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Potential oncogenic effects of basic fibroblast growth factor requires cooperation between CUG and AUG-initiated forms

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Normal adult bovine aortic endothelial cells were infected with various recombinant retroviruses expressing one, two, or three human basic fibroblast growth factor (bFGF) proteins normally synthesized by an alternative use of translation initiation codons. We show here that the constitutive expression of the AUG-initiated from (18 kDa) leads the transfected cells to form colonies in soft agar. The expression of the high molar weight (HMW) forms (22.5 and 21 kDa) initiated at one of the two CUG initiation codons allows cell immortalization, whereas the tumorigenic potential is reached when the three forms are constitutively expressed. Furthermore, we provide evidence that constitutive expression of (HMW) bFGF forms has a down-regulation effect on bFGF synthesis from the gene naturally active in parental endothelial cells.

Introduction

Basic fibroblast growth factor (bFGF) belongs to a family of potent mitogens and promotes proliferation and differentiation of neuroectodermal, mesenchymal, and epithelial cells; angiogenesis; and wound healing processes (Gospodarowicz *et al.,* 1986; Rifkin and Moscatelli, 1989). The biological activity of bFGF is mediated through a family of high-affinity cell surface receptors but also requires a low-affinity heparin-like molecule (Yayon *et al.,* 1991). bFGF is synthesized by a wide variety of cells, including capillary endothelial cells (Schweigerer *et al.,* 1987a), but the mechanism by which the molecule is secreted remains unknown. Primary subcultures of adult bovine aortic endothelial (ABAE) cells synthesize a significant amount of bFGF, but in the absence of detectable excretion, they require exogenous bFGF to proliferate (Bouche *et al.,* 1987).

In most of the producing cell lines, several forms of bFGF are detected (Renko *et al.,* 1989). They result from an alternative initiation of translation at an AUG (156 a.a.: 18 kDa) or at inframe upstream CUGs (210 a.a.: 22.5 kDa and 195 a.a.: 21 kDa, respectively). In transfected monkey COS cells or in a cell-free system, the three forms are synthesized with the same efficiency (Florkiewicz and Sommer, 1989; Prats *et al.,* 1989a,b). In these systems, the replacement of the first CUG by an AUG resulted in the production of the 210 a.a. form only (Prats *et al.,* 1989b). In different cell lines, the relative amounts of the different forms are highly different, suggesting that this alternative initiation process is highly regulated.

By immunocytology, bFGF was located both in the cytoplasm and in the nucleus of growing ABAE cells (Baldin *et al.,* 1990). In COS cells transfected with human bFGF cDNA, the two CUG-initiated forms are nuclear, whereas the AUG-initiated form is cytoplasmic (Bugler *et al.,* 1991). The signal sequence responsible for the nuclear localization of bFGF is contained in the 37 residues upstream of the AUG. Similarly, in SK-Hep1 cells and NIH 3T3 cells transfected with bFGF cDNA, the 156 a.a. form is found to be primarily cytosolic, whereas the high molar weight (HMW) bFGF are recovered in the nucleus (Renko *et al.,* 1990). It must be noted that the NH2 terminal sequence of the (HMW) bFGF contains a glycine-rich sequence with interspaced arginines, which is also recovered in a family of nuclear proteins (Sommer *et al.,* 1989).

Several sets of evidence suggest that intracellular distribution of bFGF plays a key role in cell behavior (Baldin *et al.,* 1990). Exogenous bFGF is translocated to the nucleus of ABAE cells during a specific stage of the cell cycle and is detected in the nucleus during all the S phases, whereas it is not detected in confluent cells. On the other hand, four oncogenic mem-

bers of the FGF family, *int-2* (Dickson and Peters, 1987), *hst* (Delli Bovi *et al.*, 1987), *FGF-5* (Zhan *et al.*, 1988), and *FGF-6* (Marics *et al.*, 1989) encode protein that possesses an amino terminal signal peptide and that are secreted. It has been shown that NIH 3T3 cells expressing the LMW form of bFGF fused with a signal peptide sequence are transformed in vitro and tumorigenic in vivo, whereas the over expression of the normal form does not lead to such a phenotype (Rogelj *et al.*, 1988; Blam *et al.*, 1989).

In this report we investigated the role of the larger forms of bFGF versus the smaller one when expressed in normal endothelial cells. We present evidence that the CUG-initiated forms are involved in the immortalization process and the AUG-initiated form is responsible for the anchorage-independent growth of transfected cells. Tumorigenic activity of transfected cells is reached when the three forms are constitutively expressed or when the AUG-initiated form is over expressed.

Results

Construction of cell lines constitutively expressing bFGF

Primary cultures of ABAE cells were transfected with murine leukemia virus (MuLV)-derived retroviral vectors containing various bFGF cDNAs encoding either the three bFGF initiated at the AUG and at the two CUG (PINA 4A: A4A cells), the CUG-initiated bFGF (PINA 3A: A3A cells, respectively), or the AUG-initiated bFGF (PINA 5A and 7A: A5A and A7A cells, respectively) (Figure 1). Mock cells were infected with the same retroviral vector alone or containing the chloramphenicol acetyl transferase (CAT) gene. The two-step procedure used (see Materials and methods) allowed the integration of a single retroviral copy in the recipient cell genomes.

After 2 wk in medium containing geneticin (G418), clones were selected and grown again for 1 wk in the presence of G418. Whatever the inserted bFGF cDNA, an equivalent amount of clones with similar sizes were observed. For each construction, 5 to 10 clones were picked and used for the different analysis to avoid particular effects resulting from the integration site in the genome. For each construct, the different clones presented the same phenotype and produced similar amounts of bFGF, as was shown by Western blotting analysis of total cell extracts (Figure 2a). To compare the different forms of bFGF produced by the different cell lines, large amounts of materials must be analyzed. In the following experiment, the crude extract was first run on heparin sepharose column before analysis of bFGF content. As shown in Figure 2b, this purification step did not affect the relative amount of bFGF in two different cell lines (A3A and A4A).

Protein analysis

bFGF expression in ABAE, A2A, A3A, A4A, A5A, and A7A cells was compared (Figure 2c). Each lane corresponded to bFGF extracted from 10^6 cells. In parental ABAE cells, the three forms of bFGF were detected, but the 18-kDa form represented >85% of intracellular bFGF, whereas the (HMW) bFGF forms were present in an almost equal amount (Figure 2c, lane A). The 18 kDa corresponded to the 155 residues AUG-initiated protein and not to the 146 residues form and was added to the culture medium to allow ABAE cells proliferation. This was demonstrated by replacing the 146 exogenous bFGF by a 131 residues human recombinant bFGF (hrbFGF; 16-kDa form), which was never detected in equivalent western blotting experiments. A3A cells produced less bFGF than the parental ABAE at almost one molecular species only with an apparent molecular weight of 22.5 kDa (Figure 2c, lane B) that must correspond to the 210 amino acid bFGF form encoded by the integrated bFGF cDNA. A down-regulation of the 18 and 21 kDa products detected in parental ABAE cells must be noted. A4A cells produced the three immunoreactive bFGF species with about two times more bFGF than in parental ABAE cells (Figure 2c, lane C). In A4A cells, the 21-kDa form is expressed about four times more than the 22.5 kDa form, and the 18 kDa AUG-initiated form represents 50% of the total bFGF.

A5A cells produced only one immunoreactive molecular species of 18 kDa that corresponds to the 155 a.a. bFGF form (Figure 2c, lane D). The amount of 18-kDa protein is similar to that presented in the parental ABAE cells. In addition to the bFGF 18-kDa form, A7A cells produced a large amount of a 46-kDa bFGF immunoreactive species and two forms of 19.5 and 20 kDa (Figure 2c, lane E). These aberrant bFGF forms did not result from aggregation nor intermolecular disulfure bonds nor O-glycosylation because they were resistant to strong denaturing agents (10 M Urea, 1 M DTT, βMercaptoethanol) and alcaline treatment, but from the over expression of bFGF (50-fold) resulting from the deletion of the bFGF mRNA leader sequence.

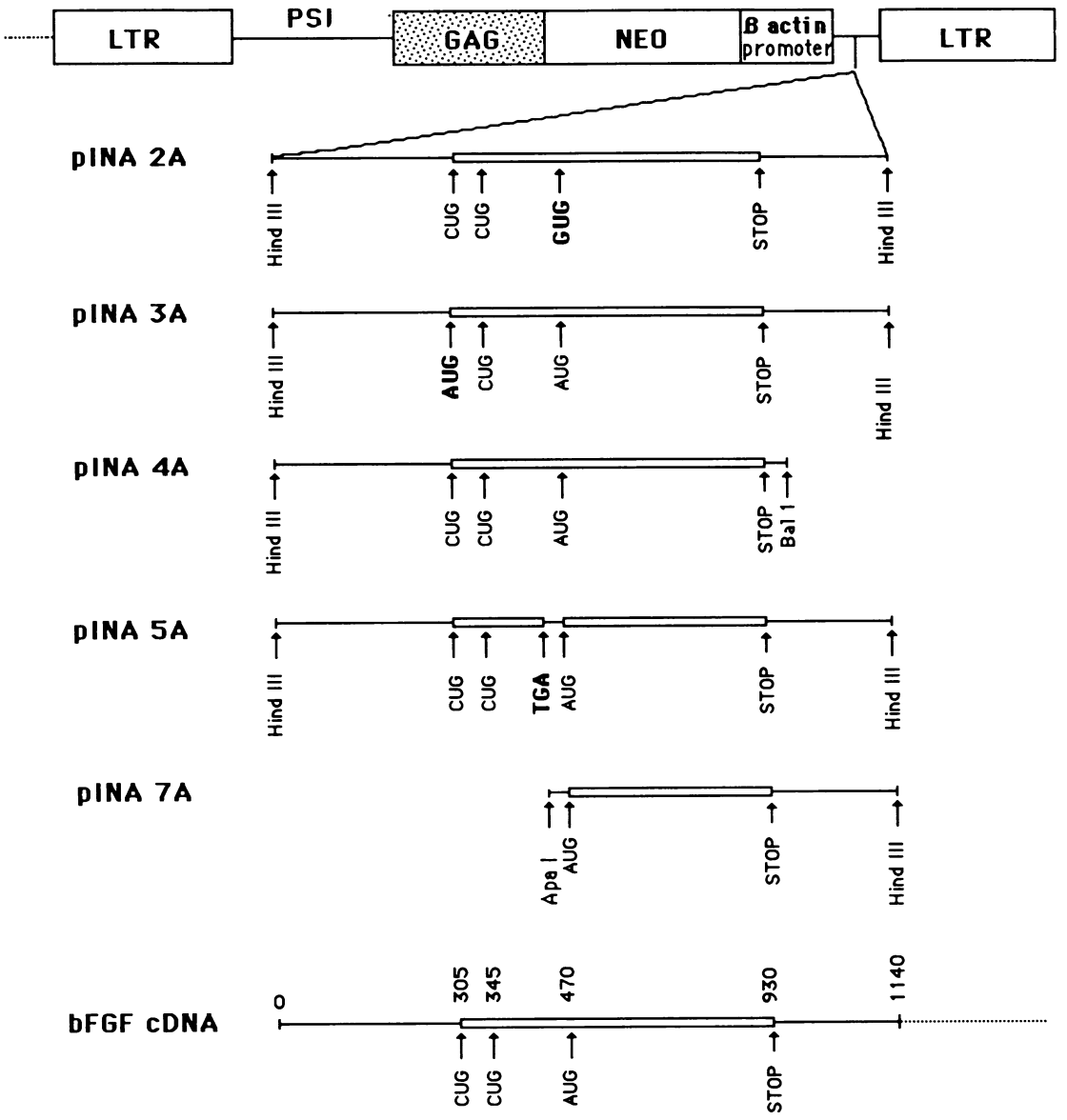

Figure 1. **Recombinant plasmid structures.** The retroviral vector Pina (Morgenstern and Land, 1990) carrying a geneticin resistance gene (neo) was used to construct the PINA plasmids. Control and mutants bFGF cDNAs were inserted into the polylinker downstream of the β actin promoter. The 1140 bp *Hin*dIII subfragment of bFGF cDNA contained the 625 bp coding region with three initiation codons (CUG, GUG, and AUG). PINA 2A, the AUG 470 was mutated in GUG encoding the 22.5- and 21-kDa proteins. PINA 3A, CUG 305 was mutated in AUG allowing the synthesis of the 22.5-kDa protein only. PINA 4A, wild-type bFGF cDNA encoded the three 22.5-, 21-, and 18-kDa proteins. PINA 5A, a termination codon TGA was added in frame upstream of the AUG, allowing the synthesis of the 18-kDa protein. PINA 7A, a deletion of the 5′ sequence (450 bp).

Phenotypes of transfected cells

Two weeks after the different clones were picked, the morphology of the cells was observed. The G418 resistant cell lines showed morphological changes that appeared to be different, according to the bFGF cDNA expressed by the cells. The cells looked smaller or larger than parental cells, spindle-shaped or rounded, depending on the cDNA introduced. A3A and A4A adhered poorly and grew in a disorganized criss-cross pattern achieving a higher density than the parental ABAE cells (Figure 3, 1b and 1c).

A5A and A7A cells (Figure 3, 1d), which presented the less marked morphological changes, showed contact inhibition as the parental ABAE cells (Figure 3, 1a). When the cells reached confluence, the parental ABAE, mock, A5A, and

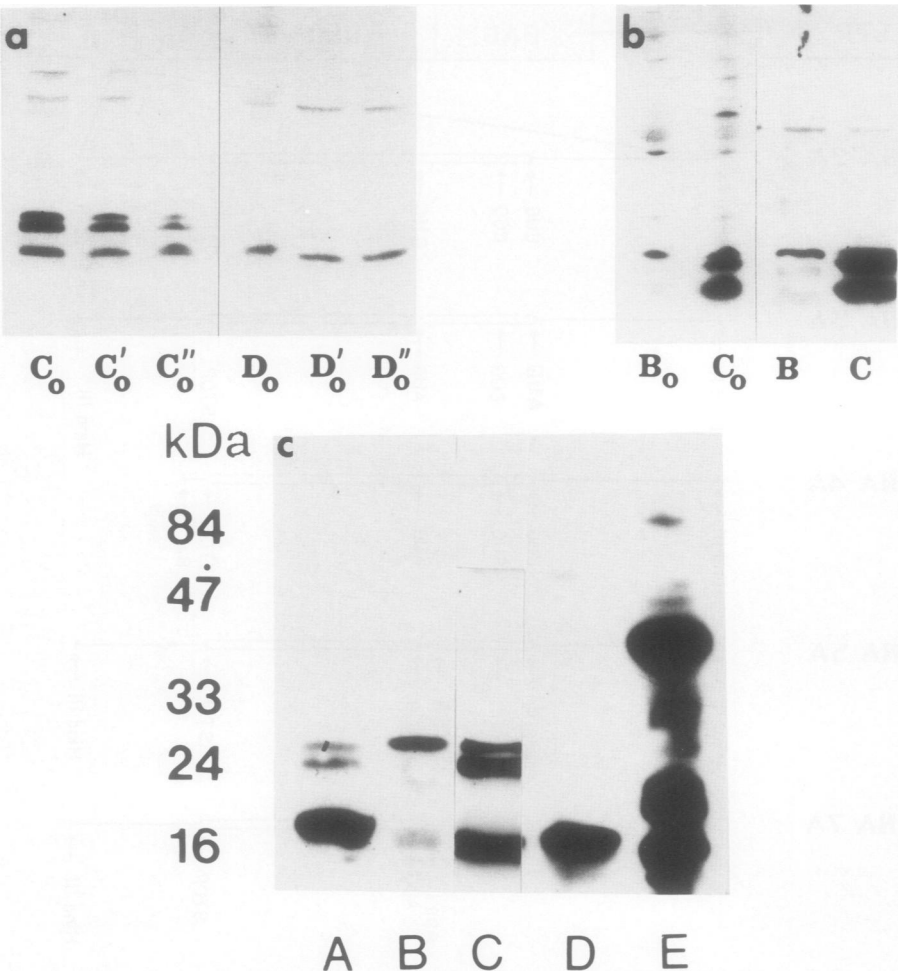

Figure 2. **Immunodetection of bFGF in mock and bFGF transfected ABAE cell lines.** Cell extracts were prepared from mock or bFGF transfected cell lines. Proteins were analyzed directly from the extracts by SDS tricine polyacrylamide gels (B_o, C_o, D_o) or after purification on heparin seraphose column (A–E). Corresponding Western blots were probed with an anti-bFGF polyclonal antibody (Cliniscience) as described in Materials and methods. Mock cells (lane A), A3A cells (lane B_o, B), A4A cells (lane C_o, C), A5A cells (lane D_o, D), A7A cells (lane E). The same aliquot of 1.10^6 cells was analyzed in each lane. Numbers on the left corresponded to the protein size markers used. C_o-C'_o-C''_o, D_o-D'_o-D''_o are three different A4A and A5A cell lines, respectively. B_o and B, C_o and C are proteins from total extract or after HS purification.

A7A cells showed the cobblestone pattern (Figure 3, 2a–d), but A5A and A7A cells, as well as A3A and A4A cells, appeared morphologically modified. It could be noticed that, at this level of investigation, differences in bFGF content among the clones A5A and A7A did not lead to different morphologies, in contrast with what was previously reported for NIH 3T3 cells (Quarto *et al.,* 1989). In addition, the numbers of bFGF high-affinity receptors by cell was estimated. Constitutive expression of bFGF induced a general decrease in infected cells compared with ABAE cells (ABAE, 80 000; A3A, 20 000; A4A, 5 000; and A5A, 9 000 sites) (Couderc, unpublished data).

Growth of transformed cells

The four cell lines transfected with bFGF DNA grew more slowly than the mock cells (generation times 26 vs. 18 h). The growth ability of the four transfected clones was examined. A3A, A4A, and A7A cells grew in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum (CS) independently of exogenous bFGF, whereas A5A and mock cells required exogenous bFGF to proliferate (Table 1). A3A, A4A, and A7A were immortalized (>110 generations), whereas A5A cells were not. The difference observed between the growth of A5A and A7A cells that were transfected with the same bFGF cDNA open reading frame can be

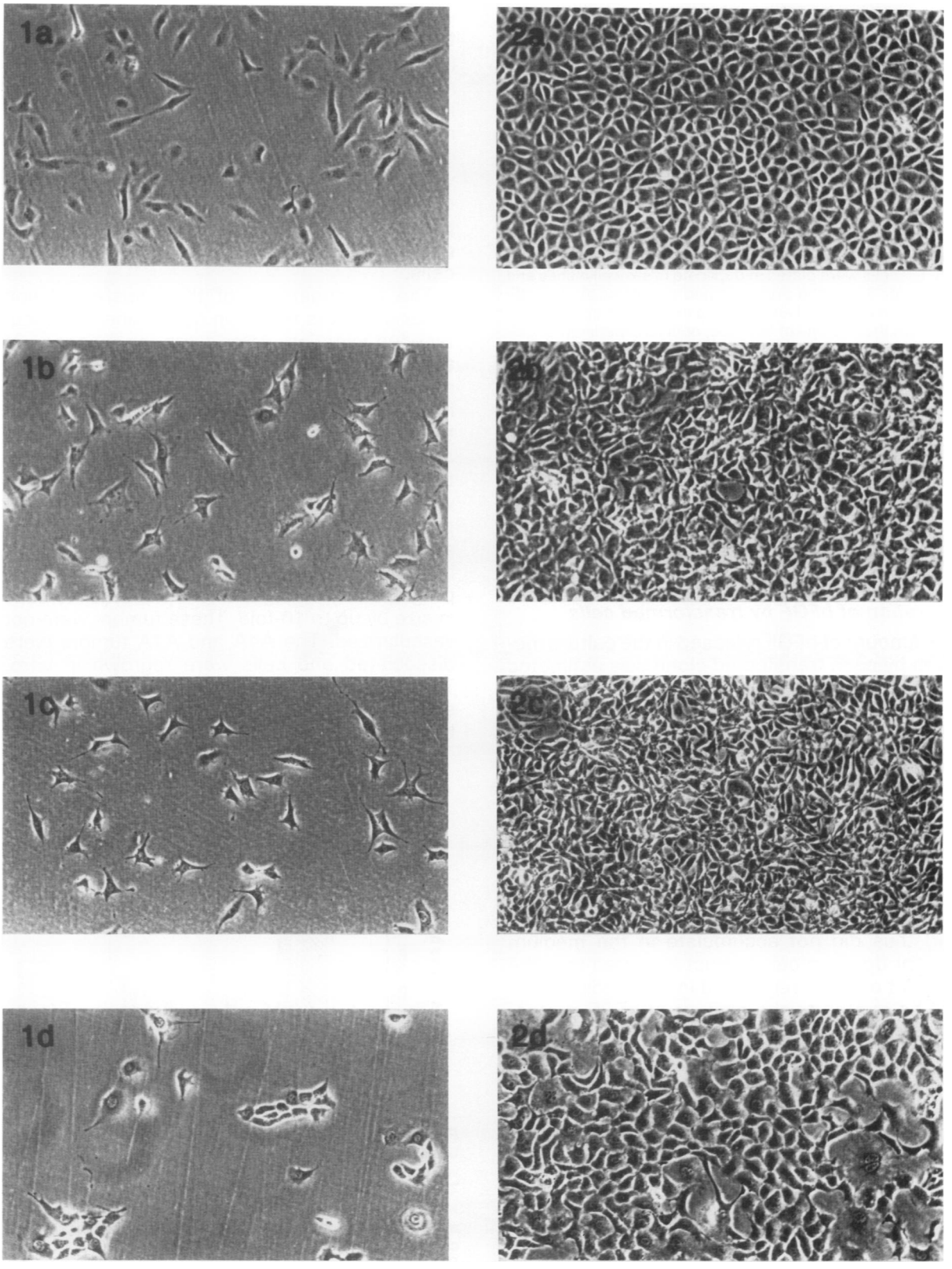

Figure 3. **Effect of endogenous expression of bFGF on cell morphology.** ABAE (a), A3A (b), A4A (c), or A5A (d) were plated on gelatinized six-wells plates. Micrographs at day 1 (1) and day 6 (2). A3A and A4A cells adhered normally to the plate but did not present contact inhibition.

Table 1. bFGF dependence, agar growth, and tumorigenicity of the different cell lines

Cell lines	bFGF dependence	Clonigenicity	Tumorigenicity
ABAE	+	−	−
A2A–A3A	−	−	−
A4A	−	++	+
A5A	+	−	−
A7A	−	+	++

Dependence of transfected cell lines to exogenous bFGF: 1×10^4 cells were plated in duplicate in six-well dishes and grown for 6 d in the presence and the absence of bFGF. Cells were harvested and counted in a coulter counter. Results are the average of five independent experiments. Colony formation in soft agar and tumorigenicity of ABAE-transfected cells. A total of 10^7 cells were infected s.c. into Swiss nude mice. Three to 10 mice per condition were injected with A3A, A4A, A5A, and A7A cells. Control mice were injected with mock cells.

related to the amount of bFGF present in the cells and the appearance of aberrant high molar mass forms.

Excretion of bFGF by transformed cells

The amount of bFGF released in the culture medium by each transfected clone was estimated by testing the ability of ABAE cells to proliferate in this conditioned medium. The culture medium of A3A, A4A, and A7A cells contained bFGF-like mitogenic activity (Figure 4, B–E, G). No such mitogenic activity was ever detected with conditioned medium from ABAE parental cells (Figure 4A) as with A5A cells (Figure 4F).

The amount of bFGF contained in the A4A-conditioned medium was estimated to be 50 pg/ml. The possibility that secreted bFGF might bind to components of the extracellular matrix and thus did not accumulate in the medium cannot be ruled out. It must be noted that the amount of bFGF recovered in the medium cannot be related to the amount of bFGF synthetized by the cells.

Growth properties of transfected cells

The growth properties of the different cell lines were then tested. A3A and A4A cells grew to densities higher than mock ABAE, A5A, and A7A cells (around 2 times). The growth in soft agar of these cell lines was checked. A4A, A5A, and A7A cells formed colonies in soft agar, whereas A3A and mock ABAE cells yielded no colonies. A5A and A7A cells, which were transfected with the 18-kDa reading frame, formed smaller colonies than A4A cells that were transfected with the three forms of bFGF DNA (Figure 5,A–C). Furthermore, the clones formation of A5A cells required exogenous bFGF in the soft agar, whereas A4A and A7A cells did not. The acquisition of anchorage-independent growth was correlated with the form and not with the amount of synthesized bFGF. A3A cells did not acquire the ability of anchorage-independent growth while they had an autonomous cell proliferation at a rate superior to parental cells.

The tumorigenicity of the transfected and mock cells was tested in nude mice by subcutaneous injection. After 1 mo, no evidence of tumors was found in the mice injected with the mock cells or with the A3A and A5A cells. Mice injected with the A4A cells that synthesized the three forms of bFGF developed small tumors after 1 wk (4 tumors for 8 injected mice). These tumors were not progressive over the next 4 wk. All the mice injected with A7A cells that produced large amounts of the 18 kDa and aberrant forms developed tumors at the site of innoculation within 6 d. The A7A tumors grew progressively during the next month to increase in size by up to 10-fold. These tumors were not vascularized. The A4A and A7A tumors were dissociated and cells were regrown in vitro. They were still resistant to G418 and synthesized in similar amounts the same forms of bFGF as the injected cells (data not shown).

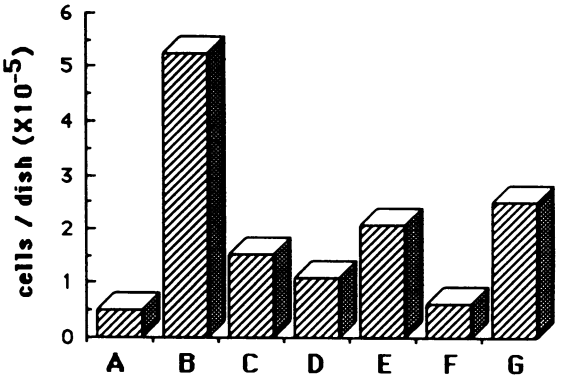

Figure 4. **Mitogenic activity of transfected ABAE-conditioned medium.** Conditioned media by the different cell lines were prepared as described in Materials and methods. These media (40%) were added to ABAE cells seeded at 1×10^4 cells per dish. Cells were trypsinized and counted after 4 d. Medium conditioned by A, mock cells; C, A2A cells; D, A3A cells; E, A4A cells; F, A5A cells; G, A7A cells. B, 1 ng/ml of *E. Coli* recombinant bFGF was added to cell culture as a control. The presented values are the average of three independent experiments.

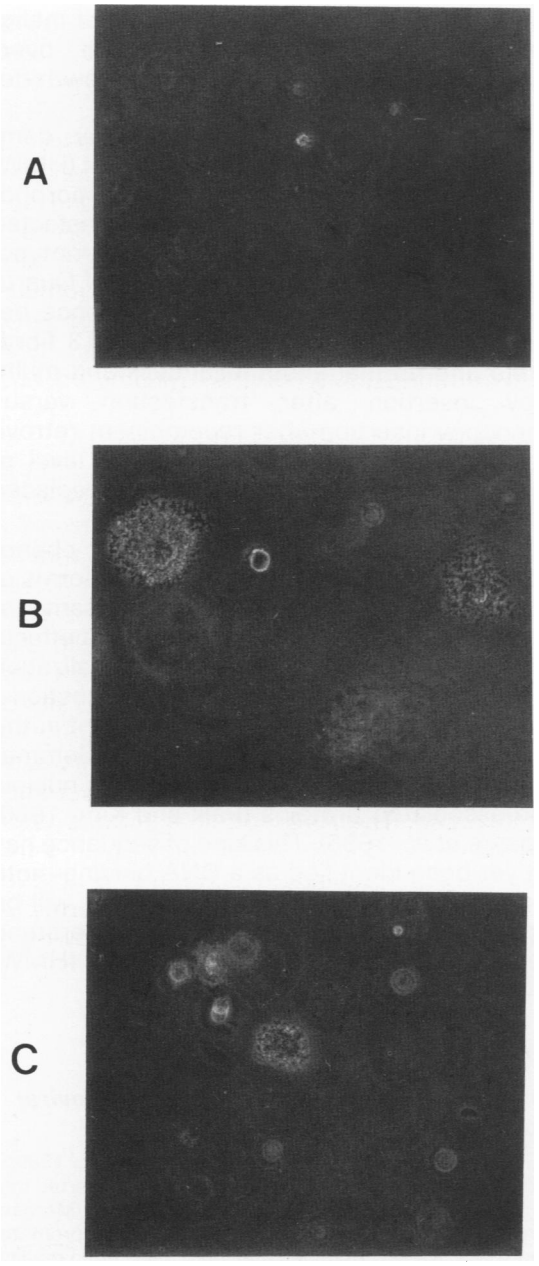

Figure 5. **Growth under agar of transfected cells.** Cells were plated and grown in soft agar as described in Materials and methods. Microphotographs at day 8. A, mock cells; B, A4A cells; C, A5A cells.

Discussion

In a growing number of cases, initiation of translation at a non-AUG codon is being reported to occur in vivo. In particular, there are three examples in which the alternative use of CUG and AUG translational start codons generates protein diversity correlated with differences in subcellular location of the products: the Mo-MuLV CUG-initiated p75 Gag protein is associated with cell membrane, whereas the AUG-initiated p65 Gag is a core virion protein (Prats *et al.,* 1989a,b) and the CUG-initiated forms of int-2 and bFGF proteins are nuclear, whereas the AUG-initiated forms are secreted (Acland *et al.,* 1990) or cytoplasmic (Bugler *et al.,* 1991), respectively. This difference in subcellular localization led us to investigate the putative role of the different forms of bFGF. The model that we have developed takes into account two criteria: 1) constitutive expression, but not an over expression of the human bFGF cDNA and 2) expressed in normal endothelial cells. We have constructed a set of recombinant retroviruses in which mutated or wild-type human bFGF cDNA is expressed under control of β-actin promoter. After each infection, at least five independent clones were isolated and in all cases the analysis failed to show any difference between the different clones in bFGF synthesis (Figure 2a). This demonstrated that the observed phenotypes are independent of the retrovirus insertion site. Furthermore, a recombinant retrovirus expressing the CAT gene was constructed and used to infect ABAE cells. The resulting clones, as well as the parental retrovirus mock-infected cells showed the same characteristics as the non-infected ABAE cells in respect of the generation number. Furthermore, the observed phenotype did not depend on the promoter used; wild-type bFGF cDNA was inserted after the Mo-MuLV long terminal repeat promoter. The cell lines infected by one or the two "wild-type" constructs show the same morphological and phenotypical characteristics (data not shown).

Although the growth of this selected parental ABAE cell line is strictly dependent on bFGF addition in the culture medium, the cells also synthesized the three forms of this factor. Why this endogenous produced factor is insufficient for an intra- or autocrine loop remains unclear. Strikingly, infected ABAE cells that constitutively expressed bFGF grew more slowly (generation time 26 h) than normal, mock-infected, or CAT-expressing ABAE cells (generation time 18h) in the absence of any cell fusion or increase in cell death. Moreover, when the 22.5 (A3A cells) form was constitutively expressed, it could be noticed that the 18-kDa form naturally synthesized from the nonretroviral bFGF gene was no longer detected. If we consider that the major difference between these genes lies in the difference between the two promoters, our results suggest that the ABAE bFGF gene is down-reg-

ulated by the products of the bFGF retroviral gene at a transcriptional level. This effect could also result from the absence of bFGF introns or from the 3′ untranslated region in the bFGF retroviral inserted gene. Moreover, the total amount of bFGF in A3A, A4A, and A5A cells is only slightly different from that observed in ABAE cells. We postulate that at least part of the observed phenotypes must be explained by the deregulation of relative amounts of the bFGF forms.

Morphological changes in ABAE cells were observed when they constitutively expressed bFGF. These changes greatly varied in respect of the form of bFGF they synthesized. Uncontrolled expression of the (HMW) bFGF (21 and/or 22.5 kDa) resulted in an immortalizing effect but did not allow colony formation in soft agar, nor tumorigenic effect. Expression of the 18-kDa form led to an in vitro transformed phenotype but failed in tumorigenic effect or in long-term established cell lines. Coexpression of the three forms allowed a full and typical malignant transformation. In full agreement with the oncogenic theory (for review see Hunter, 1991), the (HMW) bFGF that have a nuclear localization signal induced immortalization, the cytoplasmic bFGF form is transformant, and the coexpression of these forms results in a phenotype typical of oncogenes cooperation. The originality of this bFGF oncogenic process lies in the fact that the two cooperative oncogenes are generated by an alternative use of initiation codons. Our results could be compared with those obtained by Sasada *et al.,* (1988) after transfection of Balb/c 3T3 cells with a human bFGF cDNA with enough 5′ sequence to allow synthesis of the three forms of bFGF. Furthermore, LMWbFGF has tumorigenic effects when the molecule is overexpressed (this work) and/or when its secretion is induced by fusion with an N-terminal signal peptide (Rogelj *et al.,* 1988; Blam *et al.,* 1989). In both cases additional aberrant bFGF forms are recovered. It will be of interest to know if, in these LMMbFGF overexpressed cell lines, bFGF accumulates in the nucleus and nucleolus, as previously described for exogenous bFGF (Bouche *et al.,* 1987).

On the other hand, when cells expressed a bFGF cDNA truncated of the 5′ part upstream of the AUG codon, they over-expressed bFGF (A7A cells). The deleted sequence plays an important role in the translational control of bFGF synthesis (A. C. Prats, unpublished data). Western blot analysis of bFGF A7A cells content showed that aberrant forms of bFGF were found in addition to the normal 18-kDa form. The resulting phenotype was characteristic of malignant transformation, indicating that overexpression leads to another kind of growth deregulation.

Quarto *et al.,* in the preceding paper, demonstrated that the level of expression of (HMW) bFGF is also a very important data in morphological and phenotypical changes of transfected cells and can lead to a transformed phenotype. The differences between the results of Quarto *et al.* and ours may reflect the difference between our respective models: NIH 3T3 fibroblasts and normal endothelial cells and multicopy insertion after transfection versus monocopy insertion after recombinant retrovirus infection, in which the maximal level of expression is reached when CUG1 is replaced by an AUG.

In this paper we show that different phenotypes are associated with the different forms of bFGF, but we did not conclude if the same intracellular function of bFGF has different effects in respect of the different subcellular localization or if the different bFGF carry different functions. A Glycine-MethylArginine block, present in the amino terminal part of (HMW) bFGFs (Sommer *et al.,* 1989) is also found in a panel of nucleic acid-associated proteins (Paik and Kim, 1980; Lapeyre *et al.,* 1986). This kind of sequence has not yet been identified as a DNA binding motif nor a nuclear translocation signal so it will be of particular and general interest to understand the counterpart of this sequence in the (HMW) bFGF activities.

Materials and methods

Construction of mutant bFGF and retroviral expression vectors

bFGF cDNA was previously described (Prats *et al.,* 1989b). Wild-type or point-mutated bFGF cDNA were inserted into the mammalian retroviral expression vector PINA (Morgenstern and Land, 1990) downstream of the βactin promoter (Figure 1). PINA 4A contained the entire bFGF open reading frame, PINA 2A was derived from PINA 4A by inserting point mutation in bFGF at the position 467 changing ATG into GTG, PINA 3A was derived from PINA 4A by a point mutation at position 302 changing CTG into an ATG, PINA 5A was derived by a mutation at the position 440 changing GGA to TGA stop codon, and PINA 7A was derived from PINA 2A by a deletion up to position 445.

Cell cultures

Primary subcultures of ABAE cells were cloned twice and established as previously described to select cell lines that require exogenous bFGF to grow (Gospodarowicz, 1987). They are routinely maintained in DMEM supplemented with 10% CS (GIBCO, Grand Island, NY), 2 mM glutamin, 0.1 mg/ml Amphoptericin, 0.1 mg/ml gentamicin. One nanograms per milliliters of hrbFGF was added every 2 d. CRIP

cells (Danos and Mulligan, 1988) were maintained in DMEM supplemented with 4% fetal CS and 0.1 mg/ml gentamicin.

To measure proliferation, cells were plated in six-well plates at 1×10^4 cells per well and counted daily in duplicate in a coulter counter (Coulter Electronic, Coulternics S.A., France). For photomicrography, cells were grown in 35-mm dishes (Costar, Cambridge, MA) and photographed using a Nikon inverted microscope (Nikon, Garden City, NY).

Transfection and infection

CRIP cells (1.5×10^5/50-mm plate) were transfected with 2 μg/ml recombinant plasmid DNA in the presence of 10 μg of polybrene (Aubin *et al.,* 1988). After 42 h in culture the cells were split 1:8 and cultured thereafter in DMEM containing 1 mg/ml of G418 (Geneticin, GIBCO). After 2 wk, G418-resistant clones were isolated, expanded, and then released recombinant viruses in the medium. ABAE cells (1.5×10^5/50-mm plate) were infected by adding diluted medium (1/3) harvested from the virus producing cells in the presence of 8 μg/ml polybrene. Two days later cells were split 1:8 and grown in G418 selective medium. The surviving clones were individually trypsinized, expanded, and tested. Mock cells were similarly infected with PINA or recombinant retroviral particles in which bFGF insert was replaced by CAT gene.

Preparation of cell extracts and Western blot analysis

Cells were harvested from monolayer cultures by rinsing in cold phosphate-buffered saline (PBS) twice, followed by scraping with a rubber policeman. Approximately 1×10^7 cells were harvested. Cells were washed again in PBS, centrifuged at $400 \times g$ for 10 min at 4°C, frozen at −80°C, thawed and resuspended rapidly in 0.6 to 1.2 ml 0.01 M tris(hydroxymethyl)aminomethane (Tris) HCl buffer (pH 8) containing 175 μg/ml phenylmethylsulfonyl fluoride and 1 μg/ml leupeptin and aprotinine, and sonicated on ice probe setting two thirds for 10 s each. After centrifugation at 10 000 rpm for 10 min at 4°C, the final NaCl concentration was adjusted at 0.7 M. Prewashed heparin sepharose (HS) beads (Schweigerer *et al.,* 1987b) were added to the extracts and the mixture incubated for 1 h at room temperature while gently rocking. The beads were then washed with 0.01 M Tris pH 7.7, 0.7M NaCl. Proteins were eluted from the HS beads directly in polyacrylamide gel electrophoresis sample buffer containing 0.01 M Tris pH 7.7, 3 M NaCl. HS-purified extracts and prestained molecular weight size markers were run on 10% tricine-sodium dodecyl sulfate-polyacrylamide gels and electrophoretically transferred to nitrocellulose filters.

The filters were incubated for 1 h in a buffer (10 mM Tris HCl pH 8, 150 mM NaCl, 0.2% Tween (TBST), 1% nonfat milk) that contained rabbit antibFGF polyclonal antibody directed against bFGF (Oncogene Science Inc., NY). The filters were washed three times with TBST buffer and probed with ^{125}I-labeled protein A antirabbit IgG (Amersham, Arlington Heights, IL). The filters were then washed three times with TBST and exposed for autoradiography.

Preparation of conditioned medium and assay for mitogenic activity

The conditioned medium was harvested from the different cell lines grown to subconfluence in gelatinized 10 cm^2 tissue culture dishes. Forty eight hours before the test, the culture medium was removed and replaced by DMEM without bFGF and without serum. A medium change was again carried out 18 h before harvesting of the conditioned medium. To test their mitogenic activity, these media were used to grow ABAE cells seeded at a density of 10^4 cells/well. Cells were incubated in fresh medium containing 10% CS and 40% of conditioned medium. On day 4, duplicated dishes were trypsinized and cells number was determined using a Coulter counter.

Soft agar growth

DMEM supplemented with 10% CS and containing 0.8% Bacto agar (Difco, Detroit, MI) was added to each 35-mm dish and allowed to solidify. Cells were trypsinized, counted, and suspended in the same medium containing 0.4% agar/plate in quadruplicate at a density of 2×10^4 cells per dish. For the parental ABAE, the mock cells, and the PINA 5A cell line, exogenous bFGF was added to the cells suspension before the cells were plated. Every 2 d bFGF was added to the medium above the agar. These cultures were incubated at 37° at 10% CO_2 atmosphere for 14 d.

Tumorigenicity

ABAE cells, transfected with PINA 3A, 4A, 5A, and 7A, were grown to confluence. Cells were recovered by scraping, washed twice, and resuspended in 0.5 ml serum-free medium. Cell number was determined using a coulter counter and adjusted to 5×10^6 cells per 0.5 ml. Four to 6 wk female athymic nude mice (Nude/Nude) were injected subcutaneously with 5×10^6 cells. Three to 10 mice were injected per cell line and tumor formation was observed from 1 wk to 1 mo.

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