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Friend Spleen Focus-Forming Virus Induces Factor Independence in an Erythropoietin-Dependent Erythroleukemia Cell Line

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Erythroid cells from mice infected with the polycythemia-inducing strain of Friend spleen focus-forming virus ($SFFV_P$), unlike normal erythroid cells, can proliferate and differentiate in the apparent absence of the erythroid hormone erythropoietin (Epo). The unique envelope glycoprotein encoded by SFFV has been shown to be responsible for this biological effect. The recent isolation of an Epo-dependent erythroleukemia cell line, HCD-57, derived from a mouse infected at birth with Friend murine leukemia virus, afforded us the opportunity to study the direct effect of $SFFV_P$ on a homogeneous population of factor-dependent cells. The introduction of $SFFV_P$ in complex with various helper viruses into these Epo-dependent cells efficiently and reproducibly gave rise to lines which expressed high levels of SFFV and were factor independent. SFFV appears to be unique in its ability to abrogate the factor dependence of Epo-dependent HCD-57 cells, since infection of these cells with retroviruses carrying a variety of different oncogenes had no effect. The induction of Epo independence by SFFV does not appear to involve a classical autocrine mechanism, since there is no evidence that the factor-independent cells synthesize or secrete Epo or depend on it for their growth. However, the SFFV-infected, factor-independent cells had significantly fewer receptors available for binding Epo than their factor-dependent counterparts had, raising the possibility that the induction of factor independence by the virus may be due to the interaction of an SFFV-encoded protein with the Epo receptor.

The Friend strain of the spleen focus-forming virus (SFFV) induces an acute erythroleukemia in mice. Erythroid cells infected with the polycythemia-inducing variant of SFFV ($SFFV_P$) differ from normal erythroid cells in that they can proliferate and differentiate in the apparent absence of the erythroid hormone erythropoietin (Epo) (for a review, see reference 14). The unique envelope glycoprotein encoded by SFFV has been shown to be responsible for this biological effect (27). The mechanism by which SFFV alters the growth and differentiation of erythroid cells is unknown. Spleen cells from $SFFV_P$-infected mice do not secrete detectable amounts of Epo or any other erythroid growth factor (17) and show no change in the number or affinity of Epo receptors (17, 19). Although the majority of the SFFV envelope glycoprotein is expressed in the cytoplasm, the small amount of protein that is posttranslationally modified and transported to the cell surface appears to be the biologically significant form (9). Studies with chimeric envelope genes have indicated that specific sequences in the extreme 3′ end of the envelope gene which encode the transmembrane portion of the molecule are essential for conferring Epo independence (3, 4).

The recent isolation of an Epo-dependent erythroleukemia cell line derived from a mouse infected at birth with Friend murine leukemia virus (F-MuLV) (W. D. Hankins, K. Chin, and R. Dons, submitted for publication) provided us with the opportunity to examine the interaction of $SFFV_P$ with the Epo signal transduction pathway in a homogeneous population of Epo-dependent cells for the first time. Our results indicate that $SFFV_P$, but not any of a variety of other retroviruses, can efficiently abrogate the factor requirements of this Epo-dependent cell line. The induction of factor independence (FI) does not appear to involve a classical autocrine mechanism but may involve the interaction of an SFFV gene product with the receptor for Epo.

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MATERIALS AND METHODS

Cells and viruses. HCD-57 cells derived from an NIH Swiss mouse infected with F-MuLV at birth (Hankins et al., submitted) were maintained in Iscove modified Dulbecco minimal essential medium with 30% fetal calf serum, 5×10^{-5} M 2-mercaptoethanol, and 0.3 U of Epo per ml (78 pM). The Epo used was a tissue culture supernatant from fibroblasts that had been transfected with the human Epo gene. It has a specific activity of 130,000 U/mg of total weight and a molecular weight at 29,400. Thus, 1 U of Epo per ml equals 262 pM.

$SFFV_P$-containing virus preparations were obtained from $SFFV_{AP}$-L-transfected (26) NIH 3T3 cells that had been superinfected with either F-MuLV or amphotropic virus 4070. For a list of the other retroviruses used, see Table 4.

Infection. HCD-57 cells were washed and suspended at a concentration of 4×10^5/ml in virus or medium plus 4 μg of Polybrene per ml. After 1 h at 37°C, the cells were pelleted and suspended in Epo-containing medium. The cells were maintained in this medium for 3 to 7 days before being selected for FI growth in Epo-free medium. When F-MuLV pseudotypes of SFFV were used, cells were pretreated with 2.5 mM 2-deoxyglucose for 16 h before virus infection to overcome retroviral interference (13).

Agar cultures. Cells were plated in 60-mm petri dishes containing 5 ml of Iscove modified Dulbecco minimal essential medium with final concentrations of 30% fetal calf serum, 5×10^{-5} M 2-mercaptoethanol, and 0.35% SeaPlaque agarose. Cells (10^3, 10^4, or 10^5 per plate) were cultured in the presence or absence of 0.3 U of Epo per ml, and colony formation was measured after 14 days of incubation at 37°C.

Analysis of viral gene products. Cells were labeled with

[^{35}S]methionine, and cell lysates were immunoprecipitated with goat antiserum to Rauscher MuLV gp70 or to Moloney mink cell focus-forming virus that had been rendered specific for mink cell focus-inducing virus after absorption with F-MuLV (16). Precipitated proteins were analyzed by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels as previously described (16).

Cell proliferation assays. Cells (10^5/ml) were incubated for 48 h in the presence or absence of growth factors and then pulsed overnight with 1 μCi of [^{3}H]thymidine per well as previously described (15). Incorporation was measured by harvesting cells onto glass fiber filters with a microharvester and determining counts on the filters. In some experiments, rabbit anti-Epo antiserum was included during the entire incubation period at a concentration sufficient to neutralize 0.2 U of Epo.

Preparation of conditioned medium. Cells (2×10^6/ml) were cultured for 3 days in Epo-free medium. After removal of the cells by centrifugation, the conditioned medium was filtered through a filter (pore size, 0.45 μm). The IW.32 cell line (23), which produces Epo, was used as a control.

Northern (RNA) blot hybridization analysis. Total RNA was prepared by extraction of cells in 6 M urea–3 M LiCl (2). RNAs were denatured in 50% formamide–2.2 M formaldehyde, separated electrophoretically on 1% agarose gels containing 2.2 M formaldehyde, and transferred to nitrocellulose filters. RNA containing Epo sequences was identified by hybridization with a nick-translated Epo probe prepared from a 2.78-kilobase *Bam*HI-*Eco*RI fragment of plasmid DB2-5 (21) (Genetics Institute, Cambridge, Mass.). Hybridizations were performed at 42°C for 24 h in 50% formamide–5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and were followed by washing at 65°C with 0.1× SSC–0.1% sodium dodecyl sulfate.

Epo-binding assays. Epo was iodinated, and ^{125}I-Epo-binding assays were carried out at 0°C as previously described (18). Cells (10^7/ml) were incubated for 20 h in concentrations of ^{125}I-Epo ranging from 0.1 to 10.0 U/ml (26 to 2,600 pM). Bound Epo was measured by separation of the cells from the binding medium by centrifugation through dibutyl phthalate oil. Nonspecific binding was measured in the presence of 100 U of unlabeled Epo per ml and was subtracted from specific binding. The data were plotted by the Scatchard method to determine the binding affinity. Since the presence of Epo leads to the down modulation of Epo receptors on HCD-57 cells (S. Sawyer, unpublished data), these cells were grown for 24 h in the absence of Epo before being used in Epo-binding studies.

RESULTS

SFFV renders an Epo-dependent erythroleukemia cell line FI. Epo-dependent HCD-57 cells were infected with various viruses and plated in liquid culture for 3 to 7 days in Epo-containing medium. FI lines were then selected by plating the cells in medium without Epo. FI lines could be obtained at a high frequency after infection with either amphotropic MuLV or F-MuLV pseudotypes of SFFV$_P$ but never after infection with either amphotropic MuLV or F-MuLV alone (Table 1), indicating that SFFV contributed to the FI phenotype. FI lines could also be efficiently obtained when helper-free preparations of SFFV$_P$ (26) were used (data not shown).

The frequency of FI variants in the SFFV-infected HCD-57 population was determined by plating the cells before and after selection in medium with and without Epo. The frequency of FI variants in the SFFV$_P$-infected HCD-57 population was very high (3.7% cloning efficiency with Epo versus 0.4% without Epo, or ~1 FI variant per 10 cells) (Table 2).

TABLE 1. SFFV$_P$ induction of FI erythroleukemia lines

Virus(es)	No. of samples yielding FI growth/total no. of samples	No. of colonies/10^3 cells	
		With Epo	Without Epo
None	0/10	20	0
AMT[a]-MuLV	0/6	19	0
F-MuLV	0/4	20	0
AMT-MuLV–SFFV$_P$	6/6	21	20
F-MuLV–SFFV$_P$	4/4	22	21

[a] AMT, Amphotropic.

FI (SFFV$_P$) lines express high levels of SFFV envelope glycoprotein and secrete infectious virus. Each of six separately isolated FI cell lines was shown to express high levels of the envelope glycoprotein encoded by SFFV (a representative cell line is shown in Fig. 1B) and to release into the supernatant virus that induced a rapid erythroleukemia in adult mice. The Epo-dependent parental cell line neither expressed the SFFV envelope glycoprotein (Fig. 1A) nor produced virus capable of causing an acute disease in adult mice.

FI lines do not secrete detectable levels of Epo or express Epo-specific RNA transcripts. SFFV-infected FI lines proliferate to high levels in the absence of Epo, whereas the proliferation of the parental line is dependent on the addition of Epo to the medium (Fig. 2A). In order to determine if the FI lines make their own Epo, which allows the cells to grow in an autocrinelike manner, we prepared conditioned medium from the cells and assayed them for Epo production with the Epo-dependent parental HCD-57 cells. Conditioned medium from two separate SFFV-infected FI lines failed to release into the medium factors that supported the proliferation of the parental HCD-57 cells (Fig. 2B). In contrast, conditioned medium from the IW.32 cell line, which has previously been shown to secrete Epo, contained easily detectable amounts of Epo capable of stimulating the HCD-57 cells (data not shown).

Since it was still possible that the SFFV-infected FI cells synthesized Epo but that the factor was not secreted from the cells in detectable amounts, we examined the cells for the presence of Epo-specific RNA transcripts by Northern blot hybridization analysis. The Epo-producing IW.32 cell line displayed high levels of a 1.5-kilobase Epo-specific mRNA (Fig. 3, lanes 3 and 4). In contrast, no Epo mRNA could be detected in the SFFV-infected FI lines (Fig. 3, lanes 1 and 2) even when Epo sequences were amplified by the polymerase chain reaction (data not shown).

Proliferation of FI (SFFV$_P$) lines is neither density depen-

TABLE 2. Cloning efficiency of SFFV-infected HCD-57 cells in the presence or absence of Epo in agar

Cell line	% Cloning efficiency with[a]:	
	Epo	No Epo
HCD-57	2.0	<0.01
Unselected HCD-57 (SFFV$_P$)	3.7	0.4
FI HCD-57 (SFFV$_P$)	2.0	2.0

[a] Efficiencies of colony formation were independent of cell density.

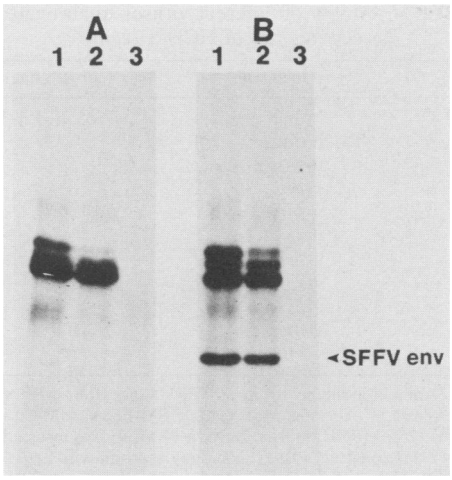

FIG. 1. Expression of SFFV envelope (env) glycoprotein in FI ($SFFV_P$) cells. HCD-57 (A) and FI ($SFFV_P$) (B) cells were labeled with [^{35}S]methionine, and lysates were prepared as described in Materials and Methods. Proteins were then precipitated with goat anti-Rauscher MuLV serum (lanes 1), a goat antiserum specific for mink cell focus-inducing virus (lanes 2), or normal goat serum (lanes 3). The 80,000- to 90,000-molecular-weight envelope glycoproteins in panel A are expressed by Friend and Friend mink cell focus-inducing viruses present in the HCD-57 cells.

dent nor inhibited by anti-Epo serum. The growth of the FI ($SFFV_P$) cells was not dependent on cell density, and cloning efficiency was the same whether 500 or 25,000 cells were plated (data not shown).

An antiserum prepared against Epo and capable of neutralizing 0.2 U of Epo had no effect on the proliferation of the FI ($SFFV_P$) lines under conditions in which the antiserum completely inhibited the proliferation of the Epo-dependent parental HCD-57 cells (Fig. 4).

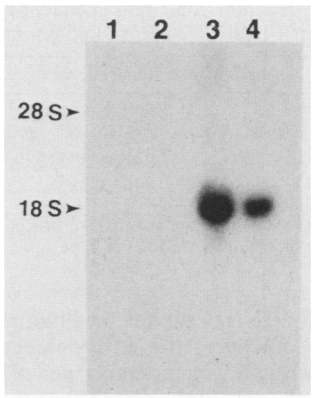

FIG. 3. Northern blot hybridization analysis of RNAs with an Epo-specific probe. RNA was extracted from either the Epo-producing IW.32 cell line (lanes 3 and 4) or a representative FI ($SFFV_P$) line (lanes 1 and 2). All lanes represent total RNA (20 μg in lanes 1 and 3 and 10 μg in lanes 2 and 4). Filters were hybridized to a ^{32}P-labeled Epo probe as described in Materials and Methods.

Binding of Epo to SFFV-infected FI cells. Previous studies have shown the existence of specific receptors for Epo on erythroid precursor cells. Some cells express two classes of receptor, one with an affinity (K_d) of 50 to 150 pM and the other with an affinity of 500 to 1,000 pM, while other cells express only the class with the lower affinity (18, 19). To determine if the expression of SFFV in the HCD-57 cells would affect the expression of Epo receptors on the HCD-57 cells, we performed equilibrium binding studies with iodinated Epo. The SFFV-infected FI cells expressed a single class of lower-affinity receptors (K_d, 500 to 650 pM) (Table 3;

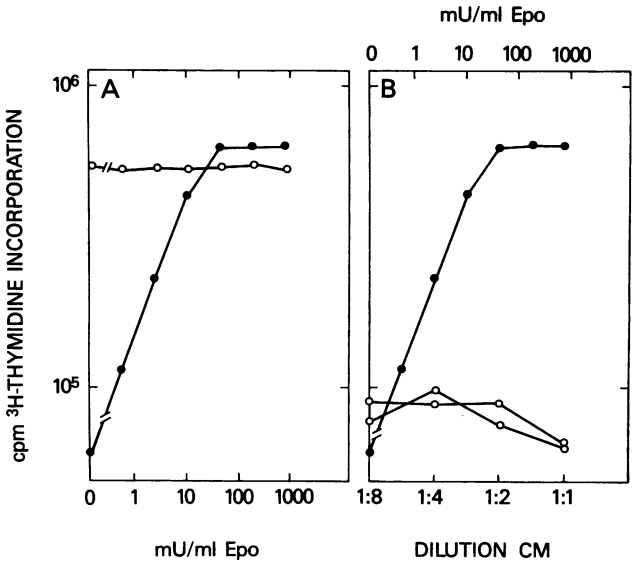

FIG. 2. Proliferation of HCD-57 and FI ($SFFV_P$) cells in the presence or absence of Epo or conditioned medium from FI ($SFFV_P$) cell lines. (A) HCD-57 (●) or FI ($SFFV_P$) (○) cells were cultured for 48 h with medium lacking Epo or medium containing the indicated concentration of Epo. (B) HCD-57 cells were cultured for 48 h in medium containing Epo (●) or conditioned medium (CM) from different FI ($SFFV_P$) lines (○). Cells were pulsed overnight with [^{3}H]thymidine and assayed for counts incorporated.

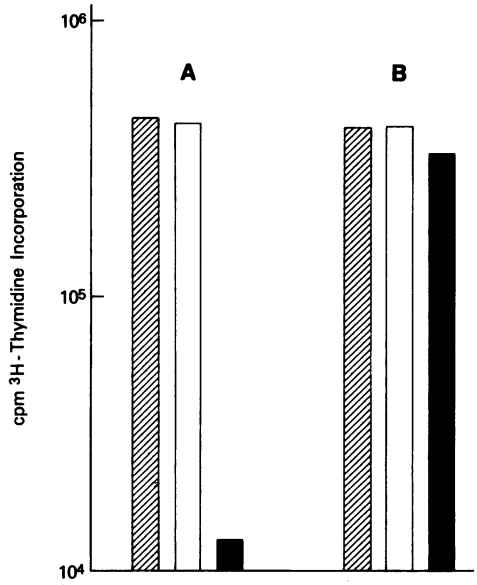

FIG. 4. Effect of anti-Epo serum on proliferation of FI ($SFFV_P$) cells. HCD-57 (A) or FI ($SFFV_P$) (B) cells were incubated for 48 h in Epo-containing medium (▨), Epo-containing medium plus anti-Epo serum (■), or normal rabbit serum (□). cpm, Counts per minute.

TABLE 3. Epo receptors on Epo-dependent HCD-57 cells and their FI, $SFFV_P$-infected counterparts

Cell type	No. of receptors/cell (mean ± SD)	K_d (pM)[a]
HCD-57	4,087 ± 652	542
FI ($SFFV_P$) 1-3	661 ± 195	575
FI ($SFFV_P$) 4-8	932 ± 334	650
FI ($SFFV)_P$ 5-2	846 ± 213	500

[a] Calculations are based on Scatchard analysis of Epo-binding data from at least two separate experiments for each cell type.

Fig. 5) comparable to those expressed by the Epo-dependent HCD-57 cells. However, the SFFV-infected FI cells expressed significantly fewer receptors per cell (a reduction of 77 to 84%) than the parental cell line expressed. Endocytosis of ^{125}I-Epo in HCD-57 and SFFV-infected FI cells was similar. In addition, down regulation of Epo receptors occurred in both SFFV-infected FI cells and HCD-57 cells when 0.6 U of Epo per ml (156 pM) was present in the medium during cell proliferation (data not shown). The structure of the receptor, as determined by cross-linking to ^{125}I-Epo, was not altered by SFFV infection (data not shown).

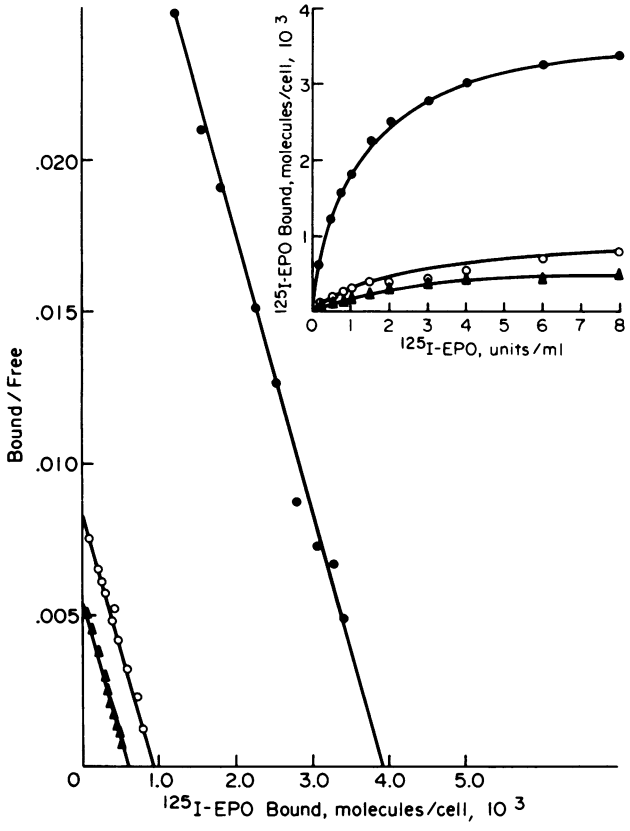

FIG. 5. Binding of ^{125}I-Epo to Epo-dependent HCD-57 cells and their FI, $SFFV_P$-infected counterparts. ^{125}I-Epo binding to cell surface receptors on HCD-57 cells (●) and FI virus-infected subclones FI ($SFFV_P$) 1-3 (▲) and FI ($SFFV_P$) 5-2 (○) was measured at 0°C with the indicated concentrations of ^{125}I-Epo as described in Materials and Methods. Specific binding of ^{125}I-Epo to the cells is shown in the inset. These data were plotted by the method of Scatchard (the intercept indicates the maximum receptor number; the slope indicates the dissociation constant).

TABLE 4. Ability of different viruses to abrogate Epo dependence of HCD-57 cells

Virus[a]	Oncogene	FI cell lines generated[b]
$SFFV_P$	None	+++++
$SFFV_A$	None	+
HaSV	v-*ras*	−
A-MuLV	v-*abl*	−
SM-FeSV	v-*fms*	−
MRSV	v-*src*	−
T1424-29	v-*erbB*	−
J2	v-*raf-/myc*	−
J3	v-*myc*	−
J5	v-*myc*	−
Cas NS-1	v-*cbl*	−

[a] $SFFV_A$, Anemia-inducing strain of SFFV (26); HaSV, Harvey sarcoma virus (20); A-MuLV, Abelson MuLV (11); SM-FeSV, SM strain of feline sarcoma virus (25); MRSV, murine retrovirus with v-*src* inserted (1); T1424-29, murine v-*erbB* construct (5); J2, Moloney sarcoma virus 3611 with v-*myc* introduced (12); J3 and J5, murine retroviruses with v-*myc* inserted (12); Cas NS-1, murine retrovirus containing v-*cbl* oncogene (8).

[b] +++++, Generated at high frequency; +, generated at low frequency; −, none generated.

Uniqueness of SFFV in rendering HCD-57 cells Epo independent. In order to determine if retroviruses other than $SFFV_P$ render HCD-57 cells Epo independent, cells were infected with amphotropic or ecotropic virus pseudotypes of a variety of other viruses. The viruses tested were chosen because they either had previously been shown to alter the Epo responsiveness of erythroid cells (for reviews, see references 6 and 7) or were capable of abrogating myeloid and lymphoid cells from dependence on growth factors (for a review, see J. H. Pierce, Biochim. Biophys. Acta, in press). Only $SFFV_P$ was capable of efficiently abrogating Epo-dependent HCD-57 cells from their factor dependence (Table 4). FI lines were also generated at considerably lower efficiency (less than 1 per 2,500 cells) from HCD-57 cells infected with the anemia-inducing strain of SFFV. However, these cells differed from those infected with $SFFV_P$ in that they could proliferate in Epo-free medium only at high density.

DISCUSSION

Our results indicate that the expression of high levels of the $SFFV_P$ envelope glycoprotein in an Epo-dependent erythroleukemia cell line, HCD-57, can alter the factor requirements of these cells. The fact that the FI lines were generated at an extremely high frequency (1 FI cell per 10 HCD-57 cells) after infection with $SFFV_P$ suggests that the expression of an SFFV gene product is the only event necessary for transformation of these cells. The low efficiency of transformation obtained after infection with the anemia-inducing strain of SFFV is consistent with previous data on the biological effects of the virus (14) and suggests that the mechanism by which this virus induces FI of the HCD-57 cells is indirect, perhaps because of integration of the virus at a specific site in the mouse genome (10, 22).

Our data also indicate that, compared with a variety of oncogene-containing retroviruses, SFFV is unique in its ability to abrogate Epo-dependent cells from their factor dependence. None of the other viruses tested, all of which have been shown to alter the Epo responsiveness of erythroid cells or render myeloid or lymphoid cells FI, had any effect on the Epo-dependent erythroleukemia cells. The fact

that Abelson MuLV was unable to abrogate the Epo-dependent cells from their factor dependence was unexpected, since the virus had previously been shown to induce FI growth of certain erythroid precursor cells in vitro (24). Results identical to those shown in Table 4 were obtained with a different stock of Abelson MuLV, and both stocks were able to abrogate FDC-P1 cells from their IL-3 dependence as previously described (11).

The mechanism by which $SFFV_P$ induces Epo independence is not known. The virus appears to exert its effect only on erythroid cells; this is supported by the observation that $SFFV_P$ had no effect on a variety of IL-3-dependent cell lines (S. Ruscetti, unpublished data). This suggests that SFFV specifically interacts with a component of the Epo signal transduction pathway. Our data indicate that induction of Epo independence by SFFV does not involve a classical autocrine mechanism, since the FI ($SFFV_P$) cells do not secrete Epo or express Epo mRNA and since their growth is not density dependent or affected by anti-Epo serum. However, it is possible that a product of the SFFV genome interacts with the Epo receptor in such a way that the need for Epo is bypassed. Such an interaction is consistent with our Epo-binding data from the SFFV-infected, FI cells. These cells contain significantly fewer Epo receptors (four- to sixfold fewer) than the parental line, suggesting that an SFFV protein may interact with the Epo receptor either at the cell surface, which could block Epo binding, or internally, which could block receptor transport to the cell surface. We failed to see this effect of $SFFV_P$ in earlier studies with spleen cells from virus-infected mice (17, 19), most likely because of the heterogeneous nature of the cells and the lack of a clear, uninfected counterpart for comparison. If $SFFV_P$ were found to abrogate Epo dependence by directly interacting with the hormone receptor, such an interaction would be a unique mechanism of retroviral transformation. Studies with SFFV-infected, FI HCD-57 cells and their uninfected factor-dependent counterparts are in progress to further examine the interaction between SFFV gene products and the Epo signal transduction pathway.

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