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 forming myeloid cells by inducing the production of this growth factor receptor (chicken myelomonocytic growth factor). Unlike the other tyrosine kinases, expressing the feline myc oncogene in chicken myeloblasts, suggesting a nonautocrine mechanism of growth factor independence.

Key Words: Oncogenes, CSF-1 receptor; transformation; cell lineage hematopoiesis

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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 [eg., 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-
quences [Fig. 1]. To test whether replicative, biologically active virus correctly expressing the \textit{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 \textit{erbB}-containing AEV-H virus construct pCR-1 was used. Table 1 shows that the \textit{v-fms}-containing virus replicated well and caused focus formation in fibroblasts. The \textit{fms}-transformed fibroblasts resembled \textit{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 proteins expected to be expressed from this construct to \textit{OH} was weaker in the partial breakdown of actin filament cables and fibronectin network (Table 1). However, expression of these proteins expected to be expressed from this construct to \textit{OH} was weaker in the

The next step was to determine whether the \textit{v-fms}-containing recombinant retrovirus was capable of transforming chicken hematopoietic cells. Chick bone marrow cells were infected with the \textit{v-fms}-containing pCR-C virus and, as a positive control for transformation, with the \textit{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 \textit{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 \textit{fms}-containing retroviral construct pCR-C, the combination of pCR-C and \textit{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 \textit{v-fms}, like other tyrosine kinase onco genes [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 \textit{fms} construct to transform myeloid cells resulted from insufficient expression or instability of the p120 \textit{fms} protein, E26-transformed myeloblasts [Beug et al. 1984] superinfected with the \textit{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 \textit{fms}-induced myeloid transformation did not result from a failure in expression of the transforming \textit{fms} protein in cells of the myeloid lin-

### Table 1. Transformation of CEFs

| Construct | Oncogene | Foci/μg DNA* | Virus-induced foci [FFU/ml]b | Actin cables [%c] | Fibronectin networkd | Hexose transport (fold stimulation) |
|-----------|----------|--------------|-------------------------------|-----------------|-----------------|----------------------------------|
| pCR-C     | \textit{fms} | 0.4 [4]      | $7 \times 10^4$               | 50              | ++              | 2/2.5e                           |
| pCR-1     | \textit{erbB} | 2 [20]       | $1 \times 10^5$              | 17              | +               | 2.5/3.5e                         |
| pBO       | —        | <0.01        | $<10^6$                      | 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].
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 investigate 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 c 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...
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* 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 cMGF-containing pCR-C virus, the *erbB*-containing AEV-ES4 A2+ virus [carrying an inactivating deletion in *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-

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**Table 2. Transformation of bone marrow cells**

| Virus       | Erythroid cocultivation | Colonies     | Myeloid cocultivation | Colonies     |
|-------------|-------------------------|--------------|-----------------------|--------------|
| pCR-C       | +                       | 1.2 × 10^3 b/23c | -                     | <2 × 10^3 b/0c |
| pCR-C       | +                       | 2.0 × 10^3 b/NDd | -                     | <2 × 10^3 b/0c |
| + v-erbA    |            |             |                       |              |
| S13         | +                       | >2.0 × 10^3 b/NDd | -                     | ND           |
| E26         | -                       | NDd          | +                     | >2 × 10^3 b/NDd |

*a* Cocultivation 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.

*b* To 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].

*b* Bone 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.

*N* (ND) Not determined.
tained with and without cMGF upon superinfection of the myb-ets-transformed myeloid cells with pCR-C.

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 × 10⁶ 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 × 10⁶ viable cells were obtained from the fms-infected macrophages, only 2.8 × 10⁵ 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 replate 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.
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-erbA, 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 cell 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 [Beug et al. 1982b]. (Insets) Typical colonies at higher magnification. (B) Growth kinetics of fms-transformed erythroblasts cultivated in the presence (Δ) or absence (O) of purified EPO. Cells were counted daily and readjusted to 1–1.5 × 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 [mammalin 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 EPO would affect myeloid and erythroid growth and differentiation. Antibodies to the avian c-fms protein would also allow clarification of the role of endogeneous 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.

2078 GENES & DEVELOPMENT
XhoI and EcoRI

in agreement with data obtained by Wheeler et al. (1986,
into the rough endoplasmic reticulum, a
ment containing the signal sequence of the envelope gene of
quirement for an activated cMGF receptor. This idea is
ducing a continuous signal, thereby bypassing the re-

At present, several other possibilities, such as produc-
tion 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 de-
tect cMGF message in factor-producing cells [A. Leutz,
pers. comm.], amplification by PCR will have to be ap-
plied 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] con-
taining the v-fms oncogene was kindly provided by Charles
Sherr. The chicken vector used was the plasmid pCR-1 con-
taining 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 BamHI at the 3' end and filled in
with Klenow polymerase, and an 8-mer EcoRI linker [Biolas]
was inserted. Cleavage of pSM-FeSV with SstII at the 5' end and
ligation of a 6-mer adapter [-TCGAGC-; EMBL, Oligonucleo-
tide Service] generated a SalI site, thereby conserving the cor-
crect reading frame of the v-fms, as determined by sequence
analysis. The SalI-EcoRI--fms fragment was inserted into the
XhoI and EcoRI 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 XhoI--BamHI frag-
ment containing the signal sequence of the envelope gene of
RSV [Prague C] and a splice acceptor site at the 5' end was in-
serted into the XhoI and BamHI sites of pUM 2. In addition, a
BamHI gag fragment of RSV was inserted into the BamHI 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 main-
tained 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 me-
dium 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 µg of virus DNA, to-
gether with 5 µg 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 me-
dium. 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 in-
fected with high-titer virus stocks and then seeded in methyl-
cellulose-containing medium with either 1% dimethylsul-
oxide 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-

| Conditioned media from myb myeloblasts superinfected with | Maximum growth-stimulatory activity* | Growth factor activity (pmol/0.1 ml) |
|-------------------------------------------------------------|--------------------------------------|----------------------------------|
| erbB                                                        | 15600, 16500                         | 80, 10                           |
| src                                                         | —, 11000                             | —, 3                             |
| fms                                                         | 6150, 6120                           | <0.5, <0.2                       |
| None                                                        | 4360, 3900                           | <0.2, <0.2                       |
| cMGF                                                        | 22500, 31300                         | 1300                             |

*Values obtained at different concentrations of the various condi-
tioned media are shown.

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

Background counts [obtained in test medium only] were 3000

cpm.

Values obtained at different concentrations of the various condi-
tioned media are shown.

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

Background counts [obtained in test medium only] were 3000

cpm.

Unit defined as in Leutz et al. (1984).
Immunoprecipitation analysis

A total of $7.5 \times 10^6$ transformed adherent fibroblasts grown on 100-mm Nunc dishes, $5 \times 10^6$ to $20 \times 10^6$ erythroblasts, and $5 \times 10^8$ to $20 \times 10^8$ myeloblasts were labeled for 2 hr with 200–300 mCi $[^{35}S]$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-myc 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 

Immunoprecipitation was carried out using a rabbit antisera 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 (Bonnei and Laskey 1974).

Assays for fibroblast transformation parameters

Assays for actin cables, fibronectin network expression, and uptake of $[^{3}H]$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 × 10⁶ myb-transformed myeloblasts were cocultivated with 2 × 10⁶ 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 × 10⁶ 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 myeloid cells. The conditioned medium (Adkins et al. 1984) was concentrated 10- to 20-fold by ultrafiltration (Amicon, YM 5-membrane) and titrated on E26-transformed factor-dependent myeloid cells. Further cocultivation was carried out as described in Beug et al. [1986].

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