Autocrine stimulation after transfer of the granulocyte/macrophage colony-stimulating factor gene and autonomous growth are distinct but interdependent steps in the oncogenic pathway

(growth factors/*mos* oncogene/myeloproliferative sarcoma virus/retrovirus vectors)

C. LAKER*, C. STOCKING*, U. BERGHOLZ*, N. HESS*, J. F. DE LAMARTER†, AND W. OSTERTAG*‡

*Heinrich-Pette-Institut für Experimentelle Virologie und Immunologie an der Universität Hamburg, Martinistrasse 52, 2000 Hamburg 20, Federal Republic of Germany; and †Biogen, 46 Route des Acacias, 1227 Geneva, Switzerland

Communicated by Manfred Eigen, August 3, 1987

ABSTRACT Autocrine stimulation of cells by aberrant synthesis of growth factor may lead to malignant transformation, either as a direct consequence of endogenous factor production or as a first step of a series of successive events. Introduction of the granulocyte/macrophage colony-stimulating factor (GM-CSF) cDNA clone into a vector based on the myeloproliferative sarcoma virus allowed efficient transfer and expression of GM-CSF in factor-dependent myeloid cell lines (FDC-P1 and FDC-P2). Factor-independent growth was acquired when the vector was introduced into the GM-CSF-responsive FDC-P1 cell line but not the multi-CSF-dependent FDC-P2 line. Nonlinear clonability in the absence of exogenous growth factor and growth inhibition by GM-CSF antiserum support a model of autocrine stimulation that requires interaction of factor and receptor at the outer membrane. However, many, but not all, infected FDC-P1 cells acquired subsequently a second mutation that abrogated the requirement of GM-CSF secretion and external interaction. The nature of the second step, which presumably leads to tumorigenicity of these cells, is not well understood, but its frequency could be correlated with the level of GM-CSF released by an individual cell clone.

There is much circumstantial evidence that links the process of malignant transformation to aberrant activation of growth factor genes in cells that express the corresponding receptor. Several tumor cell lines are known in which rearrangements of growth factor genes have occurred resulting in activation of growth factor production (1–3), but the requirement of growth factor production for factor-independent growth or malignancy could not be demonstrated.

Two retroviruses have been utilized to study the contribution of growth factor gene activation to malignancy. Infection with the simian sarcoma virus (SiSV) carrying the *sis* oncogene [homologous to the β -chain gene of the platelet-derived growth factor (PDGF)] results in transformation only in cells that express the PDGF receptor (4, 5). Treatment with PDGF antiserum inhibits cell proliferation (6), and cells infected by SiSV are neither immortal nor tumorigenic (7). Infection of a factor-dependent promyelocytic cell line (FDC-P1) with a retrovirus vector (Z-GMV) based on the Moloney murine leukemia virus (Mo-MuLV) and containing the coding region of granulocyte/macrophage colony-stimulating factor (GM-CSF) (8) also resulted in autocrine stimulation of the cell line. However, their growth was not inhibited by GM-CSF antiserum and the cloning efficiency of FDC-P1 cells infected with Z-GMV was density dependent in the presence or absence of externally supplied growth factor (autonomous cell growth). Furthermore, these cells were tumorigenic in DBA/2 mice. Both findings, autonomy of cell growth and

tumorigenicity, were interpreted to be directly linked to the production of GM-CSF by these cells (8).

We decided to reinvestigate the events that follow GM-CSF gene transfer to myeloid cell lines by using two retrovirus vectors that permit equal efficiency of gene transfer into fibroblasts and hemopoietic cells. The myeloproliferative sarcoma virus (MPSV), used as a basis for our vectors, is derived from Mo-MuLV and *mos* oncogenic sequences but has a wider host range than any of the other Mo-MuLV-derived viruses and vectors (9–11). At least part of this unique property of MPSV is due to mutations in the transcriptional control region, the U3 region of the long terminal repeat (LTR) (12, 13).

We designed our experiments to answer the following questions. (i) Does transfer and expression of the GM-CSF gene impart growth factor independence specifically to cells expressing the appropriate receptor or can hematopoietic cells normally responsive to other CSFs, but not GM-CSF, also acquire autonomous growth? (ii) Is the acquisition of autonomous growth, characterized by the loss of requirement for external stimulation, concomitant with autocrine stimulation of cell proliferation due to expression of the viral-transduced GM-CSF gene or can these two events be separated?

MATERIALS AND METHODS

Vector Construction and Virus-Producing Cell Lines. All virus recombinants were constructed by standard techniques. Transfection of recombinant DNA into ψ 2 or RAT-1 cells was performed as described (13, 14). The Z-GMV producing ψ 2 clone 5 was kindly provided by T. Gonda (Melbourne).

Hemopoietic Cell Lines. Factor-dependent myeloid cell lines used for infection and GM-CSF assays included FDC-P1 and FDC-P2 (15, 16). All cells were maintained in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal calf serum and 5% WEHI-3B conditioned medium (WEHI CM) as a source of multi-CSF.

Viral Infections. Infectious virus was collected as a 12-hr supernatant from the virus-producing ψ 2 cells. To assay for infectious virus, 10^3 fibroblasts per well were exposed to 1:5 serial dilutions of virus-containing culture supernatant. Supernatant was removed after 24 hr and virus-infected cells were selected with medium containing G418 (400–1600 μ g/ml). Colonies found resistant with G418 were counted

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: GM-CSF, granulocyte/macrophage colony-stimulating factor; GTU, G418 resistance transfer unit(s); R, resistant; LTR, long terminal repeat; Mo-MuLV, Moloney murine leukemia virus; MPSV, myeloproliferative sarcoma virus; PDGF, platelet-derived growth factor; SiSV, simian sarcoma virus; CM, conditioned medium.

‡To whom reprint requests should be addressed.

after 2 wk and titers were expressed as G418 resistance transfer unit (GTU)/ml.

FDC-P1 or FDC-P2 cells (10^7) were infected by exposure to virus-producing ψ 2 or RAT-1 cell supernatant. After an 8-hr exposure, cells were washed and plated in agar cultures (20% fetal calf serum/0.3% agar/IMDM) either without WEHI CM or with 5% WEHI CM and G418 (400–1600 μ g/ml). Cell concentrations plated ranged from 3×10^1 to 3×10^5 cells per dish. After 9 days of incubation, triplicate cultures were scored for colony formation.

RESULTS

Efficient Transfer of MPSV-Based Retroviral Vectors to Fibroblasts and Hematopoietic Cells. One of the problems in introducing and expressing genes in hematopoietic cells is the low efficiency of gene transfer either by DNA transfection or by infection with Mo-MuLV-based vectors (8, 17, 18). We therefore constructed several vectors based on *neo*^R-MPSV (14), which has an expanded host range as compared to Mo-MuLV (11, 13) and contains the Tn5 drug-resistance gene (*neo*^R) allowing quantitation of gene transfer by selecting and gene-rating G418-resistant (G418^R) colonies. In addition to deleting the *mos* oncogene as in our previously described *mos*⁻¹ *neo*^R recombinant (13), additional *pol* sequences were deleted to construct M3-*neo*^R, leaving only the splice acceptor sequences and upstream sequences found to be necessary for efficient splicing of Mo-MuLV (19). Expression of an introduced gene should occur by way of spliced subgenomic mRNA *neo*^R expression from the full-length genomic mRNA (Fig. 1). M5-*neo*^R was constructed by introducing the *neo*^R gene downstream of the 3' splice signals and replacing gag

FIG. 1. Construction of MPSV-based vectors. (A) M3-*neo*^R was generated by deleting all *pol* and *mos* sequences between the *Bam*HI site downstream of the *neo*^R gene and the *Hind*III site proximal to the 3' LTR of *neo*^R-MPSV (14) and inserting a *Bgl* II-*Sma* I fragment containing the MPSV splice consensus sequences (10) into the same site by using polylinker sequences from pUC13. (C) M5-*neo*^R was constructed by subcloning several fragments derived from the original MPSV-containing plasmid (20) and the Tn5 gene and subsequent ligation by way of polylinkers. Briefly, a *Pst* I fragment containing the 5' LTR and 5' splice signal, a *Hind*III-*Sma* I fragment containing the 3' splice signal, and a *Hind*III-*Bgl* II fragment with the *neo*^R coding region of Tn5, were ligated in the *Bgl* II-*Hind*III site of pBR322 (minus *Eco*RI site). (B) M3-GMV and M3-ΔGMV were generated by insertion of the *Bam*HI-*Eco*RI fragment of pGM5'Δ17 and the *Sac* I-*Bam*HI of pGM3'Δ1.11, respectively, into the *Bam*HI site of M3-*neo*^R by using *Bam*HI linkers. GM plasmids were kindly provided by N. Gough (Melbourne) and contain deletion mutants of the GM-CSF cDNA clone (21). The poly(A) signal was removed by BAL-31 digestion in the latter plasmid. (D) Similarly, M5-ΔGMV was generated by insertion of the *Sac* I-*Bam*HI fragment of pGM3'Δ1.11 into the unique *Bam*HI site of M5-*neo*^R. Open bars, MPSV-derived sequences; hatched bars, Tn5-derived sequences; black bars, GM-CSF cDNA sequences. Locations of splice donor (s.d.) and splice acceptor (s.a.) sequences are indicated. Δ, Location of unique restriction sites for gene insertion.

sequences by a polylinker that allows easy insertion of new genes. Expression of the *neo*^R gene should then occur by way of a spliced subgenomic mRNA and the introduced gene from the full-length genomic mRNA (Fig. 1). M3-GMV, M3-ΔGMV, and M5-ΔGMV were generated by insertion of a cDNA clone of GM-CSF with or without poly(A) signals (GM or ΔGM, respectively) into M5 or M3 vectors (Fig. 1). Virus-producing cell lines were generated either by transfection of a packaging-defective helper cell line (ψ 2; ref. 22) or by transfection of RAT-1 fibroblasts followed by superinfection with cloned Friend helper virus (12).

To test the transfer efficiency of the MPSV-based vectors into hematopoietic cell lines, RAT-1 and two myeloid cell lines, FDC-P1 and FDC-P2 (16), were infected with a limiting amount of virus and the number of G418^R colonies was determined. The resistance transfer of Z-GMV reported by Lang *et al.* (8) was very inefficient in hemopoietic cell lines as compared to fibroblasts. In contrast, all MPSV-based vectors transferred G418 resistance at equal efficiencies to fibroblasts and FDC-P1 cells, regardless of whether the *mos* oncogene was present or replaced by the GM-CSF cDNA (Table 1). However, the lower titers obtained with ψ 2 cells releasing M3-GMV (10^1 to 10^3 per ml) compared to titers of ψ 2 cells transfected with either M3-ΔGMV or M5-ΔGMV (Table 1) may reflect the presence of the poly(A) signals in the GM-CSF insert.

Production of GM-CSF in M3-GMV-Infected Fibroblasts.

To ascertain expression of the GM-CSF gene within the retroviral vectors, supernatants of infected fibroblasts were tested for release of GM-CSF by a biological assay system (Table 2). The supernatants of control cells did not stimulate indicator cells, whereas cell lines with the M3-GMV vector released stimulatory activity. This activity stimulated only cells known to respond to GM-CSF (FDC-P1) and not FDC-P2 cells that respond only to multi-CSF (16). The amount of GM-CSF that was released was lower (by a factor of 7) for ψ 2 cells infected with M3-GMV as compared to those infected with Z-GMV (Table 2), and cells infected with M5-GMV released 20-fold more GM-CSF than Z-GMV. This may be related to the site of insertion of the GM-CSF gene. In M5- and Z-GMV the GM-CSF gene is expressed directly from the genomic mRNA (8); however, expression of GM-CSF from M3-GMV occurs either from an inefficiently spliced subgenomic mRNA (as is the *mos* gene of MPSV) or, alternatively, by reinitiation of translation after termination of the first protein (23). RNA transfer blot analysis did not detect a subgenomic message in M3-GMV-infected cells. More sensitive analysis is necessary to differentiate between the two alternatives. The higher levels of GM-CSF production in M5-GMV-infected cells can probably best be explained by increased enhancer activity of the MPSV LTR in hemopoietic lines (refs. 12 and 13; G. Gräning, personal communication).

The GM-CSF Vector Is Specific in Conferring Growth Autonomy Only to GM-CSF-Responsive Cells. FDC-P1 and FDC-P2 cells were infected with M3-GMV to determine if the virus could confer factor-dependent growth to one or both of these cell lines. Although FDC-P1 responds to multi-CSF and GM-CSF, FDC-P2 has a strict requirement for multi-CSF (15, 16) and therefore one would not expect autocrine stimulation of FDC-P2 after infection with GMV.

Infected cells were selected under one of two conditions: (i) in the presence of multi-CSF and G418 or (ii) in the absence of any growth factor. Infections using control virus *mos*⁻¹ *neo*^R (13) yielded G418^R colonies in the presence of WEHI CM in FDC-P1 and FDC-P2 infections, but no colonies were detected in the absence of exogenous factors (Fig. 2A). Similarly, infection of FDC-P2 cells with M3-GMV in three separate experiments resulted in a total of 2.5×10^4 G418^R colonies when multi-CSF was supplied exogenously, but no

Table 1. Transfer efficiency of MPSV-based vectors to fibroblasts and hematopoietic cells

G418, μg/ml	Fibroblasts*				Myeloid cells* (FDC-P1)		Vector	GTU†		GTU ratio (FDC-P1/ RAT-1)
	NIH 3T3		RAT-1		GTU/ml	Ratio‡		RAT-1	FDC-P1	
	GTU/ml	Ratio‡	GTU/ml	Ratio‡						
200	Growth§	—	5.2 × 10 ³	0.31	Growth§	—	<i>neo</i> ^R <i>mos</i> ⁻¹	3.8 × 10 ⁵	8.0 × 10 ⁵	2.1
400	1.7 × 10 ⁴	1	5.2 × 10 ³	0.31	Growth§	—	<i>neo</i> ^R -MPSV	1.5 × 10 ⁴	1.9 × 10 ⁴	1.3
800	3.8 × 10 ³	0.3	1 × 10 ³	0.06	1.9 × 10 ⁴	1.1	M3-GMV	4 × 10 ²	2.6 × 10 ³	6.5
1600	2 × 10 ²	0.012	1.7 × 10 ²	0.01	1.7 × 10 ⁴	1.0	M3-GMV¶	3.4 × 10 ⁵	9 × 10 ⁵	2.7
							M3-ΔGMV	1.7 × 10 ⁴	5.2 × 10 ⁴	3.1

**neo*^R *mos*⁻¹ virus released from cell clone $\psi 2mos^{-1}4$ was used to infect fibroblasts and hematopoietic cells with a multiplicity of infection of <1. Only FDC-P1 colonies with >100 cells were scored; the calculated GTU of FDC-P1 per ml was corrected for cloning efficiency based on control infections in the absence of G418 selection.

†The relative G418^R transfer efficiency to hemopoietic cell lines as compared to fibroblasts (RAT-1) was determined for six different MPSV-based vectors transfected into $\psi 2$ cells. Resistant RAT-1 cells were selected 48 hr after infection with G418 at 400 μg/ml and FDC-P1 cells were selected 8 hr after infection with G418 at 1 mg/ml. Although the delayed onset of G418 selection in RAT-1 cells was found to influence GTU titers by a 1.4-fold increase (data not shown), a reduction (by a factor of 5–10) in G418^R colonies was observed in infected RAT-1 cells when selected with G418 at 1 mg/ml instead of 400 μg/ml. Thus, the calculated GTU ratio probably reflects a low estimate of hematopoietic gene transfer efficiency.

‡The relative titer as compared to GTU/ml obtained after infection of NIH 3T3 cells selected with G418 at 400 μg/ml.

§Cell growth was not restricted at the G418 concentration used.

¶Helper-competent cell line was used.

factor-independent colonies were obtained. However, infection with the GMV virus conferred G418 resistance and factor-independent growth to FDC-P1 cells, thus proving interaction specificity of the transduced GM-CSF gene (Fig. 2A).

Cloning Efficiency of GMV-Infected FDC-P1 Cells as a Function of Cell Density. If stimulated intracellularly by GM-CSF, M3-GMV-infected FDC-P1 cells would be expected to clone independent of cell density. Higher cell numbers, however, would favor clonal growth if stimulation required factor secretion. FDC-P1 cells were therefore plated at different cell numbers in agar under the same selective conditions as described above to test whether growth factor independence was mediated by way of autocrine stimulation by secreted growth factor or by internal stimulation. We obtained concordant results with all FDC-P1 mass cultures infected with M3-GMV (Fig. 2A). If growth factor was not supplied, clonal growth was nonlinear with increasing cell

concentration and increased disproportionately at higher cell densities. When plated in the presence of growth factor and selected for *neo*^R expression, infected cells cloned linearly with increasing cell numbers as expected.

These results were in contrast to those previously reported (8). We therefore infected FDC-P1 and FDC-P2 cells with Z-GMV obtained from the virus-producing $\psi 2$ line used in previous work (GMV clone 5; ref. 8). Results agreed with the results obtained with M3-GMV: directly after infection, mass cultures of infected FDC-P1 cloned linearly in the presence of WEHI CM and the selective pressure of G418 but cloned nonlinearly in the absence of exogenous growth factor (Fig. 2B).

We reclone six clones established from an M3-GMV-infected culture selected in the presence of growth factor and G418 to (i) confirm that clonability with growth factor selection increased disproportionately with cell numbers plated and (ii) show that *neo*^R-expressing cells also expressed the growth factor gene. Four weeks after the initial cloning all but one cell clone (FDC-P1 clone 3) cloned linearly with increasing cell numbers if G418 selection was applied in the presence of WEHI CM but cloned nonlinearly if cloned in the absence of added growth factor (WEHI CM). Recloning results of a few selected cell lines are shown in Fig. 2C and D.

Southern analysis with several restriction enzymes showed that, except for FDC-P1 clone 3, all cell lines contained one provirus genome with intact *neo*^R and GM-CSF reading frames, and biological activity confirmed expression of both genes (see below). These data demonstrate coexpression of the gene for GM-CSF and for *neo*^R in five of six independent recloning experiments.

Inhibition of Cell Growth by Anti-GM-CSF Proves That FDC-P1 Cells Infected with the M3-GMV Vector Require External Stimulation. GM-CSF antiserum was used to formally prove that external growth factor interaction is required for cell growth of GMV-infected FDC-P1 cells and that infected cells were indeed secreting GM-CSF.

Growth of uninfected FDC-P1 cells stimulated with either purified GM-CSF or supernatant of M3-GMV-infected, but not virus-releasing, FDC-P1 cells was inhibited by specific antiserum. The activity released by M3-GMV-infected cells was thus GM-CSF and, moreover, no alterations of the protein had occurred that may inhibit antibody neutralization. Finally, one representative cell clone, FDC-P1 clone 6, infected by M3-GMV and secreting GM-CSF, was treated with anti-GM-CSF. Cell growth was inhibited (see Fig. 3A),

Table 2. GM-CSF activity of GM virus-infected cells

Source of CM*			GM-CSF activity, units/ml‡
Cell type	Virus	Clone†	
Mouse lung $\psi 2$	Uninfected	—	10,000
	Uninfected	—	0
	M3-GMV	2	385
	Z-GMV	5	2,500
	M5-ΔGMV	6	50,000
FDC-P1	Uninfected	—	0
	Z-GMV	—	126
	M3-GMV	—	21
		1	0.5
		2	7
		3	0
		5	3
		4	11.7
		4§	15.1
		6	2.3
		6§	13.2

*Medium conditioned for 48 hr on cells (1 ml per 10^6 cells) 4 wk after virus infection were concentrated by a factor of 10 by Amicon filtration (exclusion $M_r > 10,000$), dialyzed, and tested for GM-CSF activity on 10^4 32D cells by [³H]thymidine incorporation.

†When no clone is specified, mass cultures were used.

‡Fifty units is defined as the GM-CSF activity per ml of MLCM at 50% maximal stimulation of 10^4 FDC-P1 cells.

§CM was analyzed 16 wk after virus infection.

FIG. 2. Clonability of infected FDC-P1 cells as a function of cell density. The number of clones obtained at different cell densities is plotted on a logarithmic-logarithmic scale; thus, a deviation from a slope of +1 reflects nonlinear cloning efficiency. Cells were plated at different cell densities and selected either in the absence of added growth factor (opened symbols) or, as control, in the presence of G418 and WEHI CM (closed symbols). (A) FDC-P1 cells infected with M3-GMV (Δ) and *neo^R mos⁻¹* ($\blacksquare$). (B) FDC-P1 cells infected with Z-GMV ($\blacklozenge$). (C and D) M3-GMV-infected FDC-P1 clones 6 and 4, respectively, recloned 4 wk ($\bullet$) and 16 wk ($\blacksquare$) after infection. Zero values were obtained with FDC-P1 cells infected with M3-GMV (A) and Z-GMV (B) in the absence of WEHI CM when 10^2 – 10^4 cells were plated.

thus proving that autocrine stimulation of growth occurred by external interaction with the secreted GM-CSF.

Nature of Autonomy of Cell Growth of FDC-P1 Cells Infected by GMV Vectors. Previous results strongly suggested that introduction of the GM-CSF gene into FDC-P1 cells leads to factor-independent growth, internal autocrine stimulation, and tumorigenicity in one step (8). How can these results be reconciled with our data? We decided to reclone not only FDC-P1 cells infected with MPSV-derived GMV but also those infected with Z-GMV to see whether clones kept

in culture for a longer period of time would be altered by the emergence of subclones that were truly growth autonomous.

In contrast to recloning results of FDC-P1 cells infected with M3-GMV (see above), only one of four clones of Z-GMV-infected FDC-P1 cells was still dependent on factor secretion (external stimulation) as tested by nonlinear clonability in the absence of exogenous growth factor (WEHI CM). In parallel, we retested two M3-GMV-infected clones (FDC-P1 clone 4 and clone 6) that had been maintained in culture either in the presence or absence of an external source of growth factor for a total of 16 wk after initial cloning. Both clones showed the emergence of a subpopulation of cells that apparently no longer required secreted growth factor (Fig. 2 C and D).

The degree of the shift to true autonomous growth for each clone was less if cells were kept without any selective pressure and more pronounced if pressure was maintained for autocrine stimulation. Overall, however, FDC-P1 clone 4 showed a relatively fast transition from dependency on external stimulation to true growth autonomy (Fig. 2C), whereas clone 6 was much slower (Fig. 2D). Growth inhibition by GM-CSF antiserum could thus not be demonstrated with the autonomous clone 4 (Fig. 3B) as had been for clone 6. Growth factor autonomy was not observed with any uninfected FDC-P1 cultures. Of 10^8 uninfected FDC-P1 cells plated in the absence of added growth factor, none exhibited factor-independent growth. These data thus strongly suggest that the transition to true autonomy was strongly favored in cells expressing the GM-CSF gene.

The relatively fast transition of Z-GMV and the slower transition of M3-GMV-infected FDC-P1 cells might be related to the higher expression of the GM-CSF gene in Z-GMV-infected cells as compared to M3-GMV-infected cells (Table 2). We therefore quantitatively tested supernatants of infected FDC-P1 cells for release of GM-CSF. Indeed, Z-GMV-

FIG. 3. Proliferation response of infected FDC-P1 cells on treatment with GM-CSF antiserum. Cells (500 or 1000) were treated for 48 hr with serial dilutions of rabbit anti-mouse GM-CSF serum ($\bullet$) or rabbit normal serum (Δ), as control, and cell proliferation was assayed by [3 H]thymidine incorporation. (A) M3-GMV-infected FDC-P1 clone 6. (B) M3-GMV-infected FDC-P1 clone 4.

infected FDC-P1 cells secreted more GM-CSF than those infected by M3-GMV. Those M3-GMV-infected cell clones that showed emergence of autonomously growing cells at a greater rate had higher levels of GM-CSF than those with a relatively slow transition to true autonomy (Table 2). This correlation suggested that the transition to autonomy and growth factor production are interdependent but independent events.

DISCUSSION

To examine the events that occur after aberrant expression of a growth factor gene in a cell normally responsive to that factor, we have constructed several retroviral vectors based on the MPSV genome and carrying the cDNA clone of GM-CSF. We have shown that MPSV is an excellent vector for introducing genes not only into fibroblasts but also into hematopoietic cells such as FDC-P1 and FDC-P2. The equal efficiency of gene transfer to fibroblasts and to many other cells, including not only myeloid but also lymphoid cells (G. Gräning, personal communication), epithelial cells (ref. 24; unpublished results), and an embryonic carcinoma cell line (11), suggests that the MPSV has distinctive features that permit high expression in a wide range of cell types (12, 13).

Low titers of M3-GMV were attributed to the poly(A) signals within the GM-CSF cDNA clone. Removal of these sequences to create M3-ΔGMV showed drastic changes in virus titer. Furthermore, Southern analysis of the M3-GMV-producing ψ 2 clone used in our studies, which was selected on the basis of its relatively high titer, showed alterations directly 5' of the GM-CSF coding region in the integrated provirus genome (data not shown). Either deletion or rearrangement of these sequences may be necessary for high virus production, as has been reported (14, 25).

Low expression of the GM-CSF cDNA inserted into M3-*neo*^R, presumably due to either inefficient splicing or inefficient reinitiation of translation by the ribosome, proved to be an unexpected advantage in analyzing the action of the GM-CSF gene products on FDC-P1 cells; two separate events involved in the acquisition of autonomous growth of FDC-P1 cells could be dissected in our studies. The first event after GM-CSF gene transfer to FDC-P1 cells was the acquisition of growth factor independence by external stimulation by endogenously synthesized GM-CSF. In many cases, but not all, this initial step was followed by a second step that abrogated the requirement for external stimulation. Concordant results were obtained with the Z-GMV vector.

Although all FDC-P1 clones infected with M3-GMV and expressing GM-CSF were dependent on secreted growth factor when recloned 4 wk after initial selection, almost all Z-GMV-infected clones (three of four) proved to grow autonomously when recloned shortly after selection. This difference in the rate of transition to true growth autonomy could be correlated to the levels of GM-CSF produced, suggesting that these two features are linked. Two hypotheses may be given to support this high correlation. (i) Binding to the GM-CSF receptor may occur internally but is less efficient in signal transduction. Higher production of GM-CSF could thus lead to sufficient levels for internal stimulation. (ii) Autonomy of external stimulation is a separate but interdependent step following GM-CSF production in cells expressing the GM-CSF receptor and signal transducers.

Our results showing that high factor release as such does not automatically make a cell grow autonomously would suggest that the second interpretation is more likely. This is most striking in Z-GMV-infected FDC-P1 cells, which, despite high levels of GM-CSF production, still require external stimulation by GM-CSF when assayed within a short time (1–3 wk) after gene transfer. Indeed, the acquisition of autonomous growth cannot be directly correlated with a

threshold level of GM-CSF production. The level of factor production of FDC-P1 clone 4, for instance, does not vary significantly when cell growth is dependent on external stimulation or 16 wk later when the cell exhibits autonomous growth (Table 2).

Although the nature of the second alteration is unknown, mutations in the GM-CSF receptor gene or mutations in any of the other genes involved in signal transmission may explain the change in requirement for external stimulation. Such mutations may have a selective advantage in cells producing high factor levels.

The data reported here are entirely in agreement with conclusions obtained recently with the SiSV. Infection of fibroblasts leads to secretion of the SiSV-encoded *sis* product (PDGF β chain) and autocrine stimulation (4–6); moreover, these cells are not tumorigenic (7).

The frequent occurrence of rearranged growth factor genes in diverse tumor cells has provided only correlative evidence that the abnormal expression of growth factor genes may lead to further oncogenic alterations. We present here a model suited for causal analysis of malignant progression initially involving autocrine stimulation.

We thank M. Kurth and A. Ohlmann for expert technical assistance. This work was supported by the DFG; construction of vectors was supported by the Deutsche Krebshilfe and the Fonds der Chemischen Industrie. C.L. is a fellow of the Boehringer-Ingelheim Fonds. The Heinrich-Pette-Institut is supported by Freie und Hansestadt Hamburg and Bundesministerium für Jugend, Familie, Frauen und Gesundheit.

- Chen, S. J., Holbrook, N. J., Mitchell, K. F., Vallone, C. A., Greengard, J. S., Crabtree, G. R. & Lin, Y. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 7284–7288.
- Duprez, V., Lenoir, G. & Dautry-Varsat, A. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 6932–6936.
- Ymer, S., Tucker, W. Q. J., Sanderson, C. J., Hapel, A. J., Campbell, H. D. & Young, I. G. (1985) *Nature (London)* **317**, 255–258.
- Huang, J. S., Huang, S. S. & Deuel, T. F. (1984) *Cell* **39**, 79–87.
- Heldin, C.-H. & Westermark, B. (1984) *Cell* **37**, 9–20.
- Johnsson, A., Betsholtz, C., Heldin, C.-H. & Westermark, B. (1985) *Nature (London)* **317**, 438–440.
- Johnsson, A., Betsholtz, C., Heldin, C.-H. & Westermark, B. (1986) *EMBO J.* **5**, 1535–1541.
- Lang, R. A., Metcalf, D., Gough, N., Dunn, A. R. & Gonda, T. J. (1985) *Cell* **43**, 531–542.
- Ostertag, W., Stocking, C., Johnson, G., Franz, T., Kluge, N., Kollek, R. & Hess, N. (1987) *Adv. Cancer Res.* **48**, 193–355.
- Stacey, A., Arbuthnott, C., Kollek, R., Coggins, L. & Ostertag, W. (1984) *J. Virol.* **50**, 725–732.
- Franz, T., Hilberg, F., Seliger, B., Stocking, C. & Ostertag, W. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 3292–3296.
- Stocking, C., Kollek, R., Bergholz, U. & Ostertag, W. (1986) *Virology* **153**, 145–149.
- Stocking, C., Kollek, R., Bergholz, U. & Ostertag, W. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 5746–5750.
- Ostertag, W., Seliger, B., Kollek, R., Stocking, C., Bergholz, U. & Smadja-Joffe, F. (1986) *J. Gen. Virol.* **67**, 1361–1371.
- Dexter, T. M., Garland, J., Scott, D., Scolnick, E. & Metcalf, D. (1980) *J. Exp. Med.* **152**, 1036–1047.
- Hapel, A. J., Fung, M. C., Johnson, R. M., Young, I. G., Johnson, G. R. & Metcalf, D. (1985) *Blood* **65**, 1453–1459.
- Keller, G., Paige, C., Gilboa, E. & Wagner, E. F. (1985) *Nature (London)* **318**, 149–154.
- Williams, D. A., Lemischka, I. R., Nathan, D. G. & Mulligan, R. C. (1984) *Nature (London)* **310**, 476–480.
- Hwang, L.-H. S. & Gilboa, E. (1984) *J. Virol.* **50**, 417–424.
- Kollek, R., Stocking, C., Smadja-Joffe, F. & Ostertag, W. (1984) *J. Virol.* **50**, 717–724.
- Gough, N., Metcalf, D., Gough, J., Grail, D. & Dunn, A. (1985) *EMBO J.* **4**, 645–653.
- Mann, R., Mulligan, R. C. & Baltimore, D. (1983) *Cell* **33**, 153–159.
- Peabody, D. & Berg, P. (1986) *Mol. Cell. Biol.* **6**, 2695–2703.
- Fusco, A., Portella, G., Di Fiore, P. P., Berlingieri, M. T., Di Lauro, R., Schneider, A. B. & Vecchio, G. (1985) *J. Virol.* **56**, 284–292.
- Shimotohno, K. & Temin, H. M. (1981) *Cell* **26**, 67–77.