the titer of the MCF virus (data not shown).

The pathogenicity of retroviruses depends on complex sets of factors. The progression of the disease, on many occasions, depends on the proliferation of lymphoid and other hematopoietic cell populations. Thus, the first step in the induction of T-cell lymphomas in mice and rats is the rapid proliferation of early hematopoietic precursors in the spleen and the bone marrow of the infected animals (12). Other retrovirus-induced diseases, such as AIDS in mice and (frequently) in humans, are also characterized by strong lymphoproliferation (16, 28). The data presented in this report provide a paradigm that may be applicable to these processes. According to this, the virus possesses the ability to induce the expression of regulatory molecules and to select for cells responding to them. Understanding the mechanisms by which such autocrine loops may be established in retrovirus-induced rodent lymphomas or in other retrovirus-induced diseases may contribute to our ability to design rational therapeutic strategies.

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REFERENCES

1. **Anderson, S., D. L. Davis, H. Dahlbäck, H. Jörnvall, and D. W. Russell.** 1989. Cloning, structure and expression of the mitochondrial cytochrome P-450 sterol 26-hydroxylase, a bile acid biosynthetic enzyme. J. Biol. Chem. **264:**8222–8229.
2. **Barker, C. S., S. E. Bear, T. Keler, N. G. Copeland, D. J. Gilbert, N. A. Jenkins, R. S. Yeung, and P. N. Tsichlis.** 1992. Activation of the prolactin receptor gene by promoter insertion in a Moloney murine leukemia virus-induced rat thymoma. J. Virol. **66:**6763–6768.
3. **Bellacosa, A., P. A. Lazo, S. E. Bear, and P. N. Tsichlis.** 1991. The rat leukocyte antigen MRC OX-44 is a member of a new family of cell surface proteins which appear to be involved in growth regulation. Mol. Cell. Biol. **11:**2864–2872.
4. **Brightman, B. K., B. R. Davis, and H. Fan.** 1990. Preleukemic hematopoietic hyperplasia induced by Moloney murine leukemia virus is an indirect consequence of viral infection. J. Virol. **64:**4582–4584.
5. **Chen, C., and H. Okayama.** 1987. High-efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol. **7:**27–45.
6. **Chen, C., and H. Okayama.** 1988. Calcium phosphate-mediated gene transfer: a highly efficient transfection system for stably transforming cells with plasmid DNA. BioTechniques **6:**632.
7. **Cloyd, M. D., J. W. Hartley, and W. P. Rowe.** 1980. Lymphomagenicity of recombinant mink cell focus-forming murine leukemia virus. J. Exp. Med. **151:**542–549.
8. **Cullen, B. R.** 1992. Mechanism of action of regulatory proteins encoded by complex retroviruses. Microbiol. Rev. **56:**375–394.
9. **Cullen, B. R., and M. H. Malim.** 1991. The HIV-1 Rev protein: prototype of a novel class of eukaryotic post-transcriptional regulators. Trends Biochem. Sci. **16:**346–350.
10. **Davis, B. R., B. K. Brightman, K. G. Chandy, and H. Fan.** 1987. Characterization of a preleukemic state induced by Moloney murine leukemia virus: evidence for two infection events during leukemogenesis. Proc. Natl. Acad. Sci. USA **84:**4875–4879.
11. **Davis, B. R., K. G. Chandy, B. K. Brightman, S. Gupta, and H. Fan.** 1986. Effects of nonleukemogenic and wild-type Moloney murine leukemia virus on lymphoid cells in vivo: identification of a preleukemic shift in thymocyte subpopulations. J. Virol. **60:**423–430.
12. **Frankel, W. N., J. P. Stoye, B. A. Taylor, and J. M. Coffin.** 1989. Genetic identification of endogenous polytropic proviruses using recombinant inbred mice. J. Virol. **63:**3810–3821.
13. **Frankel, W. N., J P Stoye, B. A. Taylor, and J. M. Coffin.** 1990. A linkage map of endogenous murine leukemia proviruses. Genetics **124:**221–236.
14. **Gilks, C. B., S. E. Bear, H. L. Grimes, and P. N. Tsichlis.** 1993. 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. Mol. Cell. Biol. **13:**1759–1768.
15. **Goldstein, D. J., T. Andresson, J. J. Sparkowski, and R. Schlegel.** 1992. The BPV-1 E5 protein, the 16 kDa membrane pore-forming protein and the PDGF receptor exist in a complex that is dependent on hydrophobic transmembrane interactions. EMBO J. **11:**4851–4859.
16. **Gougeon, M. L., V. Colizzi, A. Dalgleish, and L. Montagnier.** 1993. New concepts in AIDS pathogenesis. AIDS Res. Hum. Retroviruses **9:**287–289.
17. **Hartley, J. W., N. K. Wolford, L. J. Old, and W. P. Rowe.** 1977. A new class of murine leukemia virus associated with development of spontaneous lymphomas. Proc. Natl. Acad. Sci. USA **74:**789–792.
18. **Hiai, H., P. Morrissey, R. Khiroya, and R. S. Schwartz.** 1977. Selective expression of xenotropic virus in congenic HRS/J (hairless) mice. Nature (London) **270:**247–249.
19. **Holland, C. A., J. W. Hartley, W. P. Rowe, and N. Hopkins.** 1985. At least four viral genes contribute to the leukemogenicity of murine retrovirus MCF 247 in AKR mice. J. Virol. **53:**158–165.
20. **Holland, C. A., C. Y. Thomas, S. K. Chattopadhyay, C. Koehne, and P. V. O'Donnell.** 1989. Influence of enhancer sequences on thymotropism and leukemogenicity of mink cell focus-forming viruses. J. Virol. **63:**1284–1292.
21. **Kaplan, D. R., D. C. Pallas, W. Morgan, B. Schaffhausen, and T. M. Roberts.** 1988. Mechanisms of transformation by polyoma virus middle T antigen. Biochim. Biophys. Acta **948:**345–364.
22. **Lazo, P. A., A. J. P. Klein-Szanto, and P. N. Tsichlis.** 1990. T-cell lymphoma lines derived from rat thymomas induced by Moloney murine leukemia virus: phenotypic diversity and its implications. J. Virol. **64:**3948–3959.
23. **Levine, A. J.** 1993. The tumor suppressor genes. Annu. Rev. Biochem. **62:**623–651.
24. **Li, J. P., A. D. D'Andrea, H. F. Lodish, and D. Baltimore.** 1990. Activation of cell growth by binding of Friend spleen-focus-forming virus gp55 glycoprotein to the erythropoietin receptor. Nature (London) **343:**762–764.
25. **Li, J.-P., and D. Baltimore.** 1991. Mechanism of leukemogenesis induced by mink cell focus-forming murine leukemia viruses. J. Virol. **65:**2408–2414.
26. **McCutchan, H. J., and S. J. Pagano.** 1968. Enhancement of the infectivity of simian virus 40 deoxyribonucleic acid with diethyl aminoethyl-dextran. J. Natl. Cancer Inst. **41:**351–357.
27. **Morgenstern, J. P., and H. Land.** 1990. Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. Nucleic Acids Res. **18:**3587–3596.
28. **Morse, H. C., III, J. W. Hartley, Y. Tang, S. K. Chattopadhyay, N. Giese, and T. N. Frederickson.** 1994. Lymphoproliferation as a precursor to neoplasia: what is a lymphoma?, p. 265–291. *In* A. Minson, J. Neil, and M. McCrae (ed.), Proceedings of the 128th Meeting of the Society for General Microbiology, vol. 51. Cambridge University Press, Cambridge.
29. **Patriotis, C., A. Makris, S. E. Bear, and P. N. Tsichlis.** 1993. Tumor progression locus-2 (*Tpl*-2) encodes a protein kinase involved in the progression of rodent T cell lymphomas and in T cell activation. Proc. Natl. Acad. Sci. USA **90:**2251–2255.
30. **Petti, L., and D. DiMaio.** 1992. Stable association between the bovine papillomavirus E5 transforming protein and activated platelet-derived growth factor. Proc. Natl. Acad. Sci. USA **89:**6736–6740.
31. **Petti, L., L. A. Nilson, and D. DiMaio.** 1991. Activation of the platelet-derived growth factor receptor by the bovine papillomavirus E5 transforming protein. EMBO. J. **10:**845–855.
32. **Rosen, C. A., and G. N. Pavlakis.** 1990. Tat and Rev: positive regulators of HIV gene expression. AIDS **4:**499–509.
33. **Tsichlis, P. N., and S. E. Bear.** 1991. Infection by mink cell focus-forming (MCF) viruses confers interleukin 2 (IL-2) independence to an IL-2 dependent rat T-cell lymphoma line. Proc. Natl. Acad. Sci. USA **88:**4611–4615.
34. **Tsichlis, P. N., and P. A. Lazo.** 1991. Virus-host interactions and the pathogenesis of murine and human oncogenic retroviruses. Curr. Top. Microbiol. Immunol. **171:**95–179.
35. **Uyttenhove, C., C. Druez, J.-C. Renauld, M. Hérin, H. Noël, and J. Van Snick.** 1991. Autonomous growth and tumorigenicity induced by P40/interleukin 9 cDNA transfection of a mouse P40-dependent T cell line. J. Exp. Med. **173:**519–522.
36. **Weissman, I. L., and M. S. McGrath.** 1982. Retrovirus lymphomagenesis: relationship of normal immune receptors to malignant cell proliferation. Curr. Top. Microbiol. Immunol. **98:**103–112.