v-*erb*A Oncogene Induces Invasiveness and Anchorage-independent Growth in Cultured Glial Cells by Mechanisms Involving Platelet-derived Growth Factor[1]

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Abstract

The v-*erb*A oncogene coding for a mutated form of the thyroid hormone (T_3) receptor (TRα1) increased the invasion capacity of the mouse B3.1 glial cell line. This effect was mediated by the induction of platelet-derived growth factor (c-*sis*/PDGF B), as shown by its inhibition using an anti-PDGF BB antibody. Also, the low invasion capacity of parental B3.1 and c-*erb*A-expressing cells (B3.1 + TRα1) was enhanced by exogenously added PDGF BB. This effect was independent of the growth-promoting activity of PDGF and unrelated to the secretion of metalloproteinases. All three cell types (parental B3.1, B3.1 + v-*erb*A, and B3.1 + TRα1) secreted similar high levels of the M_r 72,000 collagenase IV (A) independently of PDGF. Anchorage-independent cell growth was also enhanced by v-*erb*A; B3.1 + v-*erb*A cells but neither parental B3.1 nor B3.1 + TRα1 cells formed foci in soft agar. The effect of v-*erb*A only happened in the presence of serum, suggesting that some serum factor(s) cooperate with PDGF to overcome the anchorage dependence of B3.1 + v-*erb*A cells. Supporting this, high doses of exogenous PDGF were much less efficient than serum, and the addition of an anti-PDGF BB antibody blocked only partially the effect of serum. Basic fibroblast growth factor was found to cooperate with PDGF to abolish anchorage dependence. Moreover, B3.1 + v-*erb*A cells detached and grew in suspension when cultured on plastic dishes. Interestingly, the transformation-competent c-*jun* and *fra*-1 oncogenes were induced by v-*erb*A in serum-free medium and are candidates to mediate v-*erb*A effects. In summary, our results show that v-*erb*A induces transformation parameters in the glial B3.1 cell line via an increase in c-*sis*/PDGF B and probably other mechanisms, suggesting a role for (autocrine) PDGF stimulation in glial cell transformation.

Introduction

The v-*erb*A oncogene is present in the genome of the avian erythroblastosis virus, a retrovirus causing rapid, fatal erythroleukemia and also sarcomas in chickens (reviewed in Ref. 1). v-*erb*A does not cause tumors by itself but potentiates the tumorigenic capacity of v-*erb*B, the second oncogene in avian erythroblastosis virus encoding a truncated epidermal growth factor receptor (2, 3). By blocking the cell differentiation program and decreasing the requirements for growth factors and conditions, v-*erb*A enhances erythroblast transformation by v-*erb*B (3, 4), endogenous c-*erb*B (5), several other tyrosine-kinase (v-*src*, v-*fps*, and v-*sea*; Ref. 6), the signal-transducer v-Ha-*ras* (6), and the nuclear v-*ets* (7) oncogenes. In culture, v-*erb*A does not transform chicken bone marrow cells or fibroblasts (3). However, it does causes an outgrowth of erythrocytic cells (8) and stimulates fibroblast growth and *in vivo* tumorigenesis of v-*erb*B-transformed fibroblasts (9).

v-*erb*A is a mutated version of the thyroid hormone (T_3) receptor α1 (TR/c-*erb*Aα1; Refs. 10 and 11), a member of the family of nuclear hormone receptors that function as sequence-specific, DNA-binding, ligand-activated transcription factors (12, 13). Due to several mutations, v-*erb*A is unable to bind hormone (10, 14) but still binds DNA, albeit with an altered affinity (15–17). Additional mutations in the carboxy-terminal domain of v-*erb*A have also destroyed the heterodimerization, gene silencing, and hormone-dependent transactivation capacities present in the normal TR/c-*erb*Aα1 receptor (18–20). As a result, v-*erb*A interferes with the normal transcriptional transactivating activity of TRα1 and perhaps other nuclear receptors, such as RAR.[3] Thus, v-*erb*A is considered a constitutive, dominant transcriptional repressor of hormone-activated specific genes (15, 21–23). In line with

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[3] The abbreviations used are: RAR, retinoic acid receptor; PDGF, platelet-derived growth factor; bFGF, basic fibroblast growth factor; TGF, transforming growth factor.

this, the arrest by v-*erb*A of chicken erythroblast differentiation induced by activated TR/c-*erb*A and RAR is at least in part a consequence of the transcriptional inhibition of three genes: carbonic anhydrase II, band 3, and δ-aminolevulinic acid synthethase (4, 18, 24).

In agreement with the broad expression of TR/c-*erb*A in the organism, v-*erb*A also has effects on the phenotype of other cell types. In rat pheochromocytoma PC12 cells, it causes a partial blockade of the neuronal differentiation induced by nerve growth factor and a potentiation of the dexamethasone-induced chromaffin differentiation and gene expression (25). Strikingly, v-*erb*A increases quail myoblast proliferation and terminal differentiation without affecting TR/c-*erb*A action (26). On the other hand, v-*erb*A cooperates with bFGF in neuroretina cell transformation (27). Recently, v-*erb*A has been shown to induce multiple alterations, including loss of adipose tissue and hepatocellular carcinomas, in transgenic mice (28). This result shows that *in vivo* v-*erb*A transforms hepatocytes, which histologically are epithelial cells. Consequently, v-*erb*A blocks brown adipose cell differentiation and gene expression *in vitro*.[4]

To study its effects in glial cells, we have expressed v-*erb*A and, in parallel, its cellular counterpart, the chicken TR/c-*erb*Aα1, in an immortal mouse glial cell line named B3.1 (29). We reported the inhibition by v-*erb*A of the expression of *CNP*, an oligodendrocytic marker gene, together with an increase in DNA synthesis and changes in the response to growth factors (29). More recently, we characterized the increase in cell survival and proliferation caused by v-*erb*A as the consequence of the induction and secretion of PDGF, and the autocrine response of v-*erb*A-expressing cells (B3.1 + v-*erb*A) (30). Given that both types of glial cells, astrocytes, and oligodendrocytes express TR/c-*erb*A *in vivo* and are the target of T_3 action (Refs. 29 and 31, and references therein), and the important role attributed to PDGF in the process of malignization of different cell types including glial cells (32–35), we have analyzed the phenotype of B3.1 + v-*erb*A cells. Here we report the effects of v-*erb*A on two transformation parameters, invasive capacity and anchorage-independent growth, and their relation to the induction of PDGF. In addition, the study of gene expression in B3.1 + v-*erb*A cells revealed that at least two other genes with oncogenic potential, c-*jun* and *fra*-1, are induced by v-*erb*A in these cells.

Results

v-*erb*A Increases Cell Invasiveness. To investigate whether the induction of PDGF B by v-*erb*A could cause phenotypic changes related to transformation in addition to the observed growth potentiation, we first analyzed the invasive capacity of B3.1 + v-*erb*A cells. As shown in Fig. 1, a clear difference was found among the different cell types. B3.1 + v-*erb*A cells exhibited a much higher capacity to invade a natural matrix (Matrigel) than the parental B3.1 or the B3.1 + TRα1 cells, and which is comparable to that of the highly malignant B16F10 murine melanoma cells used as positive control. The same result was obtained when GFR-Matrigel, containing reduced amounts of several growth factors (epidermal growth factor, PDGF, insulin-like growth factor-I) and proteases or collagen type I were used instead of normal Matrigel (data not shown).

To evaluate the role of PDGF in the increase in cell invasiveness, the effect of a neutralizing anti-PDGF BB antibody was tested. The addition of anti-PDGF BB to the B3.1 + v-*erb*A cells when they were seeded on the Matrigel reduced substantially (around 4-fold in three independent experiments) the amount of cells that invaded the matrix (Fig. 2*A*). The specificity was assessed by using an antibody directed against bFGF that was much less inhibitory, and a control antibody against the nuclear growth hormone factor-1 had no effect. The importance of PDGF in B3.1 + v-*erb*A invasiveness was further confirmed by treatment of cells with exogenous PDGF. Thus, the addition of 10 ng/ml PDGF BB led to a high increase in the invasiveness of parental B3.1 cells (Fig. 2*B*, ▨) and a less pronounced increase in the case of B3.1 + TRα1 cells (Fig. 2*B*, □). No effect of this treatment could be observed at the time of analysis (4 days after seeding) on B3.1 + v-*erb*A cells, which had mostly crossed the matrix in untreated cultures (Fig. 2*B*, ■).

Next, we investigated the possibility that the observed effect of PDGF could be a simple consequence of its growth-promoting activity. To test this, increasing amounts of cells were used in the invasion assay. Fig. 3*A* shows that the increase in B3.1 + TRα1 cell density (□) to even nearly confluency (up to 9×10^5 cells/Transwell) had no major effect on their reduced invasiveness. In the case of B3.1 + v-*erb*A cells (Fig. 3A, ■), very high cell densities resulted in a decreased number of cells attached to the porous membrane underlying the matrix; but this is due to the fact that they had even crossed the membrane and could be recovered from the lower chamber of the well. In another series of experiments, the growth-promoting activity of PDGF was abolished by treating the cells with mitomycin C, a drug which inhibits cell division. Supporting a growth-independent effect of PDGF, mitomycin C-treated B3.1 + v-*erb*A cells showed a higher invasion capacity than B3.1 + TRα1 cells (data not shown).

The effect of serum was then examined. Increasing amounts of serum greatly augmented the invasion capacity of B3.1 + TRα1 cells (Fig. 3*B*, □). In contrast, the invasiveness of B3.1 + v-*erb*A cells was not augmented by low (0.5%) serum concentrations and was doubled by higher (2–4%) concentrations (Fig. 3*B*, ■). Comparing both cell types, we observed that the difference in the invasive potential is lost at low serum and is evident again at 2–4% serum. No differences were seen at 10% serum. Together with data obtained using PDGF treatments (Fig. 2*B*), these results suggest that PDGF is a major factor responsible for the higher invasion capacity of B3.1 + v-*erb*A cells but also that other uncharacterized serum factors, either alone or in cooperation with PDGF, enhance the invasiveness of these cells. These factors are probably responsible for the increase in B3.1 + TRα1 cell invasiveness observed at 0.5% serum. However, their concentration at this low serum condition

[4] Carrolee Barlow and Björn Vennström, personal communication.

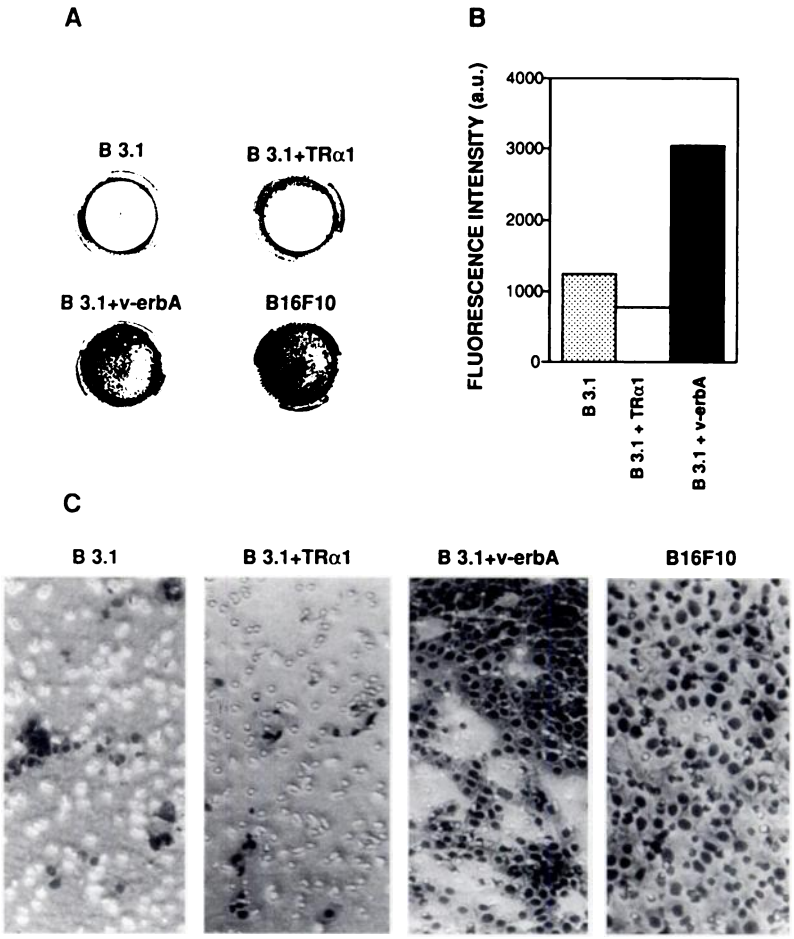

Fig. 1. Invasion capacities of parental B3.1, B3.1 + TRα1, and B3.1 + v-*erb*A cells. Cells (3×10^5/6.5-mm Transwell insert) were seeded on Matrigel in serum-free medium as described in "Materials and Methods" and incubated for 4 days at 37°C. Fibronectin was used as chemoattractant, and metastatic B16F10 cells were used as controls. *A*, photos of the Transwell inserts after Giemsa staining. *B*, spectrophotometric quantitation of cells attached to the insert by incubation with culture medium containing 6.25 μg/ml 2',7'-bis-(2-carboxyethyl)-5-(and -6)-carboxyfluorescein-acetoxymethyl ester for 10 min at 37°C. *C*, micrographs of the Transwell inserts. The attached, stained invasive cells and the clear insert pores are visible. Four separate experiments were done, giving the same results.

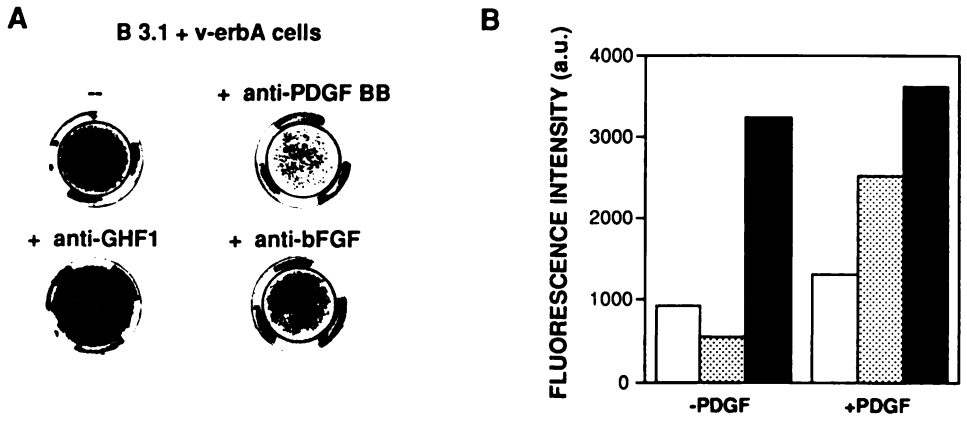

Fig. 2. Effects of PDGF on the invasive potential of B3.1 cells. *A*, inhibition of B3.1 + v-*erb*A cell invasiveness by an anti-PDGF BB antibody. Three $\times 10^5$ B3.1 + v-*erb*A cells were incubated on Matrigel as in legend for Fig. 1 in the absence or presence of antibodies against PDGF BB, GHF-1, or bFGF as indicated. Antibodies were added to the cells at the time of seeding (day 0) and 48 h later (day 2). Photos of hematoxylin and eosin-stained inserts were taken 4 days after seeding. *B*, effect of exogenously added PDGF on cell invasiveness. Quantitation of invasive cells in cultures of parental B3.1 cells (□), B3.1 + TRα1 (▨), and B3.1 + v-*erb*A (■) cells treated or not with 10 ng/ml PDGF BB at days 0 and 2. In both cases, two separate experiments were done, giving the same result.

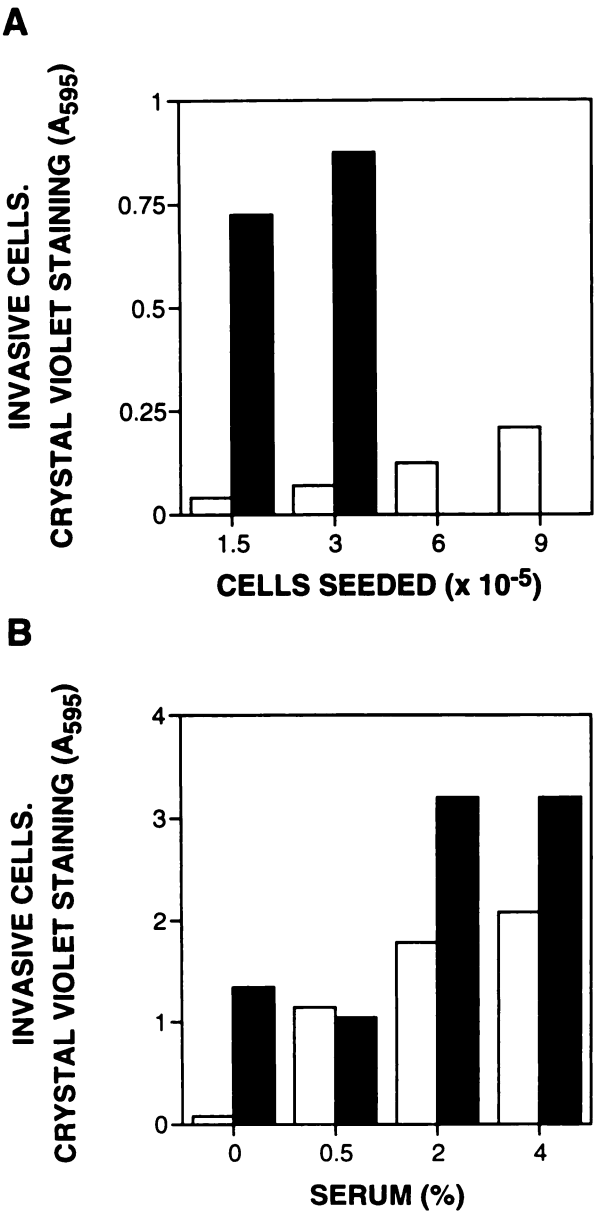

Fig. 3. Effect of cell density and serum concentration on the invasion capacities of B3.1 + TRα1 and B3.1 + v-*erb*A cells. *A*, the indicated number of cells were seeded on Matrigel as in legend for Fig. 1. Four days later, invasive cells were stained with crystal violet and quantitated as described in "Materials and Methods." *B*, 3×10^5 cells (□, B3.1 + TRα1; ■, B3.1 + v-*erb*A) were seeded in the presence of the indicated serum concentrations. Quantitation of invasive cells was as in *A*. In both cases, two experiments were done, giving the same result.

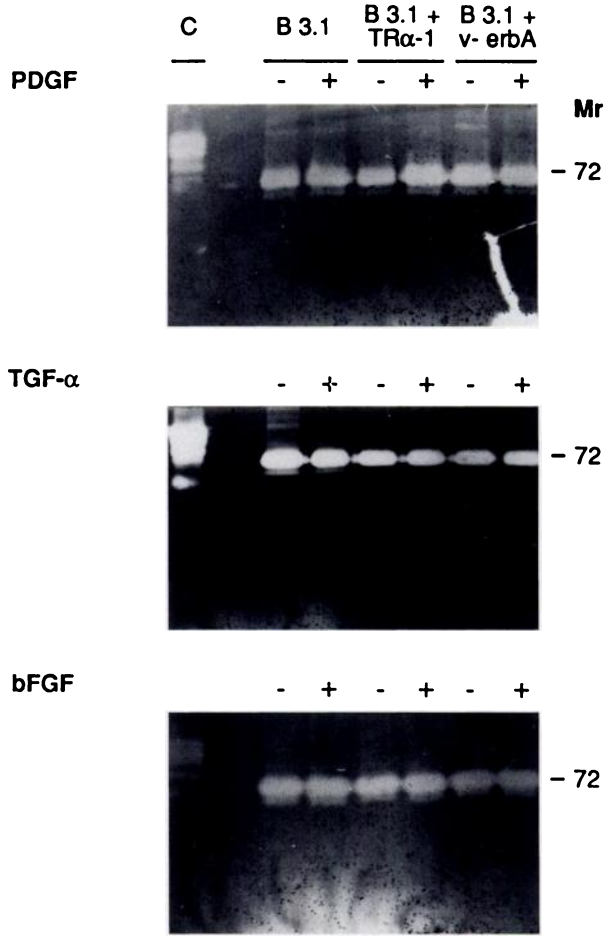

Fig. 4. Collagenase secretion by the three B3.1 cell types. Gelatin zymography of conditioned media (*CM*) from parental B3.1, B3.1 + TRα1, and B3.1 + v-*erb*A cells. Gelatin-SDS-polyacrylamide substrate gels were used to visualize the gelatinolytic activity in the CM of the respective cell types treated or not with the indicated growth factor (10 ng/ml; 2 days) as described in "Materials and Methods." Purified type XI collagenase was added to *Lane C* as a positive control. *Right*, the size of the active protein (M_r 72,000).

does not appear to be enough to potentiate the effect of PDGF.

On the other hand, we also studied the effect of PDGF as a chemoattractant. The presence or absence of PDGF (or the commonly used chemoattractant fibronectin) in the lower chamber did not change the respective invasive capacities on Matrigel or collagen type I gels described above (data not shown). Therefore, PDGF BB increases B3.1 cell proliferation and invasiveness but in contrast to its action on other cell types (Ref. 36 references therein), it does not induce a chemotactic response in these cells.

v-*erb*A Does Not Affect Proteolytic Activity. Since invasiveness is the result of the capacity of cells to attach, digest, penetrate, and cross the surrounding extracellular matrix, we examined whether B3.1 + v-*erb*A cells exhibited a higher secretion of metalloproteinases, a major group of enzymes degrading multiple matrix components. We used gelatin zymography to analyze the secretion of collagenases by the different cell types. The effect of several growth factors was also studied. As shown in Fig. 4, the three cell types secreted basically only high amounts of a gelatinase of M_r 72,000, which most probably corresponds to collagenase IV A or MMP-2. No differences were found among the parental B3.1, B3.1 + TRα1, and B3.1 + v-*erb*A cells. Furthermore, the high secretion of this collagenase was not affected by exogenous PDGF BB, TGF-α, or bFGF in any cell type (Fig.

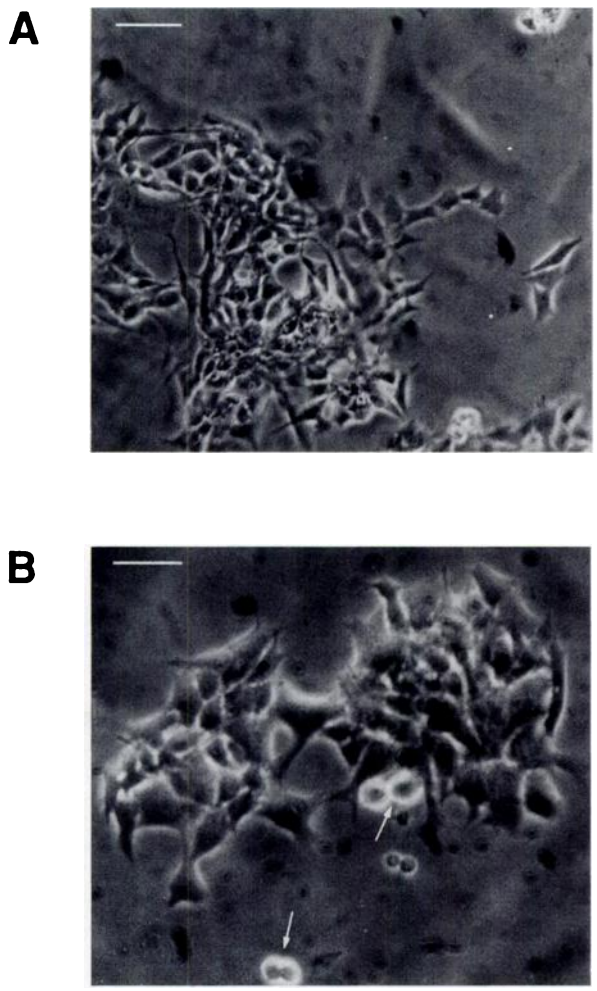

Fig. 5. Growth of B3.1 + v-*erb*A cells in suspension. Phase micrographs of arrays of floating B3.1 + v-*erb*A cells growing on top of cell monolayers 4 days after seeding. *A: bar,* 100 μm. *B: bar,* 50 μm. *Arrows,* cells in mitosis.

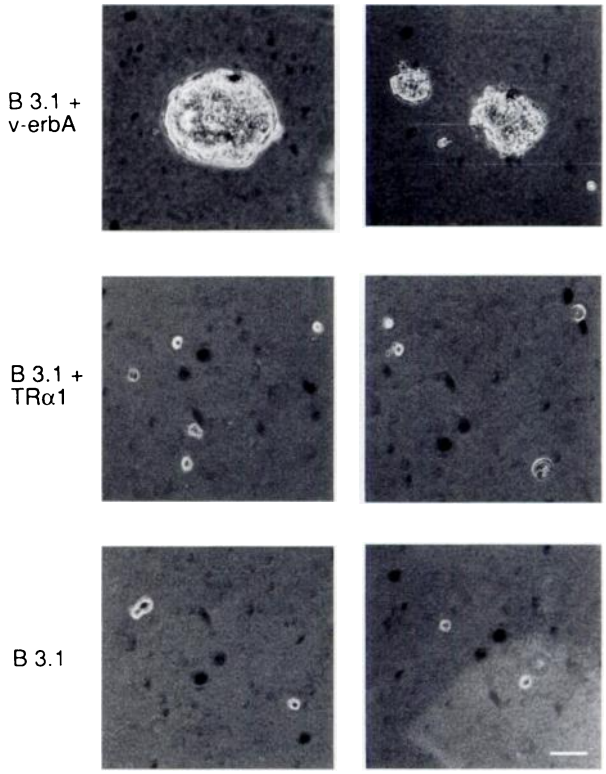

Fig. 6. Anchorage-independent growth of B3.1 + v-*erb*A cells. The three B3.1 cell types were analyzed for anchorage-independent growth in soft agar as described in "Materials and Methods." Representative foci were photographed 6 weeks after seeding. *Bar,* 100 μm.

4). In addition, low and again unchanged secretion of another gelatinase of M_r 97,000 (probably collagenase IV B or MMP-9) was found when 10-fold concentrated conditioned media were used (data not shown). On the other hand, none of the cell types treated or not showed detectable secretion of proteases of the stromelysin family, as assessed by casein zymography (data not shown).

v-*erb*A Induces Anchorage-independent Proliferation. We noticed that a percentage of B3.1 + v-*erb*A cells invariantly detached from the plastic dishes during standard culturing. An increasing number of cells formed groups floating in the medium on top of the monolayer on the days following seeding in a dish (Fig. 5). This phenomenon was not a consequence of high cell density since it started well before monolayers reached confluency. Floating cells tended to contact; therefore, many arrays of cells showing a flat morphology could be seen after 3–4 days. Furthermore, cells in suspension were able to divide, as assessed by the mitosis seen regularly (Fig. 5, *arrows* in *lower panel*).

The ability to grow in the absence of anchorage to solid substrate is another characteristic of transformed cells. We next investigated the capacity of the different cell types to form foci in semisolid agar, a typical test of cell transformation. In the presence of high (10%) serum, a clear difference was seen; B3.1 + v-*erb*A cells (270 foci counted in 6 fields) but not parental B3.1 (6 foci in 6 fields) or B3.1 + TRα1 cells (2 foci in 6 fields) formed multicellular foci after a few (4–6) weeks (Fig. 6). A high percentage of B3.1 + v-*erb*A cells were present in big multicellular foci of either well-limited, encapsulated-like (Fig. 6, *upper left panel*) or more irregular (Fig. 6, *upper right panel*) shape, and the remaining cells formed small groups. Both parental B3.1 and B3.1 + TRα1 cells remained exclusively as individual cells. However, the result was different when the cells were seeded in the absence of serum. Then, B3.1 + v-*erb*A cells were, as the parental and B3.1 + TRα1 cells, equally unable to form foci. Assuming that v-*erb*A is able to induce PDGF when the cells are cultured in semisolid agar, this result suggested that PDGF is not sufficient to induce foci formation but probably requires the cooperation with other serum factor(s). To confirm this, two types of experiments were done: (*a*) we used an anti-PDGF BB antibody to study the contribution of the v-*erb*A-induced PDGF to the formation of foci by the B3.1 + v-*erb*A cells. Only a partial (50%) inhibition of foci number was obtained, which indicated that other factor(s) must cooperate with PDGF (Fig. 7). A candidate was bFGF, which cooperates

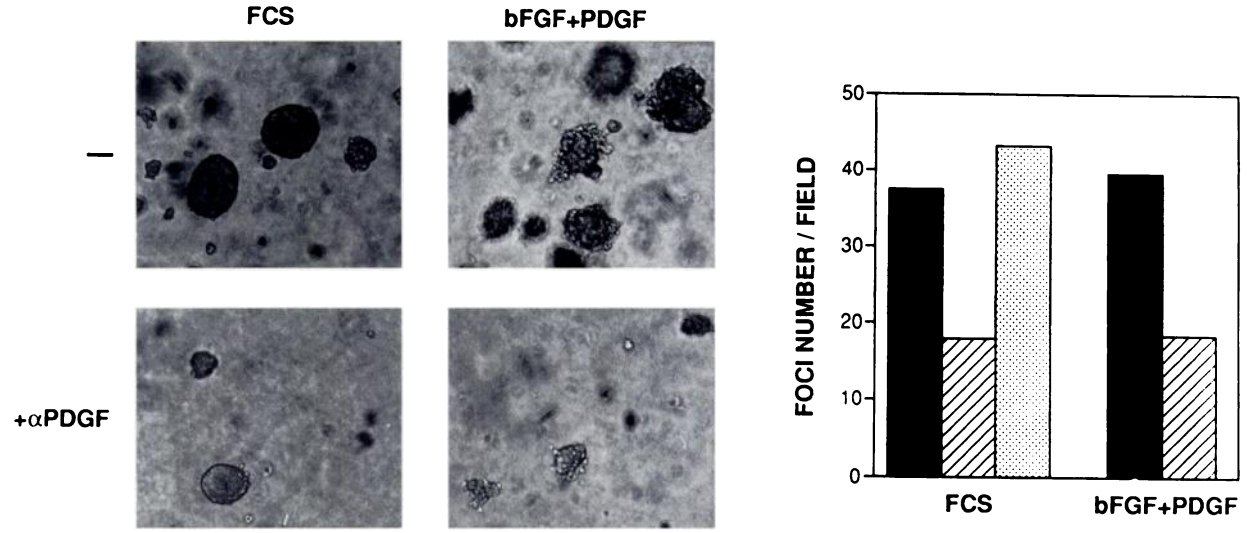

Fig. 7. Effect of an anti-PDGF BB antibody on the anchorage-independent growth of B3.1 + v-*erb*A cells. Cells were seeded in 24-well plates in the presence of serum or the indicated factors (10 ng/ml) alone or plus 10 μg/ml anti-PDGF BB antibody as described in "Materials and Methods." Foci present in six nonoverlapping fields were photographed and counted 2 weeks after seeding. Three experiments were done. Mean values correspond to one representative experiment. ■, no antibody added; ▨, anti-PDGF BB antibody; □, control anti-GHF-1 antibody. *Bar*, 100 μm.

with PDGF regulating growth and differentiation of glial O2A precursors; and (*b*) we analyzed foci formation by the B3.1 + v-*erb*A and B3.1 + TRα1 cells cultured in the presence of serum, PDGF BB, or bFGF. Although high doses of PDGF were only able to induce the formation of small groups of B3.1 + v-*erb*A cells, bFGF was basically as efficient as serum in inducing foci, which showed an irregular and less compact morphology (Fig. 8). Cell clones formed by PDGF-treated B3.1 + TRα1 cells were very small, whereas no differences were seen between bFGF-treated B3.1 + v-*erb*A and B3.1 + TRα1 cells (Fig. 8, *lower panels*). In line with this, the anti-PDGF BB antibody caused a 50% reduction in the number (and also some reduction in the size) of foci formed by B3.1 + v-*erb*A cells treated with bFGF + PDGF (Fig. 7). As expected, the anti-PDGF BB antibody abolished completely the effect of exogenous PDGF (data not shown).

Effects of v-*erb*A on Gene Expression: Induction of c-*jun* and *fra*-1. To gain insight on the basis for the described effects of v-*erb*A, we initiated a broad study of gene expression in B3.1 + v-*erb*A cells. Around 40 genes encoding proteins involved in processes more or less closely related to cell transformation or proliferation were analyzed (in addition to PDGF A and B and their receptors; Ref. 30). Northern blots containing mRNAs prepared from the three cell types growing in either complete, serum-containing growth medium, or in N2 serum-free medium (see "Materials and Methods") and specific cDNAs probes were used in this screening. Results obtained are summarized in Table 1. Three types of genes were found: (*a*) genes whose expression at the mRNA level were altered in B3.1 + v-*erb*A cells; (*b*) genes whose mRNA levels were unaffected by v-*erb*A (and also by TR/c-*erb*Aα1); and (*c*) genes that were not expressed in B3.1 cells. In the first and obviously most interesting group are two nuclear oncogenes: c-*jun* and *fra*-1. In both cases, the cellular content of their respective mRNAs (2.7 and 3.0 kb for c-*jun*; 1.6 kb for *fra*-1) was much higher in B3.1 + v-*erb*A cells than in parental B3.1 or B3.1 + TRα1 cells in serum-free conditions (Fig. 9). No big differences were seen in the presence of serum. In none of the other genes studied, including those encoding other nuclear and non-nuclear oncogenes, growth factors, and growth factor receptors, matrix proteins, membrane and nuclear receptors, and proteases and proteases inhibitors, were significant differences found (Table 1).

Discussion

In this study, we report the effects of the v-*erb*A oncogene on the phenotype of the B3.1 glial cell line. We have shown previously that v-*erb*A induced the expression and secretion of active PDGF in these cells, which were in turn stimulated to proliferate in an autocrine fashion (30). Analogous autocrine growth stimulations by PDGF have been described in other systems, including glioma cells (Refs. 37 and 38 and references therein). More importantly, autocrine PDGF stimulation induces transformation of NIH 3T3 fibroblasts (39–41). Also, the growth and *in vivo* tumorigenicity of glioma cell lines parallels their PDGF secretion (32, 34), and the existence of a PDGF autocrine loop in gliomas appears to correlate with tumor progression (35).

Here we show that v-*erb*A induces two transformation parameters in B3.1 cells: invasiveness of a complex, natural extracellular matrix; and anchorage-independent growth. These two biological effects are related, but perhaps distinctly, to the induction of PDGF B. Cell invasiveness induced by v-*erb*A is mediated to a great extent by PDGF since suppression of PDGF activity by a specific anti-PDGF BB antibody mostly abrogates this effect. The results obtained by treating the cells with exogenous PDGF or serum suggest that the v-*erb*A-induced production of PDGF is responsible

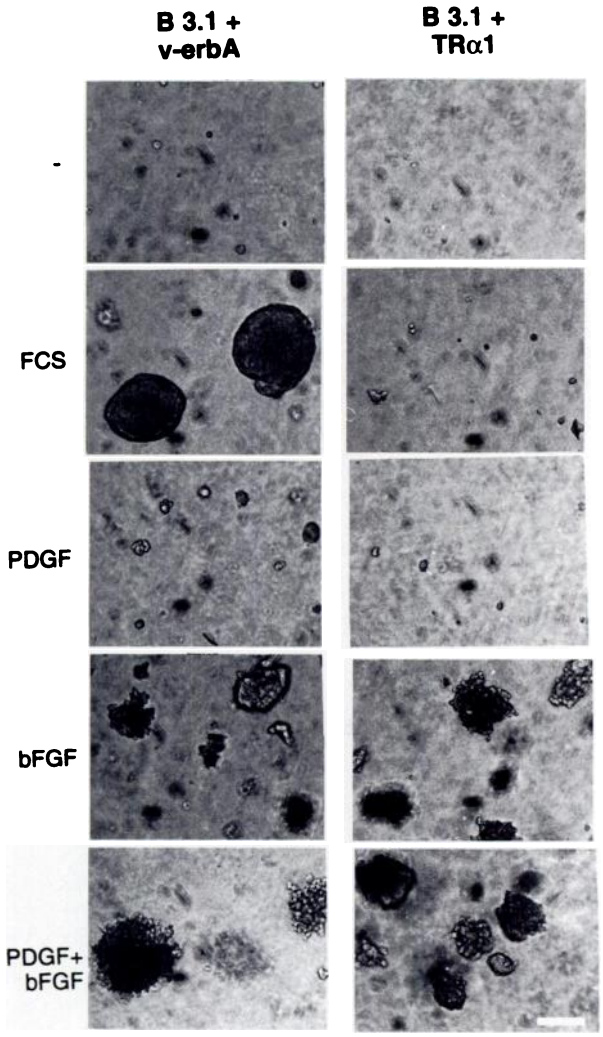

Fig. 8. Effect of exogenous PDGF and bFGF on foci formation by B3.1 + v-*erb*A and B3.1 + TRα1 cells. Cells were seeded in 24-well plates and treated with the indicated factors (10 ng/ml) as described in "Materials and Methods." Three experiments were done, giving the same results. Photos of representative foci are shown. *Bar,* 100 μm.

for a nearly maximum stimulation of cell invasiveness. However, it seems that other serum factor(s) in addition to PDGF is able to increase the invasive potential of B3.1 cells. Still, the differences observed could be a mere consequence of the (PDGF-induced) more active state of the B3.1 + v-*erb*A cells than the parental or the B3.1 + TRα1 cells. Against this possibility, replication-arrested B3.1 + v-*erb*A cells by mitomycin C treatment showed higher invasiveness than B3.1 + TRα1 cells. On the other hand, our data demonstrate that the anchorage-independent growth of B3.1 + v-*erb*A cells is the result of the cooperation between the PDGF BB produced by the cells and a serum factor(s) that may very well be bFGF.

v-*erb*A induces proliferation of chicken erythroblasts and fibroblasts by not yet well-characterized mechanisms, which may include the abrogation of the inactivation of the AP-1 (*jun/fos*) transcription factor by normal nuclear hormone receptors (42, 43). In this respect, the induction of higher levels of c-*jun* and *fra*-1 mRNAs might play a role in the phenotypic changes observed in B3.1 + v-*erb*A cells. Both are transformation competent oncogenes, the oncogenic potential of which can be achieved by overexpression (44, 45). In fact, *fra*-1 abrogates anchorage-dependent growth and induce *in vivo* tumorigenicity of rat fibroblasts (45). Therefore, although a definitive comprobation is required, c-*jun* and *fra*-1 are potential candidates to mediate at least part of the transforming activity of v-*erb*A in these cells.

On the other hand, v-*erb*A has been reported to augment the proliferative response to epidermal growth factor (46) and block RBC apoptosis by unregulated thyroid hormone receptor/RAR (47). Also, the expression the oncogenicity of v-*erb*A has been shown to require phosphorylation (48) and the cooperation with tyrosine kinase oncogenes (reviewed in Refs. 24 and 49). It is plausible, then, that the activation of PDGF-signaling pathways in B3.1 + v-*erb*A cells can contribute to transform these cells by cooperating with other mechanisms triggered by v-*erb*A. A similar reasoning would also explain the increase in v-*erb*A transforming effects seen in the presence of serum.

We have not found effects of v-*erb*A on the secreted proteolytic activity of B3.1 cells. If true, this would mean that the increase in invasiveness induced by v-*erb*A is exerted by enhancing cell motility and/or the dynamic interactions with the extracellular matrix, which are additional necessary requirements for cell invasion. However, changes in the activity of metalloproteinases or the involvement of other types of proteases undetectable by the current zymography assays cannot be totally ruled out.

An additional biological effect of PDGF is the induction of chemotaxis in several cell types (Refs. 36 and references therein). However, we have not found any chemotactic activity of exogenous PDGF on B3.1 cells. One possible explanation is the PDGF autocrine stimulation of B3.1 + v-*erb*A cells. Thus, it has been reported that an excess of early signaling inhibits the chemotactic response to PDGF of NIH 3T3 cells (50). Alternatively, the lack of chemotaxis of B3.1 cells may represent a cell-specific response.

It is also worth noting that B3.1 cells were originally established by infection of primary mouse brain cell cultures with a v-*myc*-expressing retrovirus. Therefore, the three B3.1 cell types express v-*myc*, as checked in Northern blots (29). The possibility exists, then, that part of the results here shown could be due to the cooperation between v-*erb*A and v-*myc* oncogenes.

In summary, our results indicate that v-*erb*A has transforming activity of the B3.1 glial cells through mechanisms involving an autocrine PDGF stimulation, and perhaps the induction of other nuclear oncogenes such as c-*jun* and *fra*-1. These data support a role for the normal thyroid hormone receptor/c-*erb*A and its ligand T_3 in the maintenance of the differentiated phenotype of glial cells, and the interest of the analysis of the integrity and expression of *erb*A genes in glioma tumors.

Materials and Methods

B3.1 Cell Culture. Cells were grown in Ham's F12 medium supplemented with 10% FCS, 2 mM glutamine (all from GIBCO), and 20 mM

Table 1 Gene expression in parental B3.1, B3.1 + TRα1 and B3.1 + v-*erb*A cells

Cells were grown in either complete growth medium or serum-free medium (see "Materials and Methods"). Poly(A)$^+$ RNA were prepared and analyzed in Northern blots as described in "Materials and Methods" and hybrized to specific cDNA probes. Cyclophilin was used as control gene.

Increased in B3.1 + v-*erb*A cells	Unaffected by v-*erb*A or TRα1	Undetectable expression
c-*jun* *fra*-1	*jun*B, *jun*D, c-*fos*, *fos*B, TGF-α, TGF-β1, -β2 and -β3, TIMP-1 and -2, CD44, MEP, β1-integrin, fibronectin, NURR1, NGF1B, collagen type I, glutamine synthetase, EGF-R, c-*erb*B2/neu, β1 laminin	RCC1, butyrophilin, *fra*-2, α2/α3 integrins, entactin, hepatocyte growth factor, c-*met*, S100β, SGP-2, PAI-1 and -2, N-CAM, carbonic anhydrase II, stromelysin-1 and -2

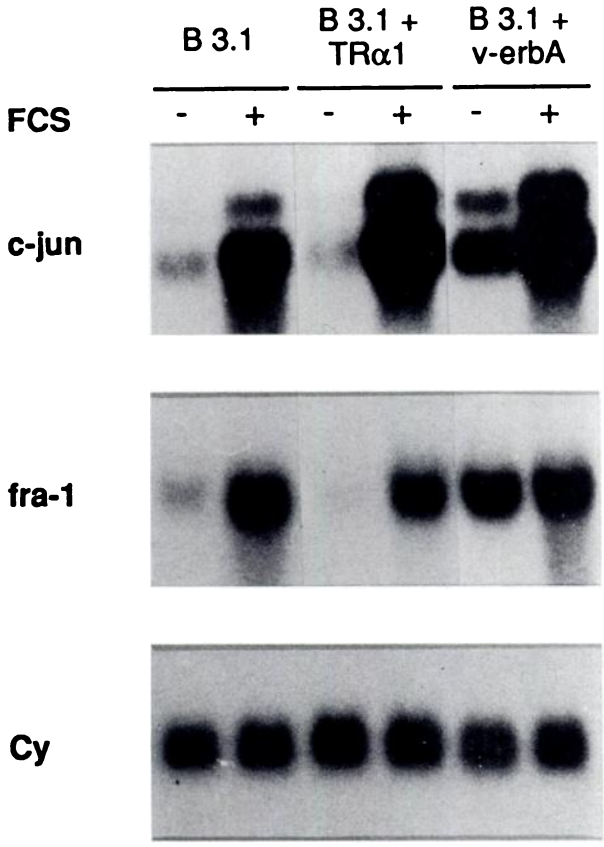

Fig. 9. Expression of c-*jun* and *fra*-1 in parental B3.1, B3.1 + TRα1, and B3.1 + v-*erb*A cells. Cells were incubated in medium containing (+) or not (−) FCS for 2 days. Poly(A)$^+$ mRNA was then purified, and 10 μg were analyzed in Northern blots as described in "Materials and Methods." Cyclophilin (*Cy*) was used as control. Two separated experiments were done, giving the same result.

glucose. For serum-free medium, we used the N2 medium described by Bottenstein and Sato (51) with the following composition: DMEM and Ham's F12 media (both from GIBCO) at a 1:1 ratio supplemented with 15 μg/ml insulin, 100 μg/ml transferrin, 20 nM progesterone, 30 nM sodium selenite, 100 μM putrescine, 2 mM glutamine (GIBCO), 0.03% BSA, and 15 mM HEPES (pH 7.3; unless specified, all from Sigma).

Growth Factors and Antibodies. Human recombinant PDGF BB was purchased from Boehringer Mannheim, and human recombinant bFGF was a generous gift from Farmitalia Carlo Erba. Rabbit polyclonal antihuman PDGF BB antibody was obtained from Genzyme, the monoclonal anti-bovine bFGF was from Upstate Biotechnology, Inc. and the rabbit polyclonal antirat GHF-1 was a gift from Dr. M. Karin (University of California-San Diego, La Jolla, CA).

Invasion Assays. Cell invasion capacity was evaluated using a modified Boyden chamber assay (52). Polyvinylpyrrolidone-free polycarbonate filter Transwell inserts (6.5-mm diameter) with 8.0 μm pores (Costar) were used. Inserts were set in 24-well culture plates (Falcon) and incubated overnight at 37°C with 0.1 mg/ml gelatin in 0.1% acetic acid/PBS solution and dried for 5 h. Matrigel, GFR-Matrigel, or collagen type I (Collaborative Research) were applied (4 μg in 50 μl cold PBS) to the upper surface of each filter and were dried at room temperature under a UV lamp inside a hood. When indicated, human fibronectin (5 μg in 500 μl culture medium) or PDGF BB (10 ng/ml) were added to the lower chamber as chemoattractants. Cells (3×10^5, unless specified) were seeded in serum-free medium (unless indicated) in the upper chamber in 200 μl medium. The highly metastatic murine melanoma B16F10 cells (53) were used as positive control. After incubation for 4 days, cells on the upper side and matrix were removed with cotton swabs. For visualization, inserts were stained with hematoxylin and eosin, cut out with a scalpel, and photographed with a Nikon FX-35 camera under an inverted IMT Olympus microscope. For quantitation of invasive cells, Transwell inserts (where attached) were incubated for 10 min at 37°C with culture medium containing 6.25 μg/ml 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester for 10 min at 37°C. When visualization was not required, cells were fixed with 1% glutaraldehyde for 10 min, washed twice with PBS, and stained with 0.1% crystal violet for 30 min. After three washes with deionized water, the cell-attached dye was dissolved in 10% acetic acid and measured at A_{595}.

Proteolytic Activity Assays. To estimate the secretion of extracellular matrix degradation enzymes by the different cell types, gelatin and casein zymograms were performed as described (54). Volumes of serum-free conditioned media normalized to protein content were electrophoresed on 10% SDS-polyacrylamide gels containing gelatin (1 mg/ml) from bovine skin (Sigma) or β-casein (0.5 mg/ml) from bovine milk (Sigma). Conditioned media were prepared from cells seeded in 60-mm dishes, which were washed twice with PBS and switched to 3 ml N2 medium for 48 h. Media were collected and spun down for 5 min in a refrigerated Sorvall centrifuge at 2500 rpm to remove cell debris. Centriprep-3 (Amicon) columns were used for 10-fold concentration when required. Purified type XI collagenase from *Clostridium histolyticum* (Sigma; 1 μg/ml) was loaded as positive control in the gelatin gels. After electrophoresis, the gels were rinsed twice in 2.5% Triton X-100/50 mM Tris (pH 7.4) for 30 min and twice in 50 mM Tris (pH 7.4) for 10 min to remove the SDS and then incubated for 22 h at 37°C in substrate buffer [50 mM Tris-HCl buffer (pH 7.5), 10 mM $CaCl_2$, 150 mM NaCl, 0.1% Triton X-100, and 0.02% NaN_3]. The gels were stained for 1 h in 30% methanol/10% glacial acetic acid containing 0.5% Coomassie Brilliant Blue and destained in the same solution in the absence of dye.

Soft Agar Foci Formation Assays. Ten thousand cells were suspended in 2 ml 0.35% Difco Noble agar in DMEM containing 10% FCS over a basal layer of 0.5% agar (3 ml). Two and 4 weeks later, the cells were refed by overlaying 2 ml of 0.35% agar in the same medium, and colonies were photographed 6 weeks after seeding. For the tests of activity of PDGF, bFGF, and the anti-PDGF BB antibody, we seeded the same number of cells in 250 μl 0.35% Difco Noble agar containing or not the indicated factor (10 ng/ml) or antibody (10 μg/ml) over a basal layer of 0.5% agar (300 μl) in 24-well plates. Cells were fed every 2 days with 100 μl N2 medium containing the corresponding factor or antibody. In these experiments, foci appeared earlier (10–14 days).

RNA Preparation and Northern Analysis. Purification of poly(A)$^+$ RNA from cells was done as described (55). Northern blots were performed on nylon membranes (Nytran; Schleicher and Schuell) according to standard protocols (56). All probes were labeled by the random priming method (57). Hybridizations were carried out overnight at 65°C in 7%

SDS/500 mM sodium phosphate buffer (pH 7.2) and 1 mM EDTA, according to Church and Gilbert (58). Filters were washed twice for 30 min each in 1% SDS and 40 mM sodium phosphate buffer (pH 7.2) at 65°C. Before rehybridizing the nylon membranes with probes for other genes, the radioactive probe was stripped off the membrane by placing it in a 75°C water bath for 5 min. Membranes were exposed to Kodak X-Omat AR films.

Probes. Probes used in this study were provided by Dr. Rodrigo Bravo (*c-jun*, *junB*, *junD*, *c-fos*, *fosB*, *fra-2*, fibronectin, and β1-integrin), Dr. M. Busslinger (*fra-1*, MEP, PAI-1, and PAI-2), Dr. D. C. Lee (TGF-α), Drs. S. W. Qian and A. B. Roberts (TGF-β1, TGF-β2, and TGF-β3), Dr. E. Wagner (TIMP-1), Dr. D. R. Edwards (TIMP-2), Dr. U. Günthert (CD44), Drs. R. M. Evans and T. Perlmann (NURR1 and NGF1-B), Dr. Y. Yamada (β1-laminin), Dr. C. Birchmeier (hepatocyte growth factor), Dr. A. E. Chung (entactin), Dr. M. E. Hemler (α2/α3 integrins), Dr. J. Newport (RCC1), Dr. S. R. Sylvester (SGP-2), Dr. I. H. Mather (butyrophilin), Dr. G. F. Vande Woude (*c-met*), Dr. A. Marks (S100β), Dr. Z. Q. Wang (collagen type I), Dr. R. E. Breathnach (stromelysin 1), Dr. L. M. Matrisian (stromelysin 2), Dr. C. Goridis (N-CAM), Dr. W. H. Lamers (glutamine synthethase), and Dr. P. J. Curtis (carbonic anhydrase II).[5]

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