

# Nuclear Activities of Basic Fibroblast Growth Factor: Potentiation of Low-Serum Growth Mediated by Natural or Chimeric Nuclear Localization Signals

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Human basic fibroblast growth factor (FGF-2) occurs in four isoforms: a low molecular weight (LMW FGF-2, 18 kDa) and three high molecular weight (HMW FGF-2, 22, 22.5, and 24 kDa) forms. LMW FGF-2 is primarily cytoplasmic and functions in an autocrine manner, whereas HMW FGF-2s are nuclear and exert activities through an intracrine, perhaps nuclear, pathway. Selective overexpression of HMW FGF-2 forms in fibroblasts promotes growth in low serum, whereas overexpression of LMW FGF-2 does not. The HMW FGF-2 forms have two functional domains: an amino-terminal extension and a common 18-kDa amino acid sequence. To investigate the role of these regions in the intracrine signaling of HMW FGF-2, we produced stable transfectants of NIH 3T3 fibroblasts overexpressing either individual HMW FGF-2 forms or artificially nuclear-targeted LMW FGF-2. All of these forms of FGF-2 localize to the nucleus/nucleolus and induce growth in low serum. The nuclear forms of FGF-2 trigger a mitogenic stimulus under serum starvation conditions and do not specifically protect the cells from apoptosis. These data indicate the existence of a specific role for nuclear FGF-2 and suggest that LMW FGF-2 represents the biological messenger in both the autocrine/paracrine and intracrine FGF-2 pathways.

## INTRODUCTION

Basic fibroblast growth factor (FGF-2)<sup>1</sup> is a member of a large family of heparin-binding growth factors. Thus far 19 members (Nishimura *et al.*, 1999) have been described. These proteins affect various biological processes ranging from cell proliferation to plasminogen activation, integrin expression, cell migration, embryonic development, and cell differentiation. FGFs also may be involved in tumor angiogenesis and malignant transformation (Burgess and Maciag, 1989; Rifkin and Moscatelli, 1989; Basilico and Moscatelli, 1992; Mason, 1994).

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<sup>1</sup> Abbreviations used: FGF-2, basic fibroblast growth factor; HMW FGF-2, high molecular weight FGF-2; LMW FGF-2, low molecular weight FGF-2.

The biological functions of the FGFs are mediated by their interaction with both high- and low-affinity plasma membrane receptors (Baird, 1994). A family consisting of four high-affinity tyrosine kinase FGF-receptors has been identified (Basilico and Moscatelli, 1992; Jaye *et al.*, 1992). The interaction of FGF-2 with its plasma membrane high-affinity receptors induces autophosphorylation of the receptor and initiates the phosphorylation of tyrosine residues in cytosolic substrates (Fantl *et al.*, 1993). The low-affinity receptors consist of heparan sulfate proteoglycans and are thought to provide a mechanism both to concentrate ligand and to present FGF dimers to tyrosine kinase receptors (Moscatelli; 1989; Baird, 1994)

The prototypic members of the FGF family, FGF-1 and -2, lack a signal sequence for secretion, although the proteins are released and have been visualized in

the ECM of different tissues (Abraham *et al.*, 1986; Jaye *et al.*, 1986). The mechanism of FGF-1 and FGF-2 release has not been elucidated. FGF-1 has been shown to be released in response to heat shock (Shi *et al.*, 1997). Evidence suggests that release of FGF-2 may involve exocytosis and require ATP because both blocking exocytosis (Mignatti *et al.*, 1992) and depleting ATP (Florkiewicz *et al.*, 1995) inhibit FGF-2 release from cells. A recent paper has proposed that the 27-kDa heat shock protein (HSP27) is involved in FGF-2 release (Piotrowicz *et al.*, 1997).

Human FGF-2 is produced naturally in four forms that originate from alternative translation initiation sites within a single mRNA species (Florkiewicz and Sommer, 1989; Prats *et al.*, 1989). The translation of the smallest molecular weight form (18 kDa, LMW) of FGF-2 is initiated at an internal AUG codon, whereas the translation of the three higher molecular weight forms (22, 22.5, 24 kDa, high molecular weight isoforms, HMW) initiates at CUG codons 5' to the AUG codon. As a result, the three HMW forms of FGF-2 contain the complete amino acid sequence of the 18-kDa form in addition to N-terminal extensions of varying lengths. Although the initiation of translation at noncanonical codons is rare (Boeck *et al.*, 1994), FGF-3 mRNA also encodes forms that initiate at both CUG and AUG codons (Acland *et al.*, 1990).

Baldin *et al.* (1990) reported that exogenous FGF-2 is translocated to the nucleus. When LMW FGF-2 is added to synchronized cultures of bovine aortic endothelial cells, a fraction of the growth factor is translocated to the nucleus and nucleolus, where it remains undegraded for up to 6 h. This process of nuclear transport may be controlled by the cell cycle because nuclear uptake is specific for the late G<sub>1</sub> phase of the cell cycle, whereas cytoplasmic uptake occurs throughout the cell cycle (Baldin *et al.*, 1990).

The subcellular distribution of the four forms of FGF-2 has been examined in cells overexpressing FGF-2 as well as in nontransfected endothelial, neuronal, and neuroendocrine cells using both immunocytochemical and subcellular fractionation techniques (Renko *et al.*, 1990; Bugler *et al.*, 1991; Yu *et al.*, 1993; Stachowiak *et al.*, 1994). These studies indicated that endogenous HMW FGF-2s were primarily nuclear, whereas LMW FGF-2 appeared to be primarily cytoplasmic. The difference in distribution of the FGF-2 forms suggested that there must be specific sequences in the N-terminal extension that targeted HMW FGF-2 molecules to the nucleus or retained them once they were in the nucleus.

Targeting of proteins to the nucleus is normally mediated by an NLS (Garcia-Bustos *et al.*, 1991), and the import process across the nuclear pore complex involves the activity of several transport factors (Nigg, 1997). The possibility that the N-terminal extension of HMW FGF-2 contains an NLS has been examined by

generating fusion constructs in which the 5' DNA encoding the N-terminal extension of FGF-2 was fused to reporter gene cDNAs encoding the non-nuclear proteins  $\beta$ -galactosidase and chloramphenicol acetyltransferase (Bugler *et al.*, 1991; Quarto *et al.*, 1991a). The resulting chimeric proteins localized to the nucleus, confirming that the amino-terminal extension of HMW FGF-2 functions as an NLS. Most NLSs share the property of being composed primarily of basic residues. The two NLS prototypes are the SV40 large T antigen (SV40 T Ag) NLS, a single cluster of basic amino acids (PKKKRKV) (Lanford and Butel, 1984), and the nucleoplasmin NLS, a bipartite signal consisting of two clusters of basic amino acids separated by a spacer region of ten amino acids (KRPAATKK-AGQAKKKK) (Robbins *et al.*, 1991). Although the majority of NLSs are members of one of these two classes, some reported NLSs do not conform to either prototype (Makkerh *et al.*, 1996; Nigg, 1997). HMW FGF-2 is an example of a protein whose nuclear targeting is based on a complex mechanism in which posttranslational methylation of arginine residues may play a critical role. Arginine methylation is a posttranslational modification found in certain nuclear proteins, including the nucleolar proteins nucleolin and fibrillarlin (Beyer *et al.*, 1977; Lischwe *et al.*, 1985a,b). The amino-terminal extension of HMW FGF-2 contains among its 55 amino acids eight potential sites for arginine methylation, according to the consensus sequence requirement reported by Najbauer *et al.* (1993), and three of these arginines have been shown to be methylated in guinea pig brain-derived HMW FGF-2 (Burgess *et al.*, 1991). Moreover, the 22- and 22.5-kDa forms of human HMW FGF-2 expressed in NIH 3T3 cells contain five dimethylarginines located in their respective amino-terminal extensions, and the 24-kDa form contains up to eight dimethylarginines (Klein, unpublished data). Pintucci *et al.* (1996) showed that the translocation of newly synthesized HMW FGF-2 into the nucleus is accompanied by posttranslational methylation and that a methyltransferase inhibitor markedly reduces the nuclear accumulation of endogenous HMW FGF-2.

Although various cells produce FGF-2, only a small amount of data concerning the differential expression and regulation of FGF-2 isoforms has been reported; however, it has been demonstrated that the relative amounts of the individual forms of FGF-2 vary among cell types and tissues during development and in adulthood (Giordano *et al.*, 1992; Liu *et al.*, 1993; Dono and Zeller, 1994; Coffin *et al.*, 1995; Riese *et al.*, 1995; Vagner *et al.*, 1996). Moreover, a recent report shows a cytokine-specific induction of HMW FGF-2 in rat astrocytes (Kamiguchi *et al.*, 1996) and a direct correlation between HMW isoform expression and stress conditions such as heat shock and oxidative stress in normal cells (Vagner *et al.*, 1996).

Different FGF-2 isoforms have been overexpressed in both transgenic mice (Davis *et al.*, 1997) and various normal and transformed cell lines (Pasumarthi *et al.*, 1994, 1996; Joy *et al.*, 1997; Dono *et al.*, 1998; Grothe *et al.*, 1998). The resulting phenotypes support the proposition of FGF-2 isoform-specific functions. In NIH 3T3 fibroblasts the overexpression of FGF-2 confers unique cellular phenotypes (Quarto *et al.*, 1991). The selective expression of either 18 kDa or HMW FGF-2 forms induces NIH 3T3 cell transformation as measured by enhanced saturation density and growth in soft agar; however, although expression of the 18-kDa form enhances cell migration, spindle-shaped morphology, FGF receptor down-regulation, and changes in integrin expression, HMW FGF-2 forms do not affect these properties. The HMW forms specifically promote growth in 1% serum (Bikfalvi *et al.*, 1995). These observations led to the suggestion that different molecular forms of FGF-2 act through distinct pathways, in particular that HMW FGF-2 acts through an intracellular, perhaps nuclear, pathway independent of cell-surface FGF receptors, whereas LMW FGF-2 acts through plasma membrane receptors. Recently, a new 34-kDa isoform of FGF-2 whose translation initiation codon is a CUG located upstream to the CUG of the 24-kDa isoform has been described in HeLa cells (Arnaud *et al.*, 1999). The 78 amino acid-long N-terminal region of this FGF-2 isoform contains an arginine-rich (PRRRPRR) nuclear localization sequence similar to the one found in the human immunodeficiency virus type-1 REV protein (Arnaud *et al.*, 1999). The existence of this new isoform reinforces the idea of a specific biological role for the nuclear localization of FGF-2.

To further investigate the role played by FGF-2 in the nucleus, we have extended our analysis of the HMW-elicited, low serum growth to NIH 3T3 fibroblasts stably overexpressing single forms of HMW FGF-2 as well as to cells expressing nuclear-targeted forms of LMW FGF-2. We provide evidence for a dose-dependent effect of all nuclear forms of FGF-2 on low serum growth. Finally, to gain insight into the mechanisms responsible for this phenotype, we analyzed the apoptotic rate and the level of DNA synthesis under low serum conditions for cells expressing both nuclear and cytoplasmic FGF-2. Cells that grew in low serum showed no change in the number of apoptotic cells but did have increased incorporation of bromodeoxyuridine (BrdU).

## MATERIALS AND METHODS

### Reagents

Recombinant human FGF-2 (18 kDa) was a gift from Scios Nova (Mountain View, CA). All other reagents were research grade.

### FGF-2 cDNA Mutations

The cDNA constructs encoding either the three HMW isoforms (365 FGF-2) or the LMW (18 kDa) isoform alone (43 FGF-2) were described previously (Quarto *et al.*, 1991). The constructs encoding the single 24-kDa and 22.5-kDa forms initiated with CUG codons were obtained by reconstituting the *XhoI*-*ApaI* region of the 365 FGF-2 cDNA with synthetic oligonucleotides carrying point mutations in each of the CTG codons (see Figure 1A). The construct encoding the AUG-initiated 24-kDa FGF-2 form was kindly provided by Marco Presta (Brescia, Italy) (Gualandris *et al.*, 1994). For the construct encoding the 22-kDa FGF-2 initiated with AUG, a CTG to ATG mutation was made in a synthetic oligonucleotide, which was substituted for the native sequence as described above. The construct encoding the three internally deleted HMW FGF-2 forms (363 $\Delta$ SL) was made by oligonucleotide-directed mutagenesis. An oligonucleotide carrying a short region 5' to nucleotide 292 and 3' to nucleotide 346 was annealed to the construct encoding the three HMW forms (365 FGF-2) looping out the intermediate region. The mutated HMW FGF-2 proteins carried a deletion spanning amino acids (-6 to -23), with respect to the 18 kDa met as (+)1 (see Figure 1A).

The construct carrying the NLS of SV40 T Ag NLS: (M)AKKKRKK in the amino-terminal region of LMW FGF-2 was kindly provided by Dr. H. Prats (Institut Louis Bugnard, Toulouse, France) (Patry *et al.*, 1994).

The construct encoding the nucleoplasmin NLS-LMW FGF-2 was obtained by PCR of the original human FGF-2 cDNA. The upstream primer carried the sequence 5'-AAGAGGCCTGCGGCTAC-CAAAAAGCAGGCCAGGCCAAAGAAGAAGAAA-3', coding for the nucleoplasmin NLS peptide KRPAATKKAGQAKKKK.

All of the constructs were sequenced to confirm that they contained only the desired mutations.

### Cell Culture

NIH 3T3 cells were transfected by calcium phosphate precipitation as described previously (Quarto *et al.*, 1991b) using the Zip-neo vectors containing each of the cDNAs mentioned above. Clones (15–20) from each transfection experiment were screened for expression by indirect immunofluorescence and Western blot analysis. Clones expressing the CUG-initiated 22.5- and 24-kDa forms of FGF-2 and the SV40 large T NLS-LMW were subcloned by limiting dilution in 96-well plates, and the resulting subclones were screened for FGF-2 expression levels.

NIH 3T3 cells overexpressing the NLS(SV40)- $\beta$ -galactosidase chimera (PXZ66) were kindly provided by Thomas Maciag (South Portland, ME) (Zhan *et al.*, 1992). This cell population was subcloned by limiting dilution. The resulting subclones were screened by X-gal staining of cell monolayers and subsequently by enzymatic assay in cell lysates. The two subclones showing the highest  $\beta$ -galactosidase activity were used for the cell growth assays in low serum.

Cells were grown in DMEM containing 5% FCS, 2% calf serum (CS), plus 300  $\mu$ g/ml Geneticin (Life Technologies, Gaithersburg, MD).

### Antibodies

Polyclonal rabbit antiserum was raised against human recombinant FGF-2 (18 kDa). This antiserum recognizes all FGF-2 forms (Quarto *et al.*, 1991b).

### Indirect Immunofluorescence

Cells grown on 12-mm glass coverslips coated with poly-D-lysine (0.1 mg/ml) (Sigma, St. Louis, MO) in 24-well plates were washed with PBS and fixed with 4% paraformaldehyde for 20 min. After a PBS wash, the cells were incubated for 5 min with PBS containing 0.5% Triton X-100, washed twice with PBS, and blocked for 30 min with 0.5% BSA in PBS. Permeabilized cells were incubated for 45 min in a humid chamber at 37°C with anti-18 kDa FGF-2 rabbit



serum (1:300 dilution). After three PBS washes and one PBS/0.2% BSA wash, the cells were stained for 30 min in a humid chamber at room temperature with anti-rabbit immunoglobulin G conjugated to fluorescein isothiocyanate (1:400 dilution in PBS/0.5% BSA). The immune complexes were visualized using a Zeiss Axiophot microscope (Zeiss, Thornwood, NY).

### Western Blot Analysis

Confluent cultures in 60-mm dishes were washed twice with ice-cold PBS, placed on ice, and scraped in cold PBS. Cell suspensions were put in Eppendorf tubes, SDS was added to each sample to reach a final concentration of 0.5%, and the Eppendorf tubes were incubated at 95°C for 5 min. After sonication (12 s at 4°C), protein content was evaluated by BCA protein assay (Pierce, Rockford IL), and 100 µg of total protein was boiled for 5 min in reducing SDS-PAGE sample buffer and electrophoresed on 12% SDS-polyacrylamide gels.

Proteins were immunoblotted using polyclonal rabbit antiserum against human recombinant FGF-2 (18 kDa). Immunoreactive bands were revealed with <sup>125</sup>I-protein A (ICN, Costa Mesa, CA), and band intensity was quantitated by PhosphorImager scanning analysis.

### β-Galactosidase Activity Assays

Cell monolayers in 60-mm plates were washed with PBS and incubated with fixative solution (0.05% glutaraldehyde in PBS) for 5 min. The monolayers were washed three times with PBS. Incubation with X-gal solution (PBS containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM MgCl<sub>2</sub>, 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) at 37°C was performed for 4 h. Positive cells were recorded by light microscopy and scored as a percentage of the total cell number.

The level of β-galactosidase expression in the NLS (sv40) β-galactosidase-expressing clones was measured in cell lysates by enzymatic assay using *o*-nitrophenyl β-galactopyranoside as a substrate. Cells grown in 60-mm dishes were detached from the plate by scraping in PBS, washed twice in the same buffer, resuspended in 200 µl of 250 mM Tris-buffer, pH 7.8, and lysed by three cycles of freezing in a dry-ice bath and thawing at 37°C. Cell lysate (10 or 30 µl) was incubated in 96-well plates in 1 mM MgCl<sub>2</sub>, 1 mg/ml *o*-nitrophenyl β-galactopyranoside, 66 mM sodium phosphate, pH 7.5, at 37°C for 30 min. Absorbance of the samples at a wavelength of 420 nm was measured in a microplate reader, and the amount of enzyme was calculated using standard amounts of β-galactosidase from *Escherichia coli* (Sigma, St. Louis, MO).

### Cell Proliferation Assay

For growth assays, 800–1200 cells/well were plated in 96-well plates and kept overnight in complete medium before substitution of the media with DMEM containing 1% fetal calf serum (day 0). At different times, cells were fixed and stained in 0.1% crystal violet solution in 200 mM 4-morpholinoethanesulfonic acid, pH 6 (Kueng *et al.*, 1989). To maintain the linearity of the assay, when the outer diameter (OD) of the wells exceeded 1 OD unit, the wells were completely destained and restained with a crystal violet solution at a lower pH (Kueng *et al.*, 1989).

### Apoptosis and BrdU Incorporation Analysis

Cells (40,000) were plated in complete medium onto 12-mm glass coverslips coated with poly-D-lysine (0.1 mg/ml, Sigma) in 24-well plates. After an overnight incubation, the medium was substituted with fresh medium containing 1% FCS. At different times, cells were washed with PBS and fixed for 30 min with a solution of 3.7% paraformaldehyde, 60 mM sucrose in PBS. Cells were washed once with PBS, permeabilized with PBS-Triton X-100 (0.5%) for 5 min, washed twice with PBS, and stained with Hoechst 33342 (1 µg/ml in PBS) for 15 min. All of the steps were performed at room tem-

perature. As a positive control for apoptosis induction, staurosporine (0.25 µM; Sigma) was added to selected wells 3 h before analysis. Coverslips were washed in distilled water by immersion, mounted on microscope slides, and analyzed using a Zeiss Axiophot microscope with a Hoechst filter. Apoptotic cells with fragmented nuclei were counted, and the result was expressed as apoptotic cell/total cell number. For BrdU incorporation analysis, cells were pulsed with BrdU (4 µg/ml) for 2.5 h before the defined time point. Cells were fixed in cold acetone:methanol 1:1 for 10 min at 4°C. To block endogenous peroxidase activity, cells were treated with methanol/0.3% H<sub>2</sub>O<sub>2</sub> for 10 min and washed twice with distilled water. DNA was partially denatured by treatment with 1.5 N HCl for 15 min at 37°C, and cells were washed twice with distilled water, incubated with sodium borate 0.1 M, pH 8.5, at room temperature, washed with PBS four times, blocked with 5% FCS in PBS for 20 min at room temperature, and incubated with α-BrdU-peroxidase (Boehringer Mannheim, Indianapolis, IN) 1:20 in PBS:5% FCS for 1 h at room temperature. After five washes with PBS, coverslips were incubated for 3 min in diaminobenzidine (Sigma). Cells were washed twice with distilled water, and nuclei were stained for 30 s with Mayer's hematoxylin solution (Sigma). Monolayers were finally washed with PBS and mounted on microscope slides before analysis by bright-field contrast microscopy.

### Growth in Soft Agar

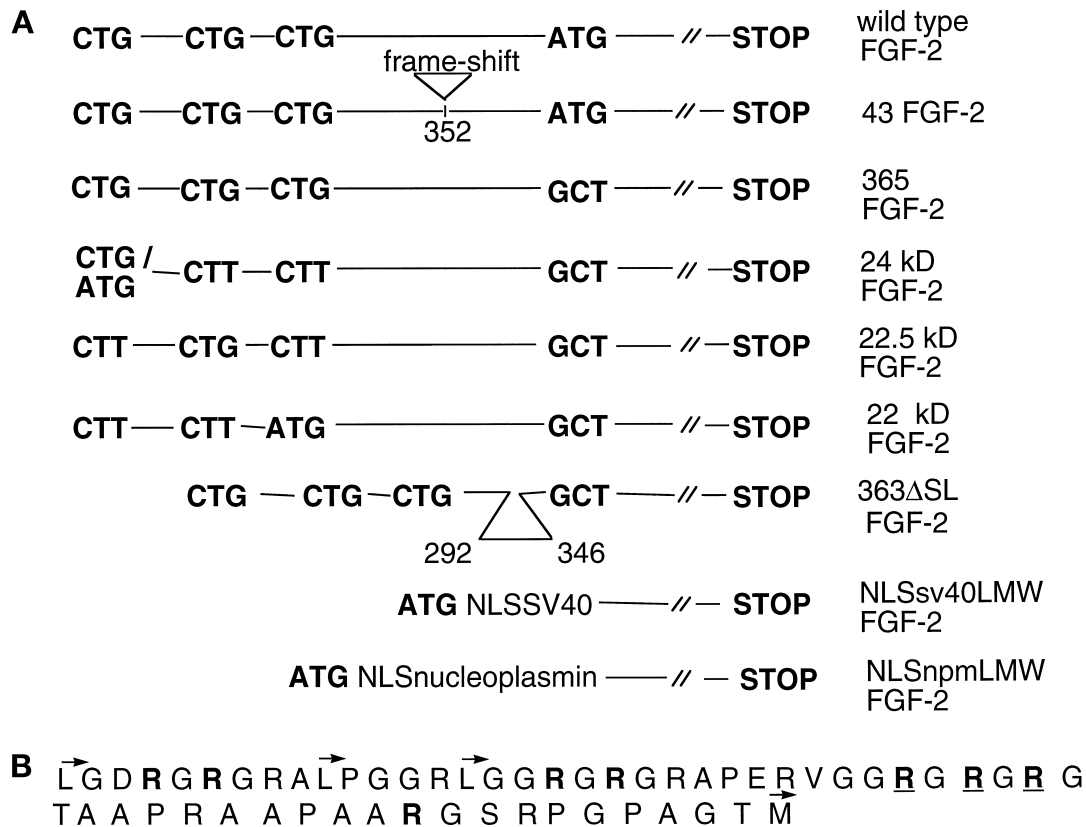
Cells (25,000–50,000 per 60-mm Petri dish) were resuspended in 2 ml of feeding solution (0.3% Difco-Bacto agar in DMEM containing 5% FCS and 2% CS) and plated on a layer (3 ml) of 0.6% agar in DMEM. After 3-h incubation, 3 ml of feeding solution were layered onto the agar. Feeding was performed every 5 d with 3 ml of feeding solution, and colonies were counted after 25 d and expressed as percentage of the total number of cells plated. Three independent experiments were made.

## RESULTS

### Production and Characterization of Clones Overexpressing Different Forms of Human HMW FGF-2

cDNAs were constructed that encoded only the 24- or 22.5-kDa FGF-2 initiated at a CUG codon, which is the natural translation initiation site for the HMW isoforms (Figure 1A). In addition, cDNAs encoding AUG-initiated 24- or 22-kDa FGF-2s were synthesized. Finally, the cDNA encoding all three HMW isoforms was mutated so that a sequence encoding 18 amino acids within the amino-terminal extension was deleted (363ΔSL). These cDNAs were transfected into NIH 3T3 cells, and clones were isolated that expressed the different forms of FGF-2. For controls we used cells overexpressing all of the FGF-2 isoforms (wild-type FGF-2), cells overexpressing the LMW form alone (43 FGF-2), and cells transfected with the empty Zip-Neo vector (Zip) (Quarto *et al.*, 1991b).

As expected from the function of AUG as the preferred translation initiation codon, Western blot analysis showed that most of the clones transfected with cDNAs encoding AUG initiation sites had higher expression levels of FGF-2 isoforms than those observed from cDNAs encoding CUG initiation codons (our unpublished results). The electrophoretic mobility of different isoforms of FGF-2 from a representative set of clones is shown in Figure 2. Indirect immunofluo-



**Figure 1.** (A) Schematic representation of the wild-type and mutated human FGF-2 cDNAs used to stably transfect NIH 3T3 cells. The strategy used to produce these constructs is described in MATERIALS AND METHODS. (B) Amino acid sequence of the human FGF-2 N-terminal extension. Eight potential sites for arginine methylation exist (bold characters) according to the consensus sequence requirements reported by Najbauer *et al.* (1993). The underlined letters indicate the three arginines that were found to be methylated in the guinea pig brain HMW FGF-2. The arrows indicate the start sites for the translation of the 24-, 22.5-, 22-, and 18-kDa FGF-2 forms.

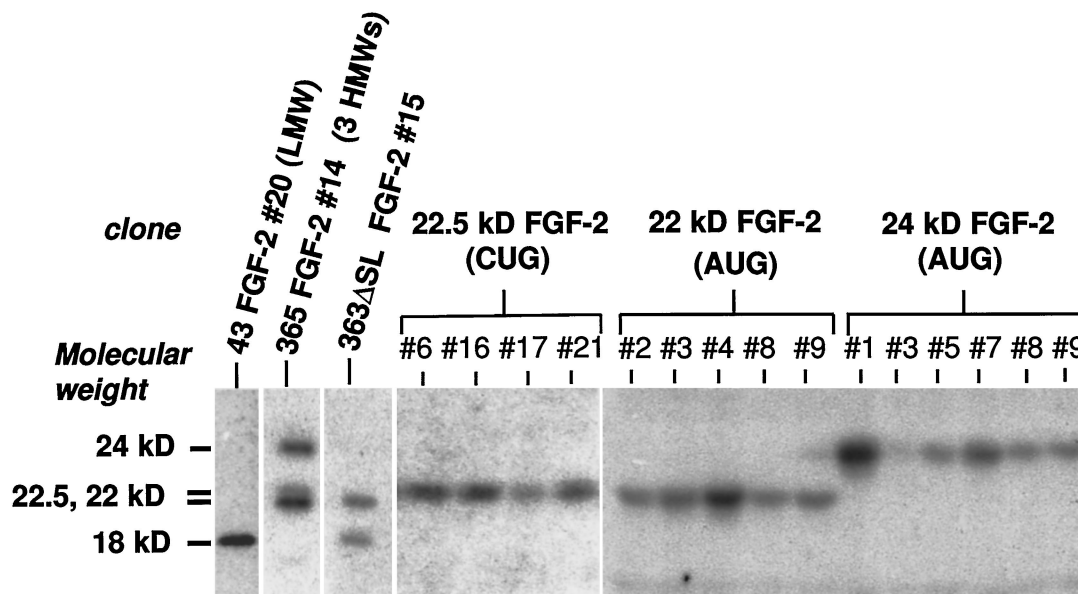
rescence with an antibody against a sequence common to all isoforms of FGF-2 demonstrated that each of the three HMW FGF-2 isoforms and the amino-terminal deletion mutant localized to the nucleus and the nucleolus (Figure 3), as observed in cells expressing the three forms together. Cells expressing LMW FGF-2 displayed only cytoplasmic staining. The nucleolar staining varied both in intensity and homogeneity depending on the clone and the individual experiment. This may relate to the position of the cells in the cell cycle and the overall degree of synchronization of each culture. The differences in efficiency of nucleolar localization between 22-, 22.5-, and 24-kDa FGF-2 forms observed in Figure 3 are probably not significant. The results indicate that even the minimal amino terminal extension is capable of directing the localization of FGF-2 to the nucleus.

#### Growth Properties of Clones Expressing HMW FGF-2 Forms

We next analyzed the growth-related effects of the different FGF-2 isoforms. A cell growth assay based on

crystal violet staining was used to examine cell proliferation in normal and low serum conditions (Kueng *et al.*, 1989). In complete medium all clones grew to saturation densities higher than those observed with cells transfected with the vector alone (our unpublished results), indicating that both HMW and LMW FGF-2 isoforms stimulated cell growth as reported (Quarto *et al.*, 1991b).

Previously, we demonstrated that cells expressing HMW FGF-2 grew in 1% serum, whereas cells expressing LMW FGF-2 did not (Bikfalvi *et al.*, 1995). To test whether single HMW forms of FGF-2 could impart this phenotype, we tested the clones expressing unique FGF-2 forms for growth in 1% serum. These data are summarized in Figure 4. Figure 4A shows growth curves in 1% serum of representative clones expressing single forms of HMW or the amino-terminal deletion mutant compared with the growth of cell expressing only 18-kDa FGF-2. The graph illustrates that all of the clones expressing HMW FGF-2 forms grew, whereas the clones producing the LMW FGF-2 (43 FGF) or transfected with the empty vector (ZIP)



**Figure 2.** Western Blot analysis of clones expressing different isoforms of FGF-2. Total protein extracts were prepared as described in MATERIALS AND METHODS. Total protein (100  $\mu$ g) was loaded and separated on 12.5% SDS-polyacrylamide reducing gels and immunoblotted with a rabbit polyclonal anti-FGF-2 antibody. The blot was developed with  $^{125}$ I-protein A and analyzed by PhosphorImager analysis.

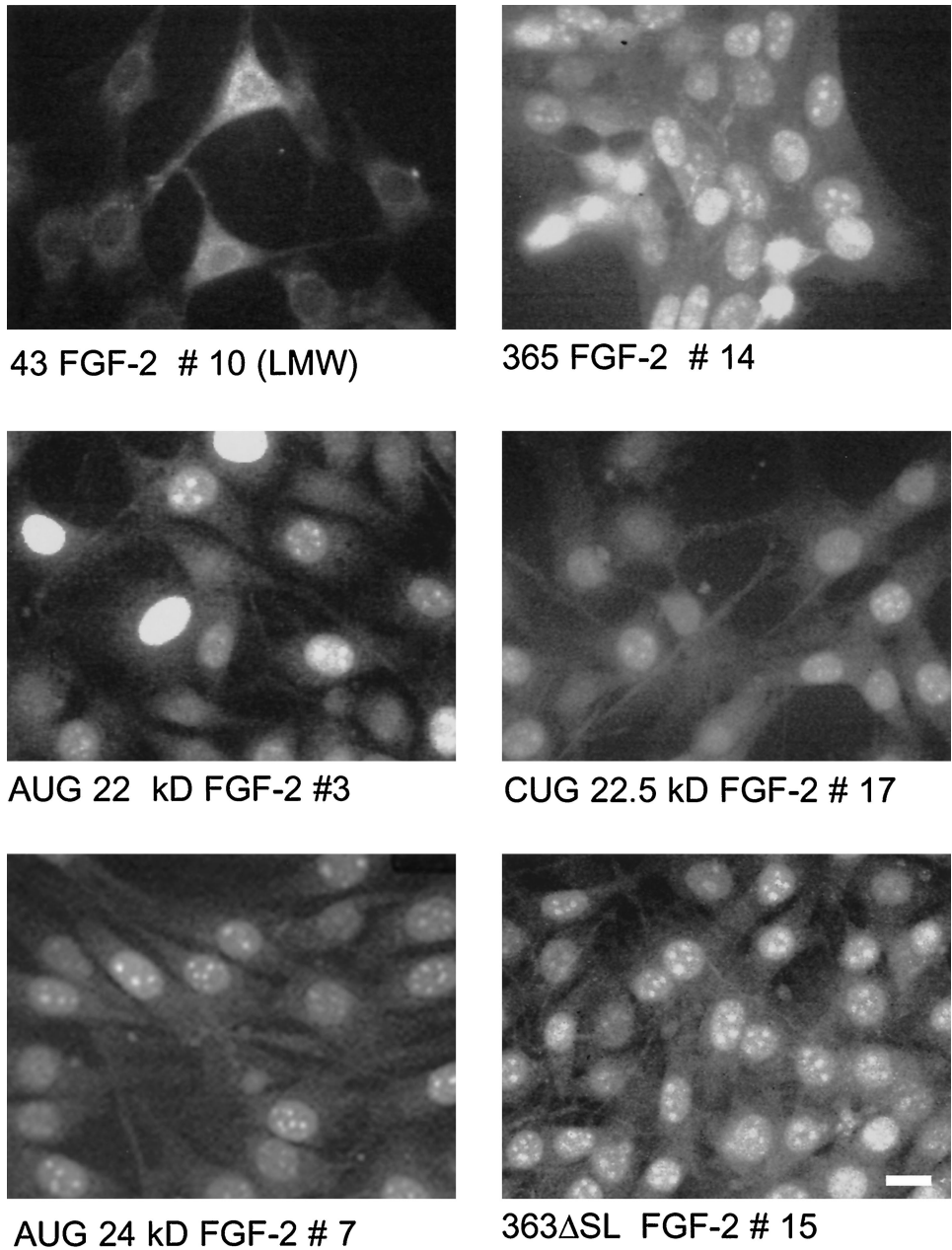
did not. The differences in growth between clones expressing different HMW forms are, in general, not significant and primarily related to differences in the expression levels between clones (see below). Although the growth of only one clone of each type is illustrated, all clones tested for each type demonstrated enhanced growth in low serum except for cells of one clone of the CUG 22.5-kDa FGF-2 group that had a very low expression level (Figure 4B). Clones expressing the wild-type cDNA or all three HMW FGF-2 forms together grew in low serum (our unpublished results) as described previously (Bikfalvi *et al.*, 1995).

In an effort to determine whether growth in low serum is dependent on the level of expression of the various FGF-2 forms, we systematically examined groups of clones for a relation between growth rate in low serum and levels of FGF-2 expression. We compared the time required by the cells to double their number (doubling time) with the amount of FGF-2 produced by the cells, as evaluated by PhosphorImager analysis of the corresponding immunoblot. Figure 4B shows that at low (1–5 ng/mg) levels of expression there appears to be an inverse relation between FGF-2 expression level and doubling time in low serum for clones expressing HMW FGF-2 forms or the deletion mutant. At expression levels >5 ng/mg, there was only a minor shortening of the doubling time, perhaps because the cells were close to their maximum growth rate for the culture conditions. LMW FGF-2-expressing clones do not show such de-

pendence because only small changes of the doubling time in low serum are present over a wide range (up to 85 ng/mg; our unpublished results) of FGF-2 production. These observations suggest that the efficiency of induction of the low serum growth phenotype is invariant among the different forms of FGF-2 that we have tested.

#### *Nuclear-targeted LMW FGF-2 (NLS-LMW FGF-2) Chimeras Mimic the HMW FGF-2 Phenotype in NIH 3T3*

The HMW FGF-2 molecule can be functionally divided into two parts: the amino-terminal extension and the LMW region. The amino-terminal extension has been demonstrated to function as an NLS (Quarto *et al.*, 1991a), whereas the LMW form has been shown to be released by cells and to exert its biological effects through plasma membrane receptors in an autocrine or paracrine manner (Bikfalvi *et al.* 1995). Thus, LMW FGF-2 may represent a ubiquitous messenger whose biological effects change with its subcellular localization, which is modulated by the presence or absence of the amino-terminal extension. To test this hypothesis, LMW FGF-2 was targeted to the nuclear compartment by an externally added signal, and the phenotype of the cells were monitored. Two different NLSs, the SV40 NLS and the nucleoplasmin (npm) bipartite NLS, were attached to the amino-terminal end of LMW FGF-2, stable transfections in NIH 3T3 were



**Figure 3.** Photos illustrating nuclear and nuclear localization of HMW FGF-2 isoforms and cytoplasmic localization of LMW FGF-2. Indirect immunofluorescence was performed as described in MATERIALS AND METHODS using a polyclonal antibody against 18-kDa FGF-2. Bar, 10  $\mu$ m.

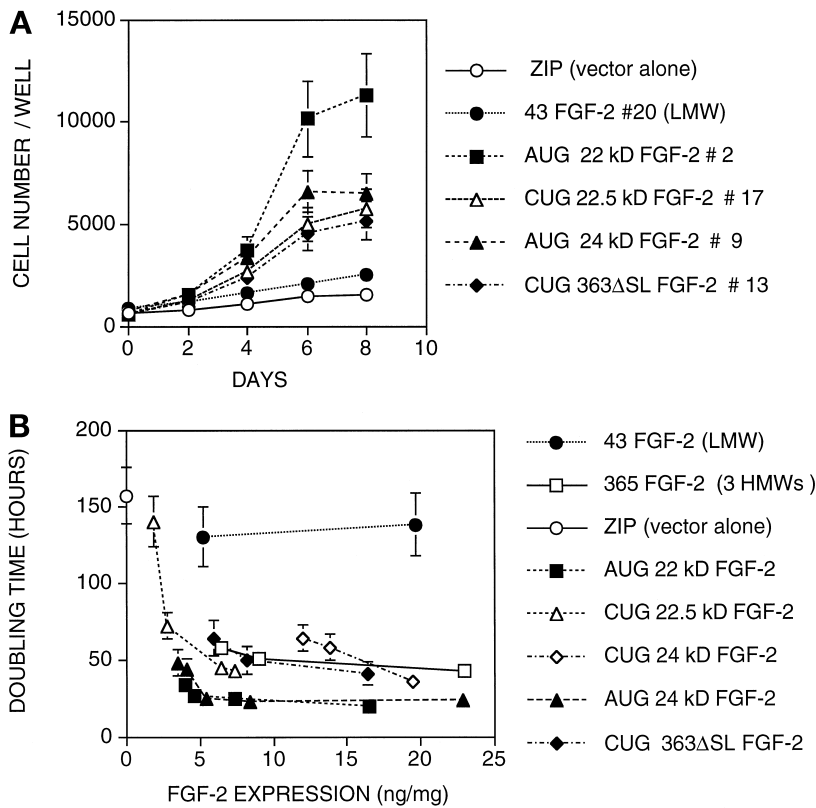
made, and clones expressing the chimeric proteins were isolated and characterized.

Cells from all of these clones grew to higher saturation densities than did cells from the control clones transfected with vector alone, indicating their transformed phenotype (our unpublished results). Figure 5 shows a Western blot analysis performed on total cell extracts obtained from different NLS-LMW FGF-2 chimera clones. PhosphorImager analysis of Western blots (Figure 5) demonstrated that cells in these clones had a low expression level of FGF-2 compared with the clones transfected with the wild-type FGF-2 cDNA

or even with the CUG- or AUG-initiated single isoforms (maximum levels of expression were approximately four times lower in cells expressing the NLS-LMW chimeras than in cells expressing HMW-FGF-2; our unpublished results). By indirect immunofluorescence, cells expressing the chimeric proteins appeared weakly and heterogeneously stained compared with the clones expressing the HMW FGF-2 forms (Figure 6); however, clear nuclear and nucleolar staining was visible.

We next tested whether the expression of the NLS-LMW FGF-2 chimeras conferred serum-independent





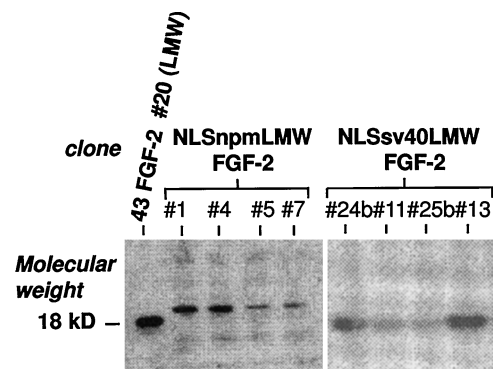
**Figure 4.** Growth of cells expressing HMW FGF-2 forms. (A) Examples of growth curves in 1% serum of cells expressing individual HMW-FGF-2 forms or an amino-terminal deletion mutant. Cells were plated in 96-well plates at 1000 cells/well; after 24 h the medium was replaced with 1% serum (day 0). At the time points indicated, cells were fixed and stained with 0.1% crystal violet solution (Kueng *et al.*, 1989) as described in MATERIALS AND METHODS. Cell number was calculated from a calibration curve (cell number/OD 600 nm) done under the same conditions. (B) Graph showing the relation between FGF-2 expression in NIH 3T3 clones and doubling time in 1% serum. Each point represents the doubling time and FGF-2 expression level of a distinct clone of the type indicated. Doubling times were calculated from growth curves in 1% serum over 6 d.

growth. As shown in Figure 7A, cells from the NLS-LMW FGF-2 clones grew in 1% serum as opposed to cells transfected with vector alone or LMW FGF-2 clones that grew only slightly. As before, we examined the relation between FGF-2 expression and growth rate in all of our clones. The growth rate in 1% serum correlated with the protein expression level in NLS-LMW FGF-2 chimeras (Figure 7B), although as mentioned, the absolute amount of NLS-LMW FGF-2 chimeric protein produced was relatively low in these cells. The expression of nuclear-targeted LMW FGF-2 in the range from 1 to 5 ng/mg mimicked the effects of HMW FGF-2 expression on the doubling time of the cells in 1% serum (Figures 4B and 7B).

To insure that overexpression of a chimeric nuclear-targeted protein did not confer a growth advantage to the cells, we determined whether the overexpression of an NLS that we used for LMW FGF-2 linked to the *E. coli*  $\beta$ -galactosidase protein could modify the cell phenotype and elicit growth in low serum. For this purpose, two clones of NIH 3T3 cells overexpressing an NLS(SV40)- $\beta$ -galactosidase chimera, NLS- $\beta$ gal s69 and s52, were assayed for growth in 1% serum. These clones expressed, respectively 2.8 and 1.1 pmol/mg total protein of  $\beta$ -galactosidase. These data were confirmed by Western blotting analysis using a polyclonal antibody raised against *E. coli*  $\beta$ -galactosidase (our

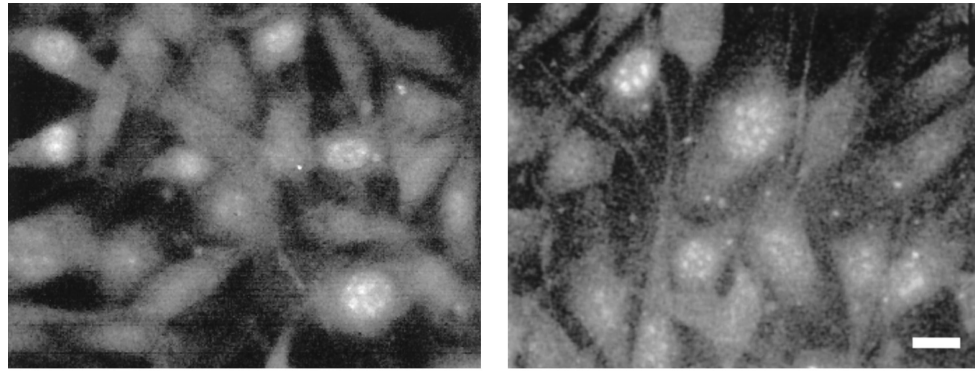
unpublished results). These levels of expression are comparable to or higher than the highest FGF-2-expressing clones (1.38 pmol/mg protein).

As shown in Figure 7B, the doubling times of the NLS- $\beta$ -galactosidase chimera expressing clones in



**Figure 5.** Western blot analysis of clones expressing the two NLS FGF-2 chimeras. Total protein extracts were prepared as described in MATERIALS AND METHODS. Total protein (100  $\mu$ g) was loaded and separated on a 12.5% SDS-polyacrylamide gel and immunoblotted with a rabbit  $^{125}$ I polyclonal anti-FGF-2 antibody. The blot was developed with protein A and analyzed by PhosphorImager. The 16 amino acids constituting the nucleoplasmic NLS account for the decreased electrophoretic mobility of NLSnplMW FGF-2.





**Figure 6.** Photos showing nuclear and nucleolar localization of NLS-LMW FGF-2 forms. Indirect immunofluorescence was performed as described in MATERIALS AND METHODS using a polyclonal antibody against LMW FGF-2. Bar, 10  $\mu$ m.

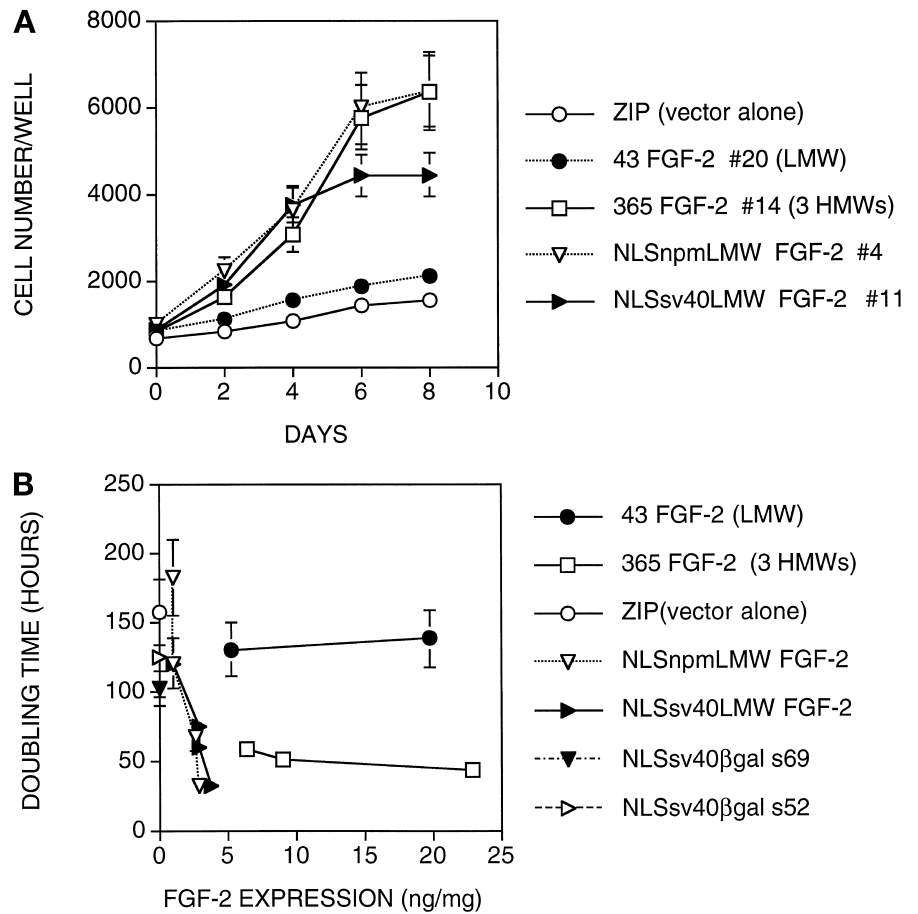
NLSnplMW FGF-2 #1

NLSsv40LMW FGF-2 #13

low serum are similar to those of cells expressing LMW FGF-2 or cells expressing the lowest levels (0.055 pmol/mg) of NLS-LMW FGF-2. Thus, the presence of the NLS by itself does not enhance growth in low serum. These results indicate, therefore, a specific effect of nuclear FGF-2 on low serum growth.

*Induced Mitogenesis and Not Increased Apoptosis Accounts for the Difference in Cell Number in Low Serum*

Our results indicate that nuclear FGF-2 imparts a specific serum-independent growth phenotype to NIH 3T3 fibroblasts as opposed to the cytoplasmic LMW



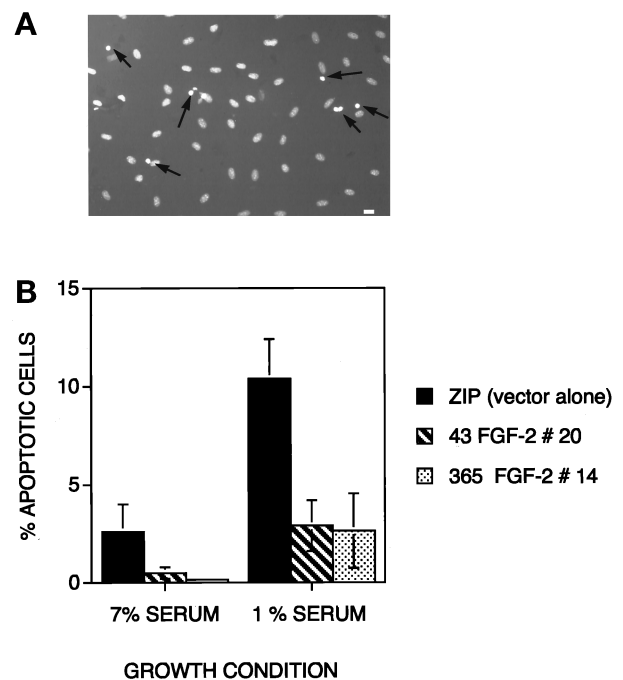
**Figure 7.** Growth properties of NLS-LMW FGF-2 chimeric proteins in 1% serum. (A) Growth curves of two representative clones. (B) Inverse correlation between NLS-LMW FGF-2 expression and doubling time in low serum. The doubling time of two clones expressing a nuclear-targeted  $\beta$ -galactosidase is also presented. Each point represents the doubling time and FGF-2 expression level of a distinct clone of the type indicated. Doubling times were calculated from growth curves in 1% serum over 6 d.

FGF-2 isoform. The difference in cell number that we detect between the clones expressing LMW or HMW FGF-2 forms may be due to an increase in the mitogenic stimulus, a decreased level of apoptosis, or a balance of the two. To gain insight into the mechanism causing low serum growth, we first studied the apoptotic rate in cells from our clones kept in 1% serum for 4 d using Hoechst 33342 staining as a method to distinguish apoptotic from normal cells (Figure 8A). As shown in Figure 8B, no differences were found in the percentage of apoptotic cells in clones expressing either LMW FGF-2 or the three HMW FGF-2 forms. All FGF-2 forms protected the cells from undergoing apoptosis under starvation conditions (as visible by comparing the apoptotic rate in the clone transfected with the vector alone). This observation suggests that increased mitogenesis is responsible for the difference in cell number in low serum.

To measure DNA synthesis, the cells were exposed to BrdU for a short period, and the cells that had incorporated the nucleotide were revealed by staining with a specific antibody to BrdU (Figure 9A). This incorporation assay showed that in low serum DNA synthesis was much higher in the cells expressing three HMW FGF-2 forms than in those expressing LMW-FGF-2 (Figure 9B). The same high level of DNA synthesis in 1% serum was observed in the cells from clones expressing single HMW or NLS-LMW FGF-2 forms (Figure 9C). These observations suggest that nuclear FGF-2 triggers a mitogenic stimulus under starvation conditions rather than preferentially protecting the cells from undergoing a cell death program.

#### *Nuclear and Cytoplasmic FGF-2 Forms Induce a Dose-dependent Enhancement of Colony Formation in Soft Agar*

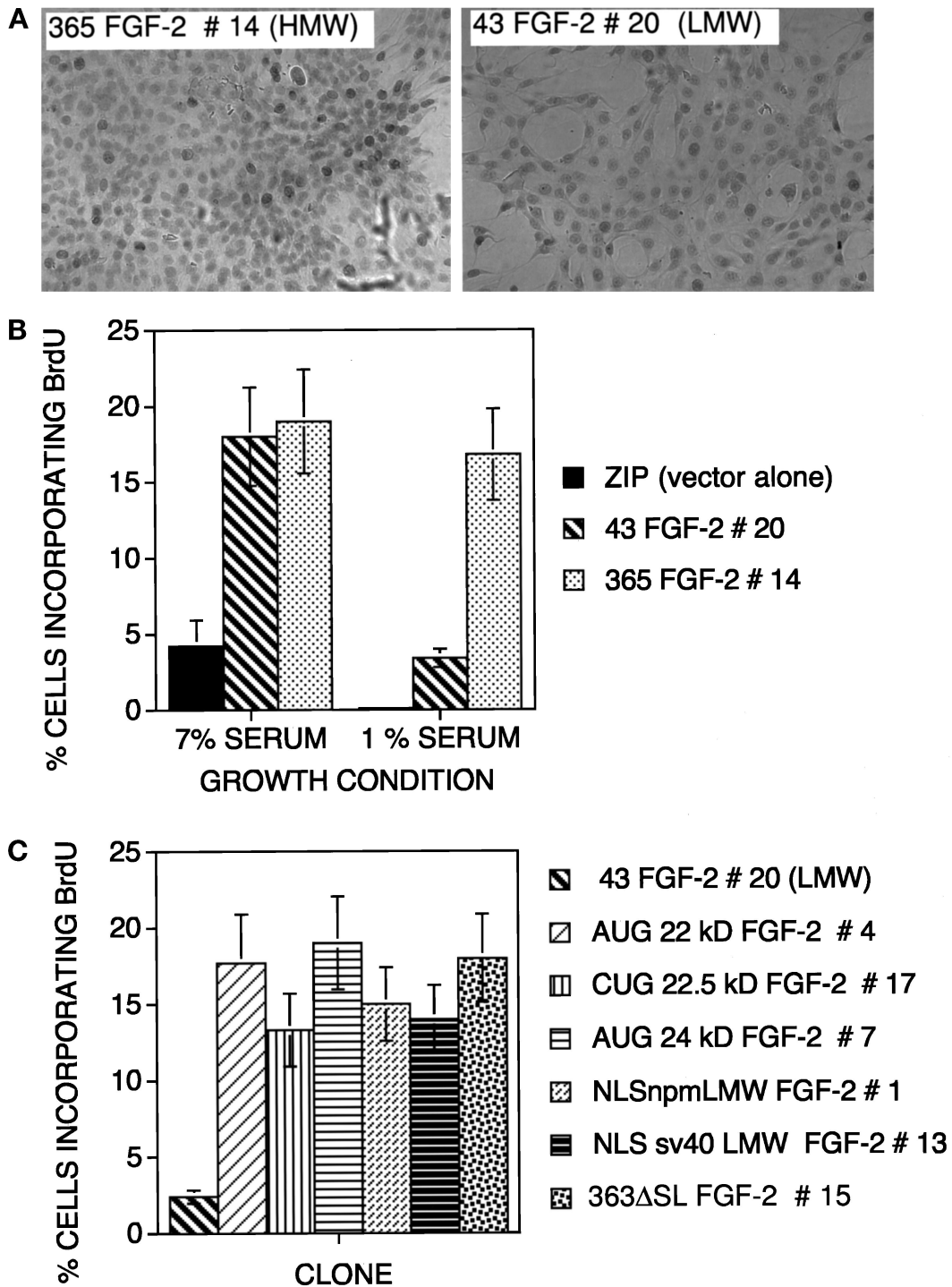
One of the fundamental effects of growth factor overexpression in normal cells is the loss of growth control and the consequent appearance of various degrees of transformation. The specific phenotype of growth in low serum elicited by nuclear-targeted FGF-2 and the enhanced saturation density in complete medium imparted by all isoforms of FGF-2 in our cells (see above and our unpublished results) are indications of cell transformation. A common phenomenon related to cell transformation is reduced anchorage dependence for growth. This has been widely used to evaluate cell transformation and is a very good *in vitro* correlate of tumorigenicity (Shin *et al.* 1975). A previous report from this laboratory (Bikfalvi *et al.*, 1995) related the overexpression of both LMW and the three HMW forms of FGF-2 to the ability of NIH 3T3 cells to grow in an anchorage-independent manner. To complete the analysis of the transformed phenotype of our cell



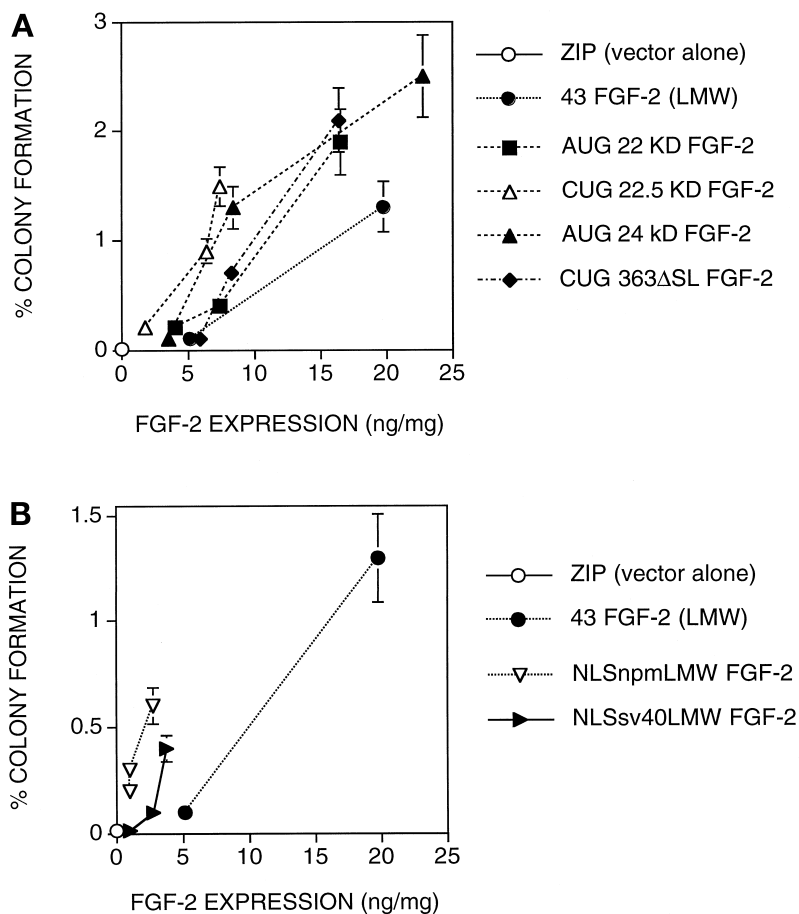
**Figure 8.** Apoptosis in cells from clones expressing LMW or HMW FGF-2 forms. (A) Representative Hoechst 33342 staining of LMW FGF-2-expressing cells maintained 4 d in 1% serum. Apoptotic nuclei are brighter and smaller than normal nuclei as a result of chromatin condensation (indicated by arrows). Original magnification, 200 $\times$ . (B) Histogram presenting percentage apoptotic cells in cultures incubated in complete and serum-depleted medium. Three fields of at least 300 cells each were counted for each sample. Data represent the mean  $\pm$  SD from triplicate samples.

clones, we tested the growth of our various cell transfectants in soft agar. Representative cell clones expressing HMW forms of FGF-2, NLS-FGF-2 chimeras, or LMW FGF-2 were plated in soft agar, and after 25 d the colonies were counted.

The results are shown in Figure 10, A and B, which shows the effects of overexpression of the HMW forms of FGF-2 as well as chimeric NLS-FGF-2 on colony formation in soft agar. A dose-dependent effect is evident for all FGF-2 forms, nuclear and cytoplasmic. The chimeric NLS-FGF-2s, even at the low levels of expression present in our clones, exhibit a significant induction of colony-forming efficiency that rapidly increases with increased protein expression. This pattern is similar to the rapid decrease in the doubling times that these clones exhibit in low serum with increased growth factor expression. These results demonstrate that transfectants overexpressing nuclear-localized FGF-2 exhibit a transformed phenotype *in vitro* and indicate that the cytoplasmic form of FGF-2, although unable to induce serum-independent



**Figure 9.** DNA synthesis in clones expressing cytoplasmic or nuclear FGF-2 forms. (A) BrdU staining of LMW or HMW FGF-2 expressing cells after 4 d in 1% serum. Nuclei that have incorporated BrdU (2.5 h, 4 mg/ml) appear brown among a population of blue nuclei. Original magnification, 200 $\times$ . (B) Histograms presenting the percentage of nuclei incorporating BrdU in the cell populations in complete or serum-depleted media for cells from vector alone, LMW, or HMW FGF-2-expressing clones. (C) Data from a similar experiment performed with cells expressing LMW or different nuclear FGF-2 forms kept in 1% serum for 4 d. Three fields of at least 300 cells each were counted for each sample. Data represent the mean  $\pm$  SD from triplicate samples.



**Figure 10.** Colony formation in soft agar of cells expressing different FGF-2 forms. Cells (25,000 or 50,000) were plated in agar (0.3%), and feeding was performed every 5 d as detailed in MATERIALS AND METHODS. After 25 d colonies were counted and expressed as percentage of the total number of cells plated. (A) Clones overexpressing HMW forms of FGF-2. (B) Clones overexpressing NLS-FGF-2 chimeras. Data represent the mean  $\pm$  SD of representative experiments of three performed.

growth, is capable of interfering with other mechanisms controlling cell growth.

## DISCUSSION

The pathway for growth factor signaling ascribes a central role to membrane-bound receptors and to the downstream signaling cascade whose eventual function is to affect gene expression in the nucleus; however, not all of the effects of cellular messengers may be produced in this manner because many reports have demonstrated a direct association of growth factors (epidermal growth factor, PDGF, FGF, nerve growth factor, Schwannoma-derived growth factor) and hormones, neurotransmitters, or cytokines (growth hormone, insulin, angiotensin II) with the cell nucleus (Burwen and Jones, 1987; Kimura, 1993; Jans, 1994; Mason, 1994; Levine and Prystowsky, 1995; Prochiantz and Theodore, 1995; Wiedlocha *et al.*, 1996; Henderson, 1997; Stachowiack *et al.*, 1997).

When added exogenously to cells, FGF-2 is reported to translocate to the nucleus in a cell cycle-dependent manner (Bouche *et al.*, 1987; Baldin *et al.*, 1990). More-

over, HMW FGF-2 contains an NLS that targets the endogenous HMW FGF-2 isoforms to the nucleus, supporting the existence of a possible alternative signal transduction pathway and/or a potential direct interaction of the growth factor with the genetic machinery of the cell. The occurrence of this NLS in HMW FGF-2 depends on an alternative translation mechanism and appears to be regulated both by cell type and environmental conditions (Giordano *et al.*, 1992; Liu *et al.*, 1993; Dono and Zeller, 1994; Coffin *et al.*, 1995; Vagner *et al.*, 1996). These data suggest that nuclear FGF-2 may ultimately initiate or execute specific genetic programs related to the cell identity or adaptive responses.

A unique phenotype related to HMW FGF-2 is growth in low serum by NIH 3T3 cells (Bikfalvi *et al.*, 1995). In NIH 3T3 cells, the phenotypic effects of HMW FGF-2 seem to be related to the level of expression. Quarto *et al.* (1991) reported that transfection of NIH 3T3 cells by the cDNA for HMW FGF-2 resulted in many clones of cells whose growth was inhibited and a few clones whose growth was rapid. The poorly growing cells usually contained only one copy of in-



tegrated HMW FGF-2 cDNA, whereas the rapidly growing cells contained multiple copies of the FGF-2 cDNA, indicating that the level of expression is of crucial importance for HMW FGF-2 effects on cell proliferation.

Other reports have related cell growth to nuclear localization of FGF-2. Recently Dono *et al.* (1998) presented evidence that the expression of the cytoplasmic form of chicken LMW FGF-2 stimulates proliferation of NIH 3T3 cells under serum starvation conditions, whereas the nuclear-targeted HMW FGF-2 interfered with mitogenic signaling. As the authors pointed out in their discussion, the low levels of expression that they achieved (similar to those of primary nontransformed chicken embryonic fibroblasts) may explain the discrepancies between their observations and the phenotype observed by Bikfalvi *et al.* (1995) and with the results in this paper. Furthermore, Dono *et al.* (1998) used a cytomegalovirus (CMV)-promoter-based vector to prepare their stable transfectants. When we used a similar strategy, we detected no FGF-2 expression by Western blot analysis with the [<sup>125</sup>I]-protein A detection technique that we normally use to analyze Western blots from ZIP vector-based transfectants. Only an enhanced chemiluminescence system was sensitive enough to reveal the FGF-2 production (our unpublished observation).

Having screened a significant number of clones with a wide range of FGF-2 expression levels, we provide additional evidence for a dose-dependent effect for low serum cell growth of all FGF-2 forms that localize to the nucleus. The absolute levels of expression of FGF-2 forms by our clones are well above the "growth-inhibiting" concentrations described previously (Quarto *et al.*, 1991b).

In agreement with our findings, Davis *et al.* (1997) reported that the nuclear-targeted FGF-2 forms were significantly more effective in increasing the rate of DNA synthesis than the released, extracellular form in vascular smooth muscle cells. Moreover, Joy *et al.* (1997) demonstrated that nuclear accumulation of FGF-2 correlated with proliferation of subconfluent normal astrocytes and was constitutively present in nuclei of continuously proliferating glioma cells independent of cell density.

In this article we demonstrate that each of the three forms of HMW FGF-2 expressed individually localizes to the nucleus and induces a serum-independent growth phenotype. The natural occurrence of the three HMW FGF-2 species suggests that each form may have a distinct function. Because the shortest form of HMW FGF-2 localizes to the nucleus, it is possible that the additional amino-terminal sequences present only in the 22.5- and 24-kDa forms are not involved in nuclear transport or retention but have additional activities whose biological effects are not detected by our low serum growth assay. On the other hand, the three

alternative CUG start codons may increase the probability of translation initiation at nonstandard codons.

A deletion mutant (363 $\Delta$ SL) lacking 18 amino acids out of 54 in the proximal (C-terminal) region of the amino-terminal extension of HMW FGF-2 localizes to the nucleus and induces a serum-independent growth phenotype. Thus, these residues must have no role in the nuclear activities of HMW FGF-2. The nuclear accumulation of these forms of FGF-2 is consistent with the putative role of arginine methylation in nuclear localization of FGF-2 because neither the 22.5- or 22-kDa HMW isoforms nor the deletion mutant (Figure 1B) lacks more than two of eight potential sites for arginine methylation (according to the consensus requirements reported by Najbauer *et al.*, 1993.),

LMW FGF-2, when artificially fused to a canonical NLS and targeted to the nucleus, mimics the effects of HMW FGF-2 on low serum growth. This finding supports the concept that nuclear activity of HMW FGF-2 is due to sequences it shares with LMW FGF-2. Further support for this idea comes from the similarities in the growth patterns in low serum of clones expressing similar amounts of FGF-2 that are visible by comparing Figures 4B and 7B. Thus, differences in the biological effects of LMW and HMW FGF-2 must be related to their subcellular localization.

We also found that the nuclear forms of FGF-2 localize to the nucleolus. In the past decade, evidence that growth factors and other regulatory proteins are present in the nucleolus has raised the intriguing possibility of unknown biological functions for this region of the nucleus (Pederson, 1998). In our cell clones, both the nucleoplasmin and the SV40 NLS-LMW FGF-2 chimeras as well as HMW FGF-2 localize to the nucleolus. One of the two sources for NLSs that we used, nucleoplasmin, has been used as a reporter protein to test the nucleolar targeting capacities of heterologous domains (Zirwes *et al.*, 1997). The second protein, SV40 large T antigen, has been shown to localize to the nucleus but not to the nucleolus in rat-1 fibroblasts (Kalderon *et al.*, 1984). On this basis, we assume that the nucleolar localization of NLS-LMW FGF-2 chimeras is driven by the LMW FGF-2 sequence. This is in agreement with the report by Quarto *et al.* (1991) that the amino-terminal extension of the long form of HMW FGF-2 targeted  $\beta$ -galactosidase to the nucleoplasm but not to the nucleolus. Apparently two separate signals, the amino-terminal extension of HMW and a putative nucleolar localization sequence within the LMW sequence, are needed for nuclear topogenesis of FGF-2; however, no identification of nucleolar association domains for FGF-2 have been reported, whereas such data exist for the viral proteins Tat and Rev (Dang and Lee, 1989; Cochrane *et al.*, 1990), the Rex protein of human T-cell leukemia virus (Siomi *et al.*, 1988), *int-2* (FGF-3) (Antoine *et al.*, 1997), and the nucleolar protein NO38 (Zirwes *et al.*, 1997). In all of

these cases, the sequences under question do not comprise an NLS-like element, supporting the general idea of separate molecular requirements for nuclear and nucleolar localization.

Finally, we show that the specific nuclear FGF-2-elicited phenotype of growth in low serum correlates with enhanced DNA synthesis and not with a differential apoptotic rate between LMW and HMW FGF-2 expressing clones. These experiments indicate that the intracrine pathway of action of FGF-2 triggers a proliferative stimulus under serum starvation conditions. We report that overexpression of both nuclear and cytoplasmic forms of FGF-2 in NIH 3T3 cells correlates in a dose-dependent manner with the efficiency of colony formation in an anchorage-free system. Thus, although both the autocrine/paracrine and intracrine pathways of FGF-2 signaling can lead to a transformed phenotype, only the latter can elicit growth in low serum, further indicating the existence of a specific role for nuclear-associated FGF-2.

In conclusion, our experiments indicate that the four forms of human FGF-2 can independently activate a biological response and impart a transformed phenotype in NIH 3T3 fibroblasts through an intracrine pathway. In particular, the nuclear localization of LMW FGF-2, whose amino acid sequence is common to all forms, is sufficient for the modulation of low serum growth, indicating that this sequence may represent the actual biological messenger of FGF-2 in both the intracrine and autocrine/paracrine pathways. The ability to elicit different cellular responses by the same molecule (LMW FGF-2) may result from differential cellular topogenesis of the protein controlled by the CUG-initiated amino-terminal extensions of FGF-2. Furthermore, we speculate that the nucleolar localization of the FGF-2 forms has a direct correlation with FGF-2 biological effects. Future studies must identify with which subnucleolar structure (i.e., dense fibrillar component, granular component, or the fibrillar center) nuclear FGF-2 forms associate. Localizing nuclear FGF-2 to these structures may help elucidate its functional role, because the dense fibrillar component is the major site of pre-RNA processing and pre-ribosome assembly, the granular component contains ribosomal subunits undergoing late assembly reactions and awaiting transport to the cytoplasm, and the fibrillar center is the site of RNA polymerase I transcription (for review, see Scheer and Benavente, 1990).

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