The mutated, myeloid cell-specific growth factor receptor *v-fms* transforms avian erythroid but not myeloid cells

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In avian hematopoietic cells, transformation by tyrosine kinase oncogenes is restricted to the erythroid lineage. To study the mechanism of this striking target cell specificity, we constructed an avian retrovirus correctly expressing the feline *v-fms* protein, an oncogenic version of the myeloid-specific CSF-1 receptor. Similar to other tyrosine kinase oncogenes (*erbB*, *src*, *fps*, *sea*), the *v-fms* oncogene induced erythroid progenitor cells to self-renew in an erythropoietin-independent manner. Spontaneous differentiation of the transformed cells was arrested by *v-erbA*. *v-fms* failed to induce transformation of myeloid cells but caused myeloid cells transformed by the *v-myb* oncogene, as well as normal macrophages, to proliferate independent of cMGF (chicken myelomonocytic growth factor). Unlike the other tyrosine kinases, *v-fms* did not induce cMGF secretion in *myb*-myeloblasts, suggesting a nonautocrine mechanism of growth factor independence.

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The *v-fms* gene, the retroviral oncogene of the McDonough strain of the feline sarcoma virus (SM-FeSV; McDonough et al. 1971), belongs to the family of *src*-related oncogenes (Hampe et al. 1984) exhibiting tyrosine-specific kinase activity (Tamura et al. 1986). Oncogenes of the tyrosine kinase family [*erbB*, *src*, *fps*, *sea* (Frykberg et al. 1983; Sealy et al. 1983; Kahn et al. 1984; Beug et al. 1985a; Palmieri 1985; Knight et al. 1988)], as well as the serine/threonine kinase *mil* (T. Graf, unpubl.) and the GTP-binding protein *Ha-ras* (Kahn et al. 1986; I. Stanley, unpubl.), are known to transform chicken embryo fibroblasts (CEFs) and chicken hematopoietic cells of the erythroid but not the myeloid lineage. In contrast, another group of oncogenes, including the nuclear oncogenes *myb* and *myc* (for review, see Graf and Beug 1978), transforms chicken myeloid cells but not erythroblasts. All oncogenes of both groups transform their respective target cells by inducing cell proliferation without completely blocking the ability of the cells to differentiate. Erythroid cells transformed by kinase-type oncogenes grow independent of erythroid growth factors [e.g., erythropoietin (EPO)]—growth factor independence probably results from a nonautocrine or bypass mechanism—whereas myeloid cells transformed by the nuclear oncogenes *myb* or *myc* are still dependent on cMGF (chicken myelomonocytic growth factor; Leutz et al. 1984). Although the kinase-type oncogenes cannot induce self-renewal in chicken myeloid cells, they can abrogate the cMGF requirement of *myc*- or *myb*-transformed myeloid cells by inducing the production of this growth factor, thus leading to stimulation of autocrine growth (Adkins et al. 1984).

In light of the idea that kinase-type oncogenes represent activated versions of growth factor receptors or other key components of the system(s) that regulate cell growth, it is difficult to understand why they are unable to induce uncontrolled cell proliferation in myeloid cells, although they do so in a number of other cell types, including erythroblasts. In an attempt to study this question, we constructed an avian retrovirus containing the feline tyrosine kinase oncogene *v-fms*. This oncogene represents an activated version of a major functional receptor [colony-stimulating factor-1 (CSF-1 receptor)] specific for myelomonocytic cells (Sherr et al. 1985). We will demonstrate that the *v-fms* oncogene exhibits transforming abilities in chicken hematopoietic cells similar to other tyrosine kinase oncogenes since it was able to transform fibroblasts and erythroblasts but was unable to stimulate continuous growth of myeloid cells. Although *v-fms* abrogated growth factor dependence of transformed and normal myeloid cells, it did not induce these cells to secrete detectable amounts of cMGF, suggesting a nonautocrine mechanism of growth factor independence.

Results

v-fms transforms chicken bone marrow cells in vitro

To investigate the transforming effects of the feline *v-fms* oncogene in chicken hematopoietic cells, we introduced the feline *v-fms* sequence into the AEV-ES4 virus genome (for details, see Materials and methods), thereby deleting both the *gag-erbA* and *-erbB* se-

Figure 1. Genomic structure of the *v-fms*-containing chicken retrovirus pCR-C. (Stippled boxes) Avian retrovirus sequences. (Δgag) RSV *gag* fragment; (sp) signal peptide sequence of RSV *env* gene. (Hatched boxes) Sequences from the SM-FeSV *fms* gene, including 400 (Δgag) from the 1600 nucleotides of SM-FeSV *gag* sequence originally encoding the *gag-fms* fusion protein. (sp) Putative signal peptide of *c-fms*. (S.D.) Splice donor; (S.A.) splice acceptor.

quences (Fig. 1). To test whether replicative, biologically active virus correctly expressing the *fms* gene could be generated from the recombinant retroviral construct, CEFs were transfected with the virus construct DNA, together with RAV-1 helper virus DNA, and a focus assay was carried out with the transfected cells. As a positive control for fibroblast transformation, the *erbB*-containing AEV-H virus construct pCR-1 was used. Table 1 shows that the *v-fms*-containing virus replicated well and caused focus formation in fibroblasts. The *fms*-transformed fibroblasts resembled *erbB*-transformed fibroblasts in their fusiform morphology (Fig. 2A–C) and in their ability to form diffuse foci. The two cell types exhibited elevated levels of hexose uptake, as well as a partial breakdown of actin filament cables and fibronectin network (Table 1). However, expression of these 'transformation parameters' (Royer-Pokora et al. 1978) appeared to be weaker in the *fms*-transformed fibroblasts than in the control CEFs transformed by the *erbB* oncogene (Table 1). Immunoprecipitation with an *fms*-specific antiserum carried out with transformed cells grown from individual foci showed that all transformed fibroblast clones expressed high levels of a 120-kD *fms* protein [Fig. 2D(1)], which corresponded to the size of the *fms* protein expected to be expressed from this construct.

The next step was to determine whether the *v-fms*-containing recombinant retrovirus was capable of transforming chicken hematopoietic cells. Chick bone marrow cells were infected with the *v-fms*-containing pCR-C virus and, as a positive control for transformation, with the *v-sea*-containing S13 virus (Beug et al. 1985b) and were cultured in methylcellulose-containing medium. To detect a possible, weak erythroid-transforming activity (Beug et al. 1985a; Kahn et al. 1986), a mixture of pCR-C virus and a retrovirus expressing the

v-erb Ar12 oncogene (Damm et al. 1987) was used for infection. In addition, bone marrow cells were cocultivated with virus-producing fibroblasts expressing the same oncogenes to increase the efficiency of infection (Beug et al. 1986). Both infected and cocultivated bone marrow cells were cultured either under erythroid (+EPO) or myeloid (+cMGF) conditions (Radke et al. 1982). Table 2 shows that transformed erythroblasts were obtained from bone marrow infected by incubation with virus or cocultivation, using the *fms*-containing retroviral construct pCR-C, the combination of pCR-C and *v-erb* Ar12r12 virus, and the S13 virus, when grown under erythroid conditions. In contrast, the pCR-C virus was unable, even after cocultivation, to induce outgrowth of myeloid cells, both under erythroid and myeloid culture conditions (Table 2). Although some relatively large clusters of macrophages were obtained from bone marrow cells infected by cocultivation and then seeded into Methocel, these cells could not be expanded after isolation and were therefore regarded as nontransformed (Table 2). These results strongly suggest that the tyrosine kinase oncogene *v-fms*, like other tyrosine kinase oncogenes [*src*, *fps*, *v-erbB*, *v-sea* (Kahn et al. 1986)], cannot induce myeloid transformation in chick bone marrow cells using the available assays.

To rule out that the inability of our *fms* construct to transform myeloid cells resulted from insufficient expression or instability of the p120 *fms* protein, E26-transformed myeloblasts (Beug et al. 1984) superinfected with the *fms*-containing virus (see below) were analyzed for p120 expression by immunoprecipitation. Figure 2D(panel 3) shows that a protein of the expected size of 120 kD could be precipitated in the myeloid cells, demonstrating that the lack of *fms*-induced myeloid transformation did not result from a failure in expression of the transforming *fms* protein in cells of the myeloid lin-

Table 1. Transformation of CEFs

Construct	Oncogene	Foci/ μ g DNA ^a	Virus-induced foci (FFU/ml) ^b	Actin cables (%) ^c	Fibronectin network ^d	Hexose transport (fold stimulation)
pCR-C	<i>fms</i>	0.4 (4)	7×10^4	50	++	2/2.5 ^e
pCR-1	<i>erbB</i>	2 (20)	1×10^5	17	+	2.5/3.5 ^e
pBO	—	<0.01	< 10^0	85	+++	1

^aFoci counted in focus assays performed with transfected CEFs.

^bFocus-forming titer of fibroblast-grown virus supernatants (Graf 1973). (FFU) Focus-forming units.

^cThe percentage of cells containing prominent actin filament bundles was determined as in Royer-Pokora et al. (1978).

^dExpression of the extracellular fibronectin network was judged as in Royer-Pokora et al. (1978).

^eData from two separate experiments.

Figure 2. Fibroblast transformation by *v-fms* and expression of the *v-fms* protein in chicken cells. (A–C) Phase micrographs of normal (A), *fms*-transformed (B), and *v-erbB*-transformed CEFs (C) are shown. (D) *fms*-transformed CEFs (1), erythroblasts (EBL; 2), and *myb*-*fms*-transformed myeloblasts (MBL; 3) were metabolically labeled with [35 S]methionine for 2 hr, and immunoprecipitation was carried out as described in Materials and methods. Proteins immunoprecipitated by anti-*fms* antiserum (gp120^{fms}), anti-*gag* antiserum (p180^{gag-pol}, p76^{gag}), and anti-*myb* antiserum (p135^{gag-myb-ets}) are indicated by arrows. Control immunoprecipitations were carried out using preimmune serum (N). (4–6) Glycoprotein processing of the *v-fms* protein is shown. *fms*-transformed erythroblasts were either metabolically labeled with [35 S]methionine in the presence of 1 μ g/ml tunicamycin (TM) for 2 hr (4) or with [14 C]mannose for 6 hr (MAN, 6), or pulse-labeled with [35 S]methionine and then chased up to 4 hr (5), as described in Materials and methods. *fms* proteins (p100, gp120, gp135, gp140^{fms}) are indicated by arrows.

age. This was further demonstrated by the fact that normal bone marrow macrophages infected with the *v-fms* virus by cocultivation (see Materials and methods) expressed the *fms* gene product in a significant fraction of the cells [Fig. 3B, see below].

Intracellular localization and processing of the expressed *fms* protein

The unexpected failure to transform myeloid cells with the avian *v-fms* retroviral construct prompted us to in-

vestigate whether or not subcellular localization and glycoprotein processing of p120 in avian cells was similar to that in mammalian cells (Anderson et al. 1984; Nichols et al. 1985; Rettenmier et al. 1985; Hadwiger et al. 1986). Because erythroblasts are easy to label with high specific activity, these cells were used for processing studies, whereas subcellular localization was studied in fibroblasts. As expected, *fms*-transformed erythroid cells expressed the 120-kD *fms* protein at high levels [Fig. 2D(panel 2)]. Immunoprecipitation of the same cells with antibody to avian *gag* proteins did not precipitate a *fms*-related protein [Fig. 2D(panel 2)], thus suggesting its expression from a subgenomic mRNA and/or cleavage at either signal peptide cleavage site (Fig. 1). Immunoprecipitation with an *fms*-specific polyclonal rabbit antiserum precipitated a 140-kD protein in addition to the 120-kD *fms* protein [Fig. 2D(panel 5)]. By pulse–chase analysis and mannose labeling, the 140-kD protein could be shown to represent the terminally glycosylated form of the *v-fms* protein [Fig. 2D(panels 5 and 6)]. From the same experiment it became clear, however, that only a small proportion of the 120-kD protein was terminally glycosylated to form the 140-kD species. The ~135-kD protein [Fig. 2D(panel 5)], which disappeared later in the chase, probably represented a partially glycosylated intermediate. As expected, treatment of the *fms*-expressing cells with tunicamycin yielded a 100-kD *fms* protein [Fig. 2D(panel 4)], confirming that the 120-kD *fms* protein is cotranslationally glycosylated. Immunofluorescence data obtained by surface staining of the *fms*-transformed erythroid cells showed that essentially all cells (>90%) were expressing the *v-fms* protein on the surface [Fig. 3A(panels e and f)]. Similar results were obtained for *fms*-transformed fibroblasts [Fig. 3A(panels a and b)], *fms*-superinfected E26 myeloblasts [Fig. 3A(panels g and h)], and normal macrophages infected with the *v-fms* virus by cocultivation (Fig. 3B). In this experiment, 15–20% of the infected macrophages, but no uninfected macrophages, were stained, suggesting that the antibodies used (SM 3.19.4; see Materials and methods) do not cross-react with the endogenous chicken *c-fms* protein.

Immunofluorescence studies carried out with fixed *fms*-transformed fibroblasts revealed a strong perinuclear staining, suggesting that most of the *fms* protein is localized on intracellular membranes. [Fig. 3A(panels c and d)]. These results show, as expected from work using mammalian cells (Anderson et al. 1984), that the *v-fms* protein is properly glycosylated and expressed at the plasma membrane in all cell types tested, although most of the *fms* protein seems to be localized at intracellular membranes.

Characterization of *fms*-transformed erythroid cells

Next we determined whether erythroid transformation by the *v-fms* oncogene was really comparable to that seen with other kinase oncogenes. Therefore, we characterized the *fms*-transformed erythroblasts for their stage of differentiation and their dependence on erythroid

Table 2. Transformation of bone marrow cells

Virus	Erythroid cocultivation ^a	Colonies	Myeloid cocultivation ^a	Colonies
pCR-C	+	1.2×10^2 ^{b/23} ^c	—	$<2 \times 10^0$ ^{b/0} ^c
pCR-C + <i>v-erbA</i>	+	2.0×10^2 ^{b/ND} ^d	—	$<2 \times 10^0$ ^{b/0} ^c
S13	+	$>2.0 \times 10^2$ ^{b/ND} ^d	—	ND
E26	—	ND ^d	+	$>2 \times 10^2$ ^{b/ND} ^d

^aCocultivation for erythroid cells was done as described in Beug et al. (1986); for myeloid cells, 10 U/ml of cMGF was added instead of EPO.

^bTo determine the colony-forming titers of virus stocks, standard erythroid and myeloid colony assays were done, as described in Graf et al. (1981). Values given are colony forming units per milliliter of virus (CFU/ml).

^cBone marrow cells were cocultivated with virus-producing fibroblasts for 2 days and seeded in Methocel containing erythroid or myeloid growth factors, respectively, as described in Materials and methods. Under myeloid conditions, some large macrophage colonies were obtained, which did not proliferate after isolation. Figures represent total number of colonies obtained per 35-mm dish.

^d(ND) Not determined.

growth factors. For this, *fms*-transformed erythroid cells from individual clones were examined with respect to their expression of several erythroid differentiation markers. Table 3 shows that the clonal cell populations represented a mixture of immature erythroblasts and more mature reticulocytes and erythrocytes, suggesting a high level of spontaneous differentiation (Beug et al. 1985a). The amount of immature and mature cells varied in the different clones examined. In contrast, all clones containing the *v-fms* oncogene (pCR-C virus) plus the *v-erbA*12 oncogene consisted almost exclusively of immature erythroblasts (Table 3), indicating that *v-erbA* arrests spontaneous differentiation in *fms*-transformed erythroblasts (Beug et al. 1985b). To study dependence of *fms* erythroblasts on EPO, cells were cultured in media lacking chicken serum components (Zenke et al. 1988), in the presence or absence of partially purified chicken EPO (Kowenz et al. 1987). Cells were grown in suspension culture and counted daily. In addition, they were seeded in Methocel-containing media, and the colonies obtained were analyzed for cell type distribution. The results of both assays clearly show that *fms*-transformed erythroblasts proliferate normally in the absence of EPO (Fig. 4). In contrast to other kinase–oncogene-transformed cells, *fms* erythroblasts grown in the presence of EPO slowed down in growth rate after 3–4 days (Fig. 4A) and formed compact colonies at high frequency containing an increased amount of mature cells (Fig. 4B,C), suggesting an induction of differentiation by EPO, as seen in mouse erythroleukemia cell lines (Klinken et al. 1988; Koury et al. 1988). Control experiments carried out with *sea*[−] and mutant epidermal growth factor receptor (EGFR)-transformed erythroblasts (Khazaie et al. 1988; Knight et al. 1988) failed to show a similar effect of EPO on these cells.

Taken together, these results indicate that *v-fms*-transformed erythroblasts correspond closely to erythroblasts transformed by other tyrosine kinase oncogenes such as *v-erbB*, *v-src*, *v-fps*, and *v-sea* (Kahn et al. 1986; Knight et al. 1988; Zenke et al. 1988).

cMGF independence of myb–fms-transformed myeloblasts

Because the *v-fms* oncogene failed to transform cells of the myeloid lineage, we investigated whether the *v-fms* oncogene would induce autocrine growth in *myb*- or *myc*-transformed chicken myeloid cells, as shown for other tyrosine kinase oncogenes (Adkins et al. 1984). Accordingly, myeloblasts expressing both the *myb* and *fms* oncogene were first assayed for cMGF-independent growth. A pool of E26 (*myb-ets*)-transformed, nonproducer myeloblast clones was superinfected with the *fms*-containing pCR-C virus, the *erbB*-containing AEV-ES4 A2[−] virus (carrying an inactivating deletion in *v-erbA*; Frykberg et al. 1983), and the *src*-containing Rous sarcoma virus (RSV), respectively. This type of superinfection experiment was repeated several times with pools of clones either transformed by wild-type E26 or ts21 E26 virus (Beug et al. 1984). Superinfection was carried out either by direct infection of *myb*-transformed, nonproducer myeloblasts with high-titer virus stocks or by cocultivation of the same cells with virus-producing fibroblasts. The superinfected cells were then cultured either in medium containing methylcellulose or in liquid culture in the presence and absence of cMGF, respectively. Figure 5 shows that a pool of E26 nonproducer clones superinfected with the *fms*-containing virus could be grown for 2 weeks in the absence of cMGF, similar to *myb*-transformed myeloblasts superinfected with *erbB*, or *src*-containing viruses. In contrast, the uninfected E26 control cells were completely dependent on cMGF for proliferation (Fig. 4). Furthermore, as determined by [³H]thymidine (TdR) incorporation into DNA, the cells of two ts21 clones superinfected with pCR-C, AEV-ES4 A2[−], and RSV, respectively, incorporated ~10 times more radioactivity in the absence of cMGF than the noninfected control cells did, whereas incorporation in the presence of growth factor was similar for all cell types (data not shown). Finally, the same number of Methocel colonies could be ob-

tained with and without cMGF upon superinfection of the *myb-ets*-transformed myeloid cells with pCR-C,

AEV-ES4 A2⁻, or RSV, respectively, whereas a >100-fold difference in colony number was observed with E26 control cells under these conditions (data not shown).

myb-fms-transformed myeloblasts do not secrete detectable cMGF

To study whether growth factor independence in *myb-fms*-transformed myeloblasts resulted from autocrine growth stimulated by secreted cMGF, 10- to 20-fold concentrated conditioned media (Beug et al. 1984) were prepared from *myb-fms*-transformed myeloblasts, from uninfected (wild-type E26, ts21 E26) control cells and, as a positive control, from *myb-erbB*- and *myb-src*-transformed myeloblasts. Then, conditioned media were tested on cMGF-dependent E26 cells to reveal factor secretion. As expected, *myb-erbB*-transformed myeloblasts and *myb-src*-transformed myeloblasts secreted a growth-promoting activity (Table 4; Fig. 6). In contrast, conditioned media from *myb-fms*-transformed myeloblasts consistently contained no or very low amounts of growth factor (Table 4; Fig. 6), suggesting that the *v-fms* oncogene does not induce cMGF production in infected myeloblasts to a level sufficient for stimulating autocrine growth.

The fms-oncogene induces normal macrophages to proliferate in a factor-independent fashion

To study whether expression of the *fms* oncogene protein in normal macrophages (Fig. 3B) would affect their proliferation and/or dependence on growth factors, two experiments were performed. First, 1×10^6 cells of both normal and *fms*-infected macrophages (see Materials and methods) were seeded in growth factor- and chicken serum-free medium, and the number of viable cells was determined 6 days later. Whereas 1.45×10^6 viable cells were obtained from the *fms*-infected macrophages, only 2.8×10^5 cells were obtained from the uninfected control culture. Furthermore, both *fms*-infected and uninfected macrophages were seeded into Methocel at different cell concentrations, both in the presence and absence of cMGF. As shown in Figure 7, normal macrophages were dependent on cMGF for cluster and colony formation (Leutz et al. 1984), particularly at low cell density. In contrast, *fms*-infected macrophages formed clusters and colonies with essentially similar efficiency in the presence and absence of cMGF (Fig. 7A). Interestingly, colony formation of uninfected macrophages in the absence of growth factor was strongly dependent on cell density, whereas *fms*-infected macrophages formed clusters and colonies at all densities tested with similar efficiencies (Fig. 7B). This again suggests that *v-fms* abrogates factor independence via a nonautocrine mechanism.

Trials to replat the macrophage colonies obtained from normal and *v-fms*-infected macrophages were unsuccessful, showing again that *v-fms* did not induce a detectable transformed phenotype in these cells.

Figure 3. Expression of the *v-fms* oncogene in living and fixed *v-fms*-infected cells. (A) Living *v-fms*-transformed fibroblasts (a), erythroblasts (e), *fms* superinfected E-26 myeloblasts (g), as well as fixed *fms*-transformed fibroblasts (c), were stained with anti-*fms* antibody plus FITC goat anti-rat antibody and visualized by indirect immunofluorescence, as described in Materials and methods. (Right) The respective control cells lacking *v-fms* (*v-erbB* erythroblasts, f; E26 myeloblasts, h; and uninfected fibroblasts, b, d). Note perinuclear staining of the *fms* protein in fixed, transformed fibroblasts (c). (B) Normal macrophages co-cultivated with *v-fms*-transformed fibroblasts (*fms* macrophages) or with uninfected CEFs [normal macrophages; see Materials and methods] were stained with anti-*fms* antibody plus FITC goat anti-rat antibody and visualized by indirect immunofluorescence (top). Note absence of cell-surface staining in the normal macrophages. (Bottom) *fms*-infected macrophages were stained with anti-*fms* antibody plus FITC goat anti-rat antibody (left) and anti-macrophage plus anti-myeloblast serum (Beug et al. 1979) plus TRITC goat anti-rabbit antibody (right) and visualized in double immunofluorescence (Beug et al. 1982b), showing the same field of cells.

Table 3. Characterization of *fms*-transformed erythroblast clones

Virus	Clone	Hemoglobin-positive cells (%) ^a	Cells classified as LR + E (%) ^b	Cells stained with c ^d (%)		
				α Ebl	α Ery	α Mbl
pCR-C (<i>fms</i>)	B 5	62	9	50	31	<0.1
	B 9	79	7	90	43	<0.1
	D 9	>95	35	ND	ND	ND
	D 10	>95	20	28	79	<0.1
pCR-C + <i>v-erbA</i>	G 3	40	0.5	90	10	<0.1

^aDetermined by staining with acid benzidine (Beug et al. 1982).^bCell types were defined as described (Beug et al. 1982) by staining with neutral benzidine plus histological dyes. (LR) Late reticulocytes; (E) erythrocytes.^c(α Ebl) Anti-erythroblast serum; (α Ery) anti-erythrocyte serum; (α Mbl) anti-myeloblast monoclonal MC 51-2.^d(ND) Not determined.

Discussion

Oncogenic spectrum of *v-fms* in chick hematopoietic cells

The results presented in this paper indicate that the *v-fms* oncogene, although representing an activated version of a growth factor receptor specific for myelomonocytic cells, is not capable of inducing self-renewal in avian hematopoietic cells of the myeloid lineage. Instead, like other tyrosine kinases, the *v-fms* oncogene transforms fibroblasts and induces self-renewal in cells of the erythroid lineage. *fms*-transformed erythroid cells closely correspond to erythroblasts transformed by *v-erbB*, *v-src*, *v-fps*, and *v-sea* (Kahn et al. 1986; Knight et al. 1988; Zenke et al. 1988), with respect to their self-renewal capacity, stage of differentiation, and *v-erbA*-induced arrest of differentiation (Beug et al. 1985b; Kahn et al. 1986). They differ, however, from erythroblasts transformed by other kinases because their spontaneous differentiation was enhanced by EPO, as seen in a variety of mammalian erythroleukemic cell lines (Klinken et al. 1988; Koury et al. 1988). Thus, not only the EGF receptor, but also the myeloid-specific CSF-1 receptor, can induce self-renewal in erythroid cells, when activated as an oncogene and overexpressed in these cells. Our results agree with the ability of the *v-fms* oncogene to induce erythroleukemia, as well as proliferative abnormalities in other hematopoietic lineages in mice (Heard et al. 1987) and suggest that tissue-specific growth factor receptors, at least in their oncogenic versions, can function in signal transduction in a rather promiscuous fashion.

Why is *v-fms* unable to transform myeloid cells?

There are several possible explanations for the observed inability of *v-fms* to induce excessive self-renewal (as caused by *v-myc* and *v-myb*) in myeloid cells. We apparently can rule out absent or low expression of *v-fms* in these cells, because the oncogene is expressed in both normal macrophages and transformed myeloblasts and

exhibits distinct biological activity in both cell types (Fig. 7). So far, the *v-fms* oncogene has not been reported to transform normal mammalian myeloid cells in vitro. In addition, activation of the CSF-1 receptor by ligand not only induces survival and proliferation in immature myeloid progenitors but also stimulates the differentiation and activation of more mature myelomonocytic cells (Stanley et al. 1983). Exactly which type of growth factor effect predominates may depend on the concentration of free ligand, the density of receptors at the cell surface, and the stage of maturation of the myeloid cell. Immature myeloid bone marrow cells express much fewer CSF-1 receptors per cells than mature macrophages do (Bartelmez et al. 1985; Gilbert and Stanley 1986). Furthermore, CSF-1 receptor numbers on GM-CFU increase prior to their differentiation into adherent mononuclear phagocytes (Bartelmez et al. 1985). Thus, overexpression of the constitutively active *v-fms* oncogene in macrophage progenitors may induce their rapid differentiation into adherent, nonproliferating macrophages in addition to stimulating their proliferation. It is also conceivable that *v-fms* expression induces production of other cytokines like tumor necrosis factor (TNF), γ -interferon, and/or interleukin-1, which induce macrophage differentiation and activation (Warren and Ralph 1986).

Our failure to induce transformation in avian myeloid cells by the feline *v-fms* oncogene could also be caused by species-specific differences between the avian and mammalian *fms* oncogenes, the heterologous mammalian receptor being able to induce some, but not all, effects produced by the homologous receptor.

Finally, the normal function of the CSF-1 receptor may involve stimulation of both proliferation and differentiation, similar to EPO receptor function in erythroid cells (Kowenz et al. 1987). This idea is supported by the finding that recombinant human CSF-1 induces differentiation of human monocytes into macrophages but does not stimulate prolonged growth of these cells (Becker et al. 1987). In addition, CSF-1 was found to stimulate the production of nondividing, adherent cells from proliferating acute myeloblastic leukemia (AML)

Figure 4. Growth and differentiation of *fms*-transformed erythroblasts in the presence and absence of EPO. (A) Cells were seeded into CFU-E-Methocel in the absence (–EPO) or presence (+EPO) of purified chicken EPO plus REV-factor (Kowenz et al. 1987), and colonies were photographed 3 days later (top). Alternatively, cells were grown in suspension culture (Zenke et al. 1988) in the presence and absence of the above factors. After 3 days at 37°C, cells were cytocentrifuged onto slides, stained with neutral benzidine plus histological dyes, and photographed under blue light, as described previously (bottom; Beug et al. 1982b). (Insets) Typical colonies at higher magnification. (B) Growth kinetics of *fms*-transformed erythroblasts cultivated in the presence (△) or absence (○) of purified EPO. Cells were counted daily and readjusted to $1\text{--}1.5 \times 10^6$ cells/ml with fresh medium. Cumulative cell numbers obtained were plotted. (C) Relative percentages of erythrocytes (Ery), late reticulocytes (LR), early reticulocytes (ER), and erythroblasts (Ebl), as defined in Beug et al. (1982b), were counted using the benzidine-stained cytocentrifuge preparations shown in A.

cells (Miyauchi et al. 1988). It is tempting to speculate that *v-fms*, as well as the other kinase oncogenes, do not induce self-renewal in myeloid cells because they are unable to reduce expression of myeloid differentiation genes, as observed for erythroid-specific genes in erythroblasts transformed by these oncogenes (Knight et al. 1988).

We hope to resolve some of these questions using the avian *c-fms* proto-oncogene that has recently been cloned (U. Fuhrmann et al., in prep.). After insertion into a suitable retrovirus vector and identification of the avian CSF-1 ligand [mammalian CSF-1 is completely inactive in chicken cells (A. Leutz, T. Graf, and H. Beug, unpubl.)], we intend to investigate how overexpression

of the avian *c-fms* gene in the presence and absence of ligand would affect myeloid and erythroid growth and differentiation. Antibodies to the avian *c-fms* protein would also allow clarification of the role of endogenous receptor in CSF-induced growth and differentiation of chick macrophages.

How does the v-fms oncogene abrogate growth factor dependence in myeloid cells?

Similar to other oncogenes of the tyrosine kinase family (Adkins et al. 1984), the *v-fms* oncogene can abolish the growth factor requirement of *myb*-transformed myeloid cells. Whereas superinfection of *v-myb*-transformed myeloid cells with the *v-erbB*, *v-src*, and *v-fps* oncogenes resulted in the induction of cMGF, which then stimulated autocrine cell growth (Adkins et al. 1984), little, if any cMGF-like activity could be detected in the supernatant of proliferating, factor-independent *myb-fms*-transformed myeloblasts. Similarly, colony formation in the absence of exogenous factor of normal bone marrow macrophages [that constitutively produce low amounts of cMGF (H. Beug et al. unpubl.)] was strongly dependent on cell density, whereas colony formation of *v-fms*-infected macrophages under the same conditions was not affected by cell density (Fig. 7). Thus, the *v-fms* oncogene, in contrast to other tyrosine kinase oncogenes, seems to abrogate factor independence by a nonauto-

Figure 5. Factor-independent growth of *myb-fms*-transformed myeloblasts. E26 myeloblasts superinfected with RSV, AEV-ES4 A2⁻, or pCR-C were grown in the absence of cMGF. Cells were counted at the times indicated using a Coulter counter and plotted as cumulative cell numbers.

Table 4. Growth-stimulating activity in conditioned media from myb-myeloblasts superinfected with tyrosine-kinase oncogenes

Conditioned media from myb myeloblasts superinfected with	Maximum growth-stimulatory activity ^{a-c} ([³ H]TdR cpm)	Growth factor activity (units) ^{b,d}
<i>erbB</i>	15600, 16500	80, 10
<i>src</i>	—, 11100	—, 3
<i>fms</i>	6150, 6120	<0.5, <0.2
None	4360, 3900	<0.2, <0.2
cMGF (positive control)	22500, 31300	1300

^aValues obtained at different concentrations of the various conditioned media are shown.

^bResults from two different experiments using different batches of conditioned media and different test cells.

^cBackground counts (obtained in test medium only) were 3000 cpm.

^dUnit defined as in Leutz et al. (1984).

crine mechanism. As a working hypothesis, we assume that *v-fms* stimulates myeloid cell proliferation by producing a continuous signal, thereby bypassing the requirement for an activated cMGF receptor. This idea is in agreement with data obtained by Wheeler et al. (1986, 1987), showing that the *v-fms* oncogene abrogates the growth factor requirements of interleukin-3-dependent FDC-P1 cells and a CSF-1 dependent SV40-transformed macrophage cell line via a nonautocrine mechanism.

At present, several other possibilities, such as production of low amounts of cMGF that are nevertheless able to stimulate growth of hypersensitive *fms*-transformed E26 myeloblasts or intracellular action of cMGF, cannot be excluded. Because conventional methods to detect cMGF mRNA were not sensitive enough to reliably detect cMGF message in factor-producing cells (A. Leutz, pers. comm.), amplification by PCR will have to be applied to settle this question.

Materials and methods

Construction of an avian retrovirus expressing the feline v-fms oncogene

The SM-FeSV provirus cloned in pBR 325 (pSM-FeSV) containing the *v-fms* oncogene was kindly provided by Charles Sherr. The chicken vector used was the plasmid pCR-1 containing a permuted form of the genome of the AEV strain ES4 (Vennström et al. 1980; Jansson et al. 1987) in pSV2 *neo* (Southern and Berg 1982).

pSM-FeSV was cleaved with *Bam*HI at the 3' end and filled in with Klenow polymerase, and an 8-mer *Eco*RI linker (Biolabs) was inserted. Cleavage of pSM-FeSV with *Sst*II at the 5' end and ligation of a 6-mer adapter (-TCGAGC-; EMBL, Oligonucleotide Service) generated a *Sal*I site, thereby conserving the correct reading frame of the *v-fms*, as determined by sequence analysis. The *Sal*I-*Eco*RI-*fms* fragment was inserted into the *Xho*I and *Eco*RI sites of pCR-1, thereby deleting both the *gag-erbA* and *-erbB* sequences of this plasmid. This basic vector was denoted pUM 2. For transferring the *v-fms*-encoded protein into the rough endoplasmic reticulum, a *Xho*I-*Bam*HI fragment containing the signal sequence of the envelope gene of RSV (Prague C) and a splice acceptor site at the 5' end was in-

Figure 6. Conditioned media prepared from *myb-fms* myeloblasts do not stimulate growth of cMGF-dependent myeloblasts. Conditioned media were prepared and tested on cMGF-dependent E26 cells, as described in Materials and methods. In the cMGF control (top left), purified cMGF dissolved in test medium at a concentration of 1300 U/ml was used.

serted into the *Xho*I and *Bam*HI sites of pUM 2. In addition, a *Bam*HI *gag* fragment of RSV was inserted into the *Bam*HI site of pUM22, providing the packaging signal for the virus and a splice donor site.

Cells and cell culture

All cells were derived from our SPAFAS chicken flock maintained in Heidelberg. CEFs were prepared according to Graf (1973) and grown in standard growth medium, consisting of modified Dulbecco's modified Eagle medium (DMEM) plus 8% fetal calf serum, 2% chicken serum, and 10 mM HEPES (pH 7.3). Transformed erythroblasts were cultivated in CFU-E medium without anemic serum (Radke et al. 1982), unless stated otherwise. Transformed myeloblasts were grown in standard growth medium (see above), with or without 3% crude cMGF (Beug et al. 1982a).

Transfection of CEFs

Primary CEFs were transfected with 10 μ g of virus DNA, together with 5 μ g of RAV-1 DNA (Frykberg et al. 1987) to allow for virus spread. A focus assay with the transfected CEFs was performed 2 days after transfection, as described by Frykberg et al. (1983). Foci were isolated by scraping after discarding the agar overlay, and cells were expanded in standard growth medium. Virus titer was determined by carrying out a subsequent focus assay (Graf 1973) using fibroblast supernatant.

In vitro transformation of bone marrow cells

Bone marrow was prepared from 3- to 10-day-old SPAFAS chicks, as described (Graf 1973). Bone marrow cells were infected with high-titer virus stocks and then seeded in methylcellulose-containing medium with either 1% dimethylsulfoxide and 5% anemic chicken serum as a source for EPO or 2% cMGF as described earlier (Graf 1973, 1981). Alternatively, bone marrow cells were cocultivated with virus-producing fi-

Figure 7. Colony formation of normal and *fms*-infected macrophages in the presence and absence of cMGF. (A) Photographs of colonies and clusters obtained 10 days after seeding 5×10^4 *fms* macrophages or normal macrophages per 35-mm dish in the presence (+ cMGF) or absence (- cMGF) of 10 U/ml of purified factor. (Insets) Typical colonies at higher magnification. (B) Clusters (5–10 cells) and colonies (>30 cells) were counted in cultures similar to those shown in A, 10 days after seeding, and plotted against the cell numbers seeded. (GF) cMGF.

broblasts either under erythroid conditions (Beug et al. 1986) or myeloid conditions (10 U/ml of cMGF being added instead of EPO). After 2 days, 2×10^6 cocultivated bone marrow cells were seeded in CFU-E Methocel, consisting of a 1 : 1 mixture of pH 8.1 and pH 7.2 Methocel (Zenke et al. 1988; Methocel was made up using NaCl/NaHCO₃-free medium instead of the respective liquid medium). For erythroid conditions, 1% anemic serum, insulin, and an optimal dose of REV-T-virus induced-erythroid factor (Kowenz et al. 1987) was added, whereas for myeloid conditions, cMGF and a myeloid growth factor produced by a chicken fibroblast cell line (CEF-32; H. Kaaden, pers. comm.) were added at optimum concentrations (as determined on E26 myeloblasts).

Immunoprecipitation analysis

A total of 7.5×10^6 transformed adherent fibroblasts grown on 100-mm Nunc dishes, 5×10^6 to 20×10^6 erythroblasts, and 5×10^6 to 20×10^6 myeloblasts were labeled for 2 hr with 200–300 mCi [³⁵S]methionine, respectively, as described (Beug et al. 1981). Cells were lysed in RIPA buffer (Hayman et al. 1979, 1983); cell lysates were immunoprecipitated with a rat monoclonal against the v-*fms* protein (SM 3.19.4, a kind gift of Charles Sherr; Anderson et al. 1982). An anti-*myb* antibody (Ness et al. 1987) and an anti-*gag* monoclonal antibody (IG10, a kind gift of D. Boettiger) were used to precipitate the viral *myb* protein and virus structural proteins, respectively.

Labeling of erythroblasts in the presence of tunicamycin (1 μ g/ml) was initiated after a 30-min preincubation period with the antibiotic at this concentration. For pulse-chase analysis, 50×10^6 cells were labeled with 1 mCi of [³⁵S]methionine in 2 ml of labeling medium (Beug et al. 1981) for 20 min and then chased up to 4 hr with complete, methionine-containing medium. For mannose labeling, 30×10^6 *fms* erythroblasts were labeled for 6 hr with 250 μ Ci [³H]mannose in medium containing one-tenth the normal amount of glucose [4.5 g/liter (Beug and Hayman 1984)].

Immunoprecipitation was carried out using a rabbit anti-serum against bacterially expressed *fms* protein (Furman et al. 1986). The immunoprecipitates were analyzed by SDS-PAGE, according to the method of Laemmli (1970), on 7–15% linear gradient slab gels. Protein bands were detected by fluorography (Bonner and Laskey 1974).

Assays for fibroblast transformation parameters

Assays for actin cables, fibronectin network expression, and uptake of [³H]deoxyglucose were performed as described previously (Royer-Pokora et al. 1978; Palmieri et al. 1983). Focus assays were performed according to Graf (1973).

Immunofluorescence

The virus-transformed fibroblasts, erythroblasts, and myeloblasts were analyzed for their expression of v-*fms* protein at the cell surface by indirect live cell immunofluorescence, as described earlier (Hayman et al. 1983; Beug and Hayman 1984; Beug et al. 1986), using a rabbit antiserum against bacterially expressed *fms* protein (Furman et al. 1986) plus FITC-labeled goat anti-rabbit IgG or the SM 3.19.4 monoclonal antibody (see above) plus FITC-labeled goat anti-rat antibody. For intracellular staining, transformed fibroblasts seeded on glass coverslips (Royer-Pokora 1978) were fixed and permeabilized according to Anderson et al. (1982) prior to antibody treatment.

Assays for erythroid differentiation markers

Hemoglobin content was determined by benzidine staining at acid or neutral pH (Beug et al. 1982b). Rabbit anti-erythroblast, anti-erythrocyte, anti-macrophage, and anti-myeloblast antisera (Beug et al. 1979) and mAb MC51-2, specific for myelomonocytic cells (Kornfeld et al. 1983), were used in indirect immunofluorescence, as described (Beug et al. 1979).

Infection of normal bone marrow macrophages with the *fms* retrovirus

Bone marrow cells were cocultivated for 2 days with pCR-C-infected fibroblasts or, in the control, with RAV-1-infected fibroblasts. Thereafter, the nonadherent cells were cultivated for 3 days on bacterial dishes in the presence of cMGF to remove any

residual fibroblasts and on tissue culture dishes for another 2 days. Adherent macrophages were harvested using EDTA (Hayman et al. 1983) and stained with anti-*fms* antibodies, as described above, or seeded in liquid medium or CFU-E-Methocel (minus erythroid factors, plus and minus 10 U/ml cMGF). Colonies were counted after 10–14 days at 37°C.

Superinfection experiments

E26- or ts21-transformed myeloblasts were either superinfected with virus directly or cultured in methylcellulose-containing medium (Graf et al. 1981) or in liquid culture with and without 2% cMGF, respectively. In addition, 3×10^6 *myb*-transformed myeloblasts were cocultivated with 2×10^5 virus-producing fibroblasts seeded on 35-mm Nunc dishes the day before, with and without 0.5% purified cMGF. Further cocultivation was carried out as described in Beug et al. (1986).

Preparation and testing of conditioned medium

To obtain conditioned medium from the superinfected myeloblasts, 3×10^6 cells were grown overnight in serum-free monoclonal medium (Adkins et al. 1984). The conditioned medium was concentrated 10- to 20-fold by ultrafiltration (Amicon, YM 5-membrane) and titrated on E26-transformed factor-dependent myeloblasts, as described in Leutz et al. (1984).

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