MOLECULAR AND CELLULAR BIOLOGY, Aug. 1995, p. 4364–4374 Vol. 15, No. 8
0270-7306/95/$04.00+0

Nucleolar Association of Fibroblast Growth Factor 3 via Specific Sequence Motifs Has Inhibitory Effects on Cell Growth

PAUL KIEFER AND CLIVE DICKSON*

Imperial Cancer Research Fund, London 3PX, United Kingdom

Received 17 March 1995/Returned for modification 19 April 1995/Accepted 10 May 1995

The dual subcellular fate of fibroblast growth factor 3 (FGF3) is determined by the competing effects of amino-terminal signals for nuclear localization and secretion (P. Kiefer, P. Acland, D. Pappin, G. Peters, and C. Dickson, EMBO J. 13:4126–4136, 1994). Mutation analysis has implicated additional basic domains in the carboxy-terminal region of the protein as necessary for nuclear uptake and the association of FGF3 with the nucleoli. Immunogold electron microscopy shows that FGF3 is predominantly within the dense fibrillar component of the nucleolus. A form of FGF3 that localizes exclusively in the nucleus and nucleolus was generated by removing signals for secretion, and expression of this nonsecreted FGF3 in a mammary epithelial cell line resulted in slowly growing colonies of enlarged cells. Thus, nuclear import and nucleolar association of FGF3 are determined by the concerted interaction of several distinct motifs, and the exclusive production of the nuclear isoform can inhibit DNA synthesis and cell proliferation.

Fibroblast growth factors (FGFs) are structurally related proteins encoded by at least nine distinct genes in mammals (31, 44; for reviews, see references 8 and 17). The prototypic members of this protein family, FGF1 and FGF2, have been ascribed a variety of properties, including the stimulation of cell proliferation and motility, induction or inhibition of cell differentiation, and enhanced neuron survival (8, 17). Paracrine and autocrine signalling by FGFs appears to be mediated through binding to high- and low-affinity cell surface receptors (for reviews, see references 22 and 25). The heparan sulfate glycosaminoglycans attached to proteoglycans function as low-affinity receptors and facilitate signalling by the high-affinity receptors. Four FGF receptor genes (FGFR1 to FGFR4) have been identified, but alternative splicing produces multiple isoforms, some of which show altered ligand binding specificities (reviewed in references 21 and 22). Signal transduction involves receptor autophosphorylation which mediates the activation of second-messenger pathways, leading to changes in gene expression and, ultimately, cell phenotype.

We have been interested in the properties of FGF3 both as an oncoprotein in mouse mammary tumorigenesis and as a signalling molecule during embryogenesis (12, 35, 45, 46). The secreted form of FGF3 binds with high affinity to FGF receptor 2 and triggers a signalling cascade that includes activation of the mitogen-activated protein kinase pathway (29). However, FGF3 biosynthesis is unusual in that translation initiates at two codons, a conventional AUG and an upstream CUG (13). We and others recently showed that transcripts originating from the P2 promoter of the mouse *Fgf-3* gene initiate predominantly at CUG and that the CUG-initiated product has a dual fate, being distributed in both the secretory pathway and the nucleus (1, 23). The dual fate of FGF3 is achieved by the competition between two classical signals within the same polypeptide: a hydrophobic signal peptide for vectorial transport across the endoplasmic reticulum and a bipartite nuclear localization signal (NLS) (23). Once in the nucleus, most of the protein associates with the nucleolus.

* Corresponding author. Mailing address: Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London 3PX, United Kingdom. Phone: 44-171-269-3336. Fax: 44-171-269-3094.

In this paper, we show that sequence motifs near the carboxy terminus of FGF3 contribute to nuclear localization and are necessary for its association with the nucleolus. These sequences are different from the NLS previously identified. We also present evidence that FGF3 localized to the nucleus causes a distinct effect on cell growth: cells become enlarged and grow more slowly, consistent with a suppression of DNA synthesis. These data provide the first indication of a biological function for nuclear FGF3 which is different from that induced by the secreted form and could have important implications for the signalling potential of this protein in mammalian development.

MATERIALS AND METHODS

Cell culture and DNA transfection. COS-1 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (FCS) and passaged weekly at a ratio of 1/10. For transient DNA transfections, 20 μg of purified plasmid DNA was introduced into 5×10^5 COS-1 cells by electroporation (450 V, 250 μF) using a Bio-Rad Gene Pulser. Between 48 and 72 h after transfection, the cells were harvested for immunoblot or immunofluorescence analysis. HC11 mammary epithelial cells were maintained in RPMI 1640 medium supplemented with 8% FCS, 10 ng of epidermal growth factor (EGF) per ml, and 5 μg of insulin per ml as described previously (4).

Immunofluorescence. COS-1 cells grown on glass coverslips were transfected with the appropriate plasmids, and 48 h later the cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min. The cells were permeabilized with 0.2% Triton X-100 in PBS for 4 min and then treated with 3% bovine serum albumin (BSA) in PBS (3% BSA–PBS) to block nonspecific binding of antibodies. The coverslips were then exposed to primary antibodies and fluorescently labelled secondary antibodies diluted in 3% BSA–PBS. After being washed, the stained cells were mounted in 90% glycerol containing *p*-phenylenediamine and viewed with a 100× oil immersion lens on a Zeiss microscope equipped with the appropriate barrier filters for Texas red optics. Confocal images were taken on a Zeiss confocal laser scan microscope. For FGF3 detection, a 1:1,000 dilution of a carboxy-terminal antipeptide serum was used (13).

Immunogold electron microscopy. Transfected COS-1 cells were fixed with 1% monomeric glutaraldehyde in 0.1 M Sorensen's phosphate buffer (pH 7.4) for 1 h at room temperature. After fixation, the cells were treated with 0.5 M NH_4Cl in phosphate buffer for 4 h and then washed in buffer overnight. The cells were dehydrated in a graded series of methanols at progressively lower temperatures and infiltrated with Lowicryl HM20 at −50°C. For immunogold studies, ultrathin sections were mounted on carbon-coated grids and were labelled as follows. After preincubation on a drop of 5% normal goat serum–5% BSA in PBS, the grids were incubated on a drop of C-terminal polyclonal anti-FGF3 serum diluted 1:2 in preincubation medium overnight at 4°C. The sections were washed in PBS and then incubated with immunogold conjugates of goat anti-rabbit immunoglobulin G–10-nm-diameter gold particles for 2 h. After three rinses in PBS followed by one in distilled water, the sections were air dried and then

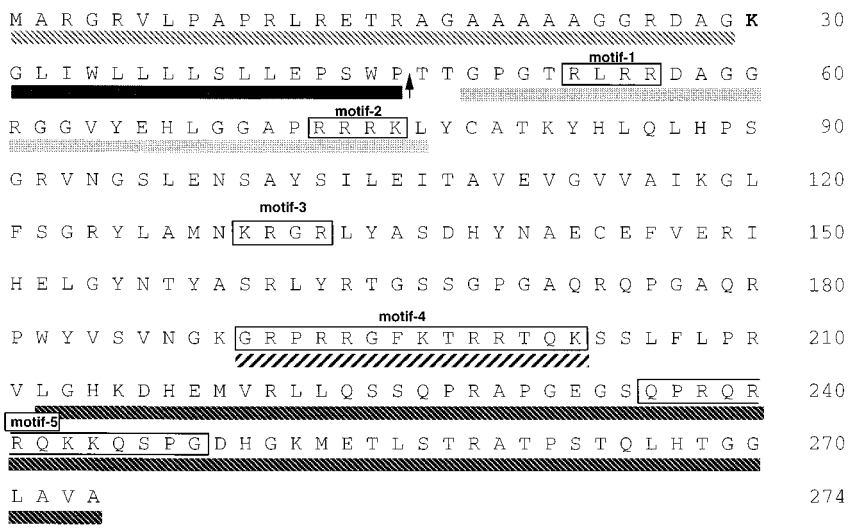


FIG. 1. Schematic of FGF3 showing motifs implicated in protein targeting. The predicted amino acid sequence of mouse FGF3 mutant pKC4.12 optimized for translation from the CUG codon is shown in single-letter code. Alternative initiation at a downstream AUG triplet was prevented by changing AUG to AAG (boldface lysine [K]). The five basic domains in the mouse FGF3 sequence (motifs 1 to 5) are indicated. The bipartite NLS is composed of motifs 1 and 2. The amino-terminal domain (light diagonal stripes) signal peptide (heavy stippling) bipartite NLS (light stippling) and additional domains (shaded bars) are identified in subsequent figures with the same shadings. The signal peptide cleavage site (arrow) is also shown.

contrasted with uranyl acetate and lead citrate and examined with a Zeiss 10C electron microscope. As a staining control, the primary antibody was omitted from the procedure to visualize any nonspecific binding of gold particles on cell organelles. A further control of COS-1 cells transfected with pKC4.24 was also included.

Plasmid constructions. The simian virus 40-based vectors pKC4.12 and pKC4.16, which express full-length mouse FGF3 and a mutant FGF3 lacking the signal peptide, respectively, have been described previously (23) (see Fig. 2 for schematic illustration). Mutant plasmids pKC4.16(1N,2N) and pKC4.16(Δ1,2) were constructed by replacing the sequences of pKC4.16 (between the *Apa*I and *Not*I sites) with the corresponding fragments of the previously described plasmids pKC4.12(1N,2N) and pKC4.12(Δ1,2), respectively (23). The C-terminal deletion clone pKC4.19 was constructed by PCR amplification using mismatched oligonucleotides, creating a stop codon after codon 212 and introducing point mutations in the last 12 amino acids to allow detection of the mutant protein with a C-terminal FGF4 polyclonal antibody (16).

pKC4.20 was produced by changing Lys-130 to Thr by PCR amplification using mismatched oligonucleotides. To replace the Arg-191 and Lys-197 codons with Asn codons to make pKC4.21, two PCR products were amplified by using mismatched oligonucleotides to encompass the desired mutations and to introduce a single *Sac*II site which allowed fusion of the two PCR products. By a similar strategy, two PCR products were derived from *Fgf-3* cDNA, 3′ of codon 237 and 5′ of codon 247, and joined to create a new *Sma*I site resulting in a deletion of codons 238 to 247. This deletion was introduced into plasmids pKC4.22, pKC4.23, and pKC4.24 by replacing the carboxy terminus by using appropriate restriction enzyme fragments of pKC4.20 or pKC4.21. The N-terminal deletion mutant pKC4.18 has been previously described (23). Plasmids pKC4.25 to pKC4.27 were generated by PCR amplification so that codons 102, 137, and 200 were converted to an optimized translation start site, effectively deleting upstream sequences and introducing an amino-terminal methionine. The mutant pKC4.28 was generated by replacing the *Kpn*I-*Eco*RI fragment of pKC4.16 with the corresponding fragment of pKC4.24.

The expression plasmids pHst1 and pHst1.1 have been described previously (16) as kFGFwt and NSP, respectively (kindly provided by F. Fuller-Pace). To produce pHst1.2, a *Sac*I-*Eco*RI fragment of pHst1.1 was replaced by the corresponding region of the previously described plasmid pKC5.2 (24). A retrovirus vector based on Moloney murine leukemia virus was used to construct pDobs4.16 by inserting the FGF3 cDNA from pKC4.16 as a blunted *Hin*dIII fragment into the blunted *Bam*HI site of the pDobs vector (32).

Cell proliferation assay. A cell proliferation kit supplied by Amersham was used. Briefly, cells were incubated for 1 h at 37°C in complete medium containing 5-bromo-2′-deoxyuridine (BrdU). After being labelled, the cells were fixed for 30 min in 90% ethanol–5% acetic acid–5% water at room temperature. To block endogenous peroxidases, the cells were washed for 30 min in 2% hydrogen peroxide in methanol. The incorporated BrdU was detected with a monoclonal antibody and visualized with a second, peroxidase-conjugated anti-mouse immunoglobulin G antibody.

RESULTS

The bipartite NLS is not necessary for nucleolar localization. To investigate the basis for the nucleolar association of FGF3, we have used the standard approach of expressing mutant *Fgf-3* cDNAs in COS-1 cells and monitoring subcellular localization by indirect immunofluorescence as described previously (24). A schematic illustration of FGF3 showing the identified functional domains and basic motifs which might participate in nucleolar association is presented in Fig. 1. Most of the mutant cDNA constructs were based on two derivatives, pKC4.12 and pKC4.16, described in an earlier report (23). In pKC4.12, the upstream CUG initiation codon was changed to AUG and the adjacent sequence was altered to match a consensus for optimal translation initiation (GGCAUGG) (26). In addition, the second initiation codon was changed from AUG to AAG to suppress internal initiation. Expression of this plasmid in COS-1 cells produces full-length FGF3 which is distributed to the nucleus, nucleolus, and secretory pathway (Fig. 2). In contrast, pKC4.16, in which the hydrophobic domain of the signal sequence has been deleted (codons 30 to 46), produces a protein that is exclusively nuclear (Fig. 2). We had previously found that R-to-N mutations in the bipartite NLS of pKC4.12 block nuclear localization and lead to an exclusively secreted protein (23). However, when analogous mutations were introduced into pKC4.16, the protein was still nuclear (Fig. 2). This result implies that in the absence of the hydrophobic signal peptide, it was possible for FGF3 to accumulate in the nucleus and nucleolus even when the bipartite NLS was mutated. Moreover, replacement of the entire bipartite NLS domain with an unrelated linker sequence also allowed nuclear and nucleolar localization of FGF3 protein (construct pKC4.16Δ1.2; Fig. 2). Two important conclusions can be drawn from these experiments: first, the previously defined bipartite NLS is not necessary for nucleolar association, and second, other regions of the protein contribute to the nuclear and nucleolar locations. However, for the wild-type protein, which contains a signal

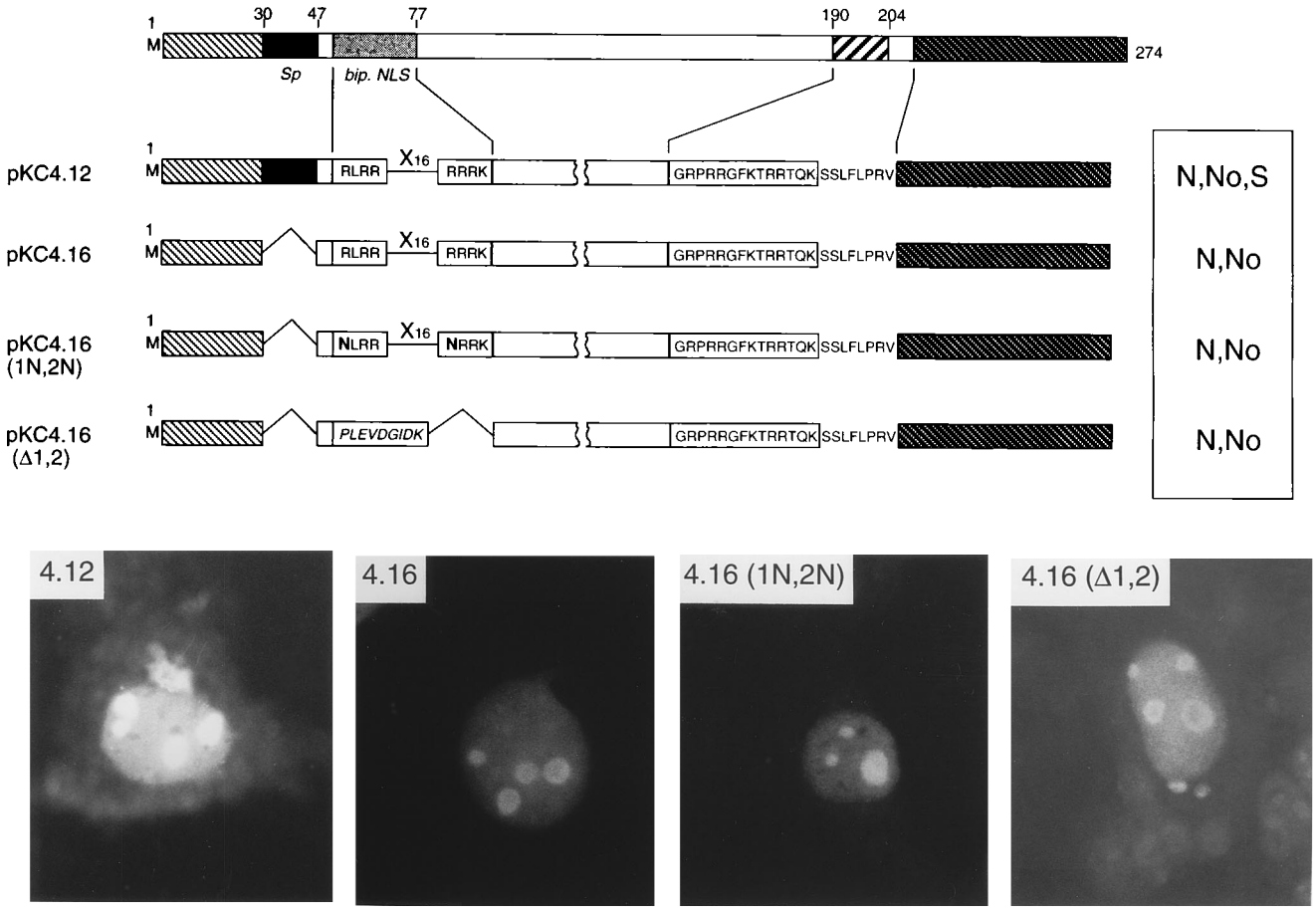
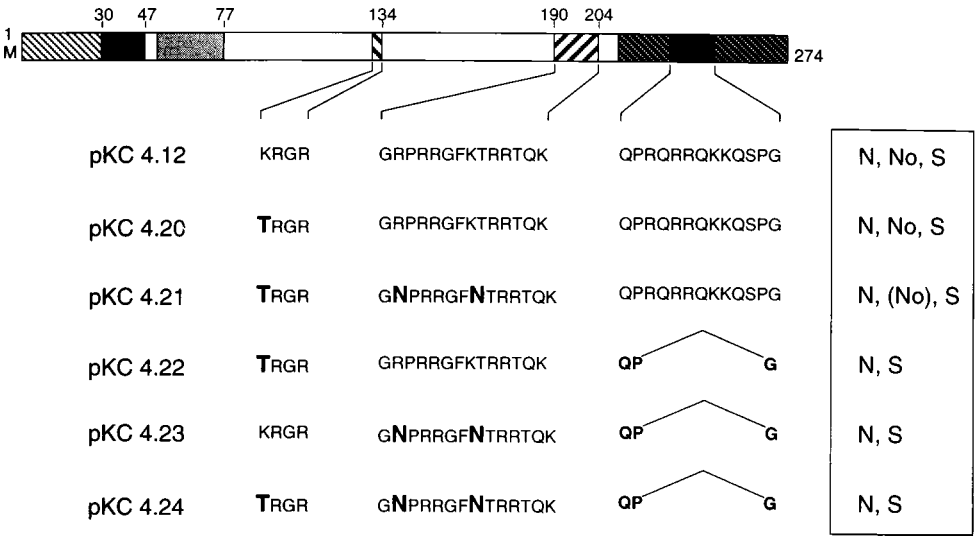


FIG. 2. Influence of the bipartite NLS on nucleolar association. A series of mutant cDNAs with alterations of the bipartite NLS is depicted schematically with the shading convention used in Fig. 1. The subcellular localization of FGF3-related proteins in COS-1 cells transiently transfected with these cDNAs is shown below. The FGF3-related proteins were detected by immunostaining as described in Materials and Methods. Representative examples are shown, and the overall staining patterns are summarized to the right of each depicted cDNA as follows: N, nuclear; No, nucleolar; and S, juxtanuclear and reticular staining indicative of the secretory pathway. Sp, signal peptide; M, position of the translational start site; bip., bipartite. Amino acids in italics are non-FGF3 amino acids.

peptide for entry into the secretory pathway, the bipartite NLS is essential for nuclear uptake (23).

Identification of elements necessary for nucleolar association. In addition to the previously defined bipartite NLS, FGF3 contains several clusters of basic residues depicted as motifs 3, 4, and 5 in Fig. 1. To test the effects of these motifs on nucleolar accumulation, a series of point and deletion mutations were introduced into pKC4.12, which disrupted these putative motifs singly or in combination (Fig. 3). Changing the **K**RGR (motif 3) to **T**RGR (construct pKC4.20) had no detectable effect on the subcellular distribution of FGF3 (compare Fig. 2 and 3). However, further amino acid substitution in motif 4 [G(**R**→**N**)PRRGF(**K**→**N**)TRRTQK] reduced but did not completely abolish the accumulation of the mutant protein in the nucleoli (pKC4.21 in Fig. 3). The absence of nucleolar FGF3 in a significant proportion of cells was verified by confocal imaging (Fig. 3). In contrast, deletion of the sequence RQRRQKKQSP, which shows some similarity to proposed retroviral nucleolar targeting motifs (10, 18, 27, 42), leads to a virtual disappearance of nucleolar staining (pKC4.22). Combining the mutations in all three motifs (pKC4.24) still resulted in staining of the nucleus but with exclusion of the nucleoli (Fig. 3). Interestingly, none of these mutations significantly changed the staining for FGF3 in the secretory pathway, which is clearly seen as strong perinuclear staining of the Golgi complex. These experiments suggest that motif 5 is necessary for nucleolar association and that motif 4 has a contributory effect.

The FGF3 C-terminal domain is necessary but not sufficient for nucleolar accumulation. Having implicated the C terminus of FGF3 as involved with nucleolar association, we were interested in determining the minimal structural domain of protein necessary for this interaction. For this purpose, several amino-terminal deletion mutants were generated as illustrated in Fig. 4, and their subcellular localization was analyzed as described above. Deleting the signal peptide and the sequence upstream of the signal sequence (pKC4.18 in Fig. 4) excluded entry into the secretory pathway but did not significantly affect the nuclear and nucleolar localization of the encoded protein. More-extensive deletion to remove exon 1 (pKC4.25) also did not change the subnuclear localization of the product, although a low level of cytoplasmic staining was also detected. By contrast, deletion of a further 35 amino acid residues (pKC4.26), representing the highly conserved domain of exon 2, prevented the protein from localizing to the nucleoli. With this construct there was still some staining of the nucleus, but cytoplasmic staining was predominant. Expression of the carboxy-terminal domain containing the last 73 amino acids (pKC4.27) resulted in a similar distribution of protein between cytoplasm and nucleus but with exclusion of the nucleoli. These results suggest that the carboxy-terminal domain of FGF3, which contains



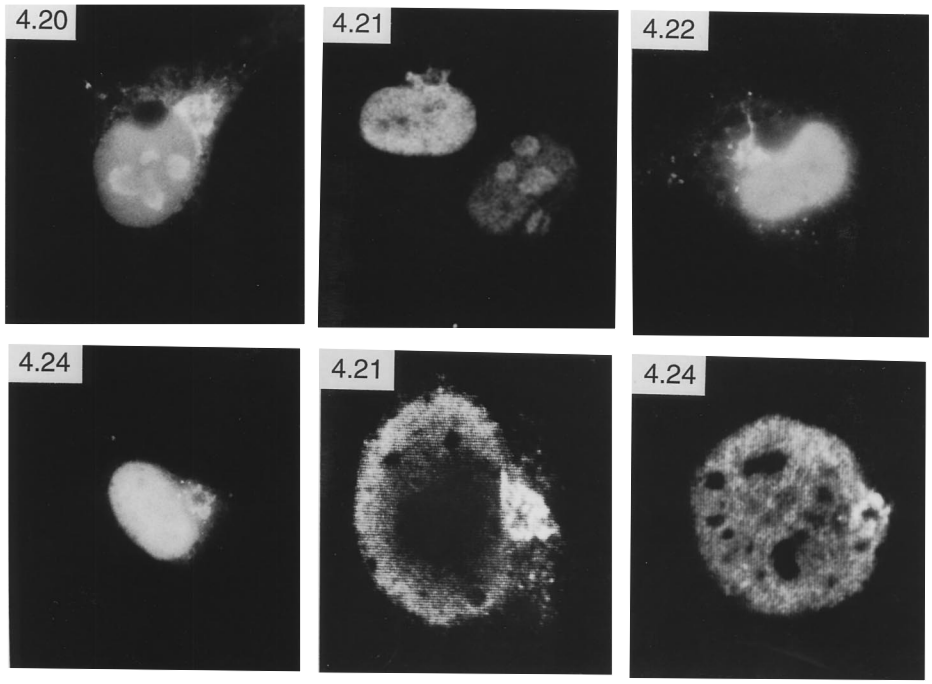


FIG. 3. Mutations affecting nucleolar accumulation of FGF3. Mutations introduced into pKC4.12 are depicted schematically, with the changed nucleotides in boldface. The different plasmids were introduced into COS-1 cells by electroporation, and 48 h later, the subcellular distribution of FGF3-related proteins was analyzed by immunofluorescence. Representative examples of the staining are shown, and the overall staining patterns are summarized to the right of each cDNA as follows: N, nuclear; No, nucleolar in some cells; and S, secretory pathway. Confocal microscopy images are shown for pKC4.21 and pKC4.24 (last two panels of the bottom row). Only the nuclei and the bright perinuclear fluorescence of the Golgi complex (24) are stained. M, position of the translational start site.

basic motifs 4 and 5, was not sufficient for association with the nucleoli.

Sequence context affects the function of the C-terminal motifs. As an alternative strategy to test whether different regions of the FGF3 protein could confer nucleolar association, we linked various combinations of the different motifs to a marker protein, β-galactosidase. Surprisingly, although motifs 4 and 5 together were able to confer a nuclear localization upon β-galactosidase, none of the combinations, including one with all five motifs, was able to direct β-galactosidase to the nucleoli (data not shown). This suggests that the context of these motifs may be important for their ability to target protein to the nucleolus.

To address this idea, we questioned whether the FGF3 motifs would alter the subcellular location of another FGF which does not normally show a nuclear distribution. Two classes of mutants were tested. In the first, the carboxy-terminal sequences of FGF3 were joined to a cDNA clone of FGF4, and in the second, the carboxy-terminal sequences containing motif 5 were deleted from pKC4.12 and replaced by those of FGF4 (Fig. 5). In both cases, the hybrid protein was detected by using an antiserum against the C-terminal peptide of FGF4 (16).

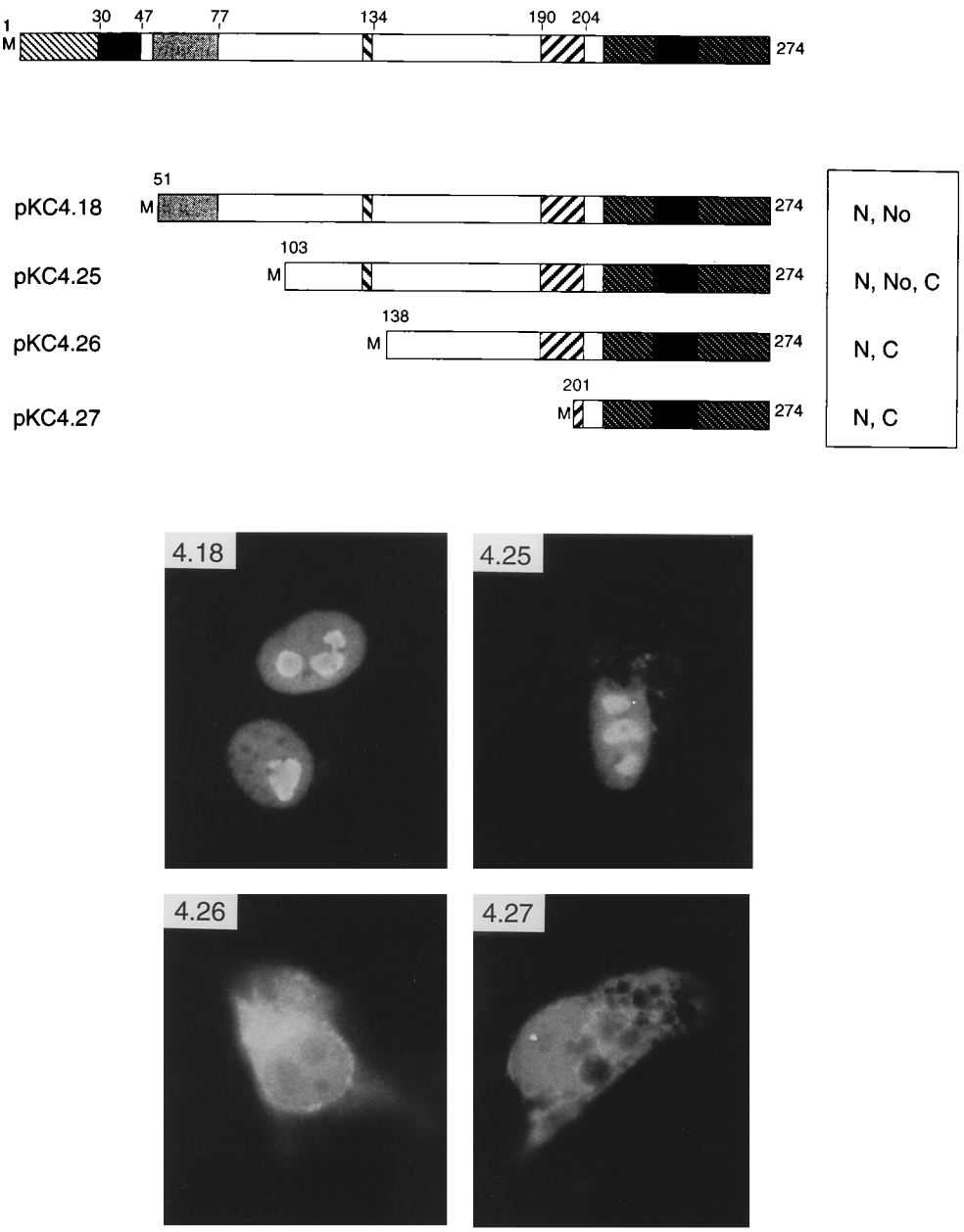


FIG. 4. Carboxy-terminal motifs are not sufficient to target FGF3 to the nucleolus. Deletion mutants derived from pKC4.12 are depicted schematically with domains shaded as in Fig. 1. The positions of the new translational start sites (M) are indicated. The different plasmids were introduced into COS-1 cells, and the subcellular distribution of FGF3-related products was monitored by immunofluorescence. Representative examples of the staining are shown, and the overall patterns are summarized to the right of each cDNA as follows: N, nuclear; No, nucleolar; and C, cytoplasmic.

Since FGF4 is normally an exclusively secreted protein, the signal peptide was deleted to generate plasmid pHst1.1 (Fig. 5). After transfection into COS-1 cells, the resultant protein was retained in the cytoplasm, as expected (16). When the carboxy terminus of FGF3 was appended to pHst1.1 to generate pHst1.2, the resultant hybrid protein was transferred to the nucleus and accumulated in the nucleoli (Fig. 5). Conversely, the product of pKC4.19, in which the FGF4 C terminus is substituted for that of FGF3, was not detected in the nucleoli, although there was still strong staining of the nucleus and the secretory pathway. These findings strongly support the idea that motif 5 is necessary for nucleolar accumulation but functions only in a restricted structural context.

FGF3 is associated with DFCs of nucleoli. In the electron microscope, the nucleolus appears as a membrane-free organelle containing one or more fibrillar centers bounded by a dense fibrillar component (DFC) and surrounded by the granular components that dominate the overall structure (for a review, see reference 41). To determine whether FGF3 distributes to a subcompartment of the nucleolus, COS-1 cells were transfected with pKC4.12 and processed for immunogold staining and electron microscopy as described above. A survey of approximately 200 nuclei showed gold labelling associated specifically with the DFC substructure (Fig. 6).

Nuclear FGF3 suppresses cell growth. To assess the biological properties of nuclear FGF3, the pKC4.16 cDNA was trans-

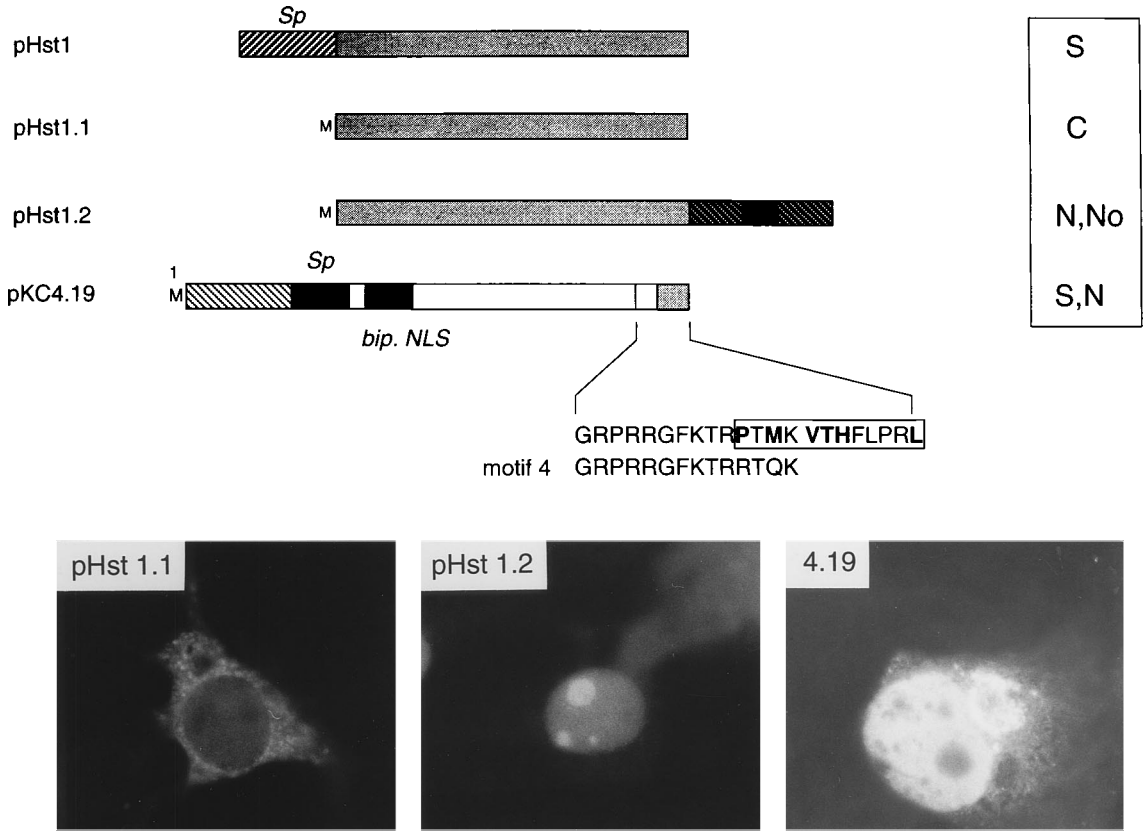


FIG. 5. Effect of the FGF3 carboxy-terminal domain (motif 5) on subcellular localization. Conversion of the carboxy-terminal domain of FGF3 to that of FGF4 (pKC4.19), which effectively deletes motif 5, results in the loss of nucleolar staining. FGF4 expressed without a signal peptide (pHST1.1) remains as a cytoplasmic protein, whereas fusion of the C-terminal region of FGF3 to FGF4 (pHST1.2) confers a nucleolar staining pattern. Representative staining patterns are shown. S, secretory pathway; C, cytoplasmic; N, nuclear; No, nucleolar; bip., bipartite; Sp, signal peptide; M, position of the translational start site.

ferred to a Moloney murine leukemia virus-based vector (pDOBs [32]) and transfected into mammary epithelial (HC11) cells (4). Transfected cells were selected by resistance to G418, and several colonies were chosen and passaged in complete medium supplemented with FCS, EGF, and insulin. Colonies of HC11 cells transfected with the empty-vector DNA were used as controls. At the outset, it was clear that pDobs4.16-transfected colonies grew at a much lower rate than the control or parental cells. In Fig. 7a, the growth rates of six independent pDobs4.16-transfected colonies are compared with those of two vector-only controls and the parental cell line. Cells were seeded at low density, and cell numbers after 3 and 5 days of growth in complete medium were measured. An even more pronounced effect was obtained when rates of growth at low concentrations of serum were compared. Cells were seeded at low density in medium supplemented with 2.5% serum, EGF, and insulin. At 10 days, the cells containing the empty vector were nearly confluent, while the cells expressing the nuclear form of FGF3 had grown into only small colonies, although there was no significant difference in the number of colonies (Fig. 7b). The morphologies of a control line containing the empty vector (HCDobs-2) and a transfected line (HC4.16-16) expressing nuclear FGF3 growing under reduced-serum conditions were compared, as shown in Fig. 8a. The cells expressing a nucleolus-associated form of FGF3 appear larger and grow to a lower density than the empty-vector-transfected control cells. The expression of FGF3 was confirmed for each of the cell clones by Northern (RNA) blotting and Western blotting (immunoblotting) (data not shown). The Western blots showed that all the clones were expressing FGF3 but at a relatively low level. Interestingly, in the original selection, there were many very small, slowly growing colonies that were not selected for practical reasons and which might have resulted from higher FGF3 expression levels causing an even more potent growth-inhibitory effect.

To assess cell proliferation, subconfluent cell lines were pulse-labelled with BrdU and incorporation was detected by using a specific monoclonal antibody. The results show that more than 50% of the control cells were positive for BrdU incorporation, compared with less than 10% of $HC_{4.16}$-16 cells (Fig. 8b). Therefore, it appears that the nuclear isoform of FGF3 inhibits the entry into S phase of the cell division cycle.

DISCUSSION

In a previous report, (23), we and others demonstrated that mouse FGF3, initiated at a CUG codon, contains two classical signals at the amino terminus for protein distribution, a hydrophobic signal sequence for secretion and a juxtaposed bipartite NLS. In the presence of the signal peptide, the NLS is essential for nuclear localization. However, the data presented here (Fig. 2 and 3) indicate that FGF3 must contain at least one additional NLS element, since deletion of the entire amino-terminal region does not prevent nuclear uptake. This may be an advantageous arrangement of signals, since the amino-terminal bipartite NLS is necessarily weak, to allow a proportion

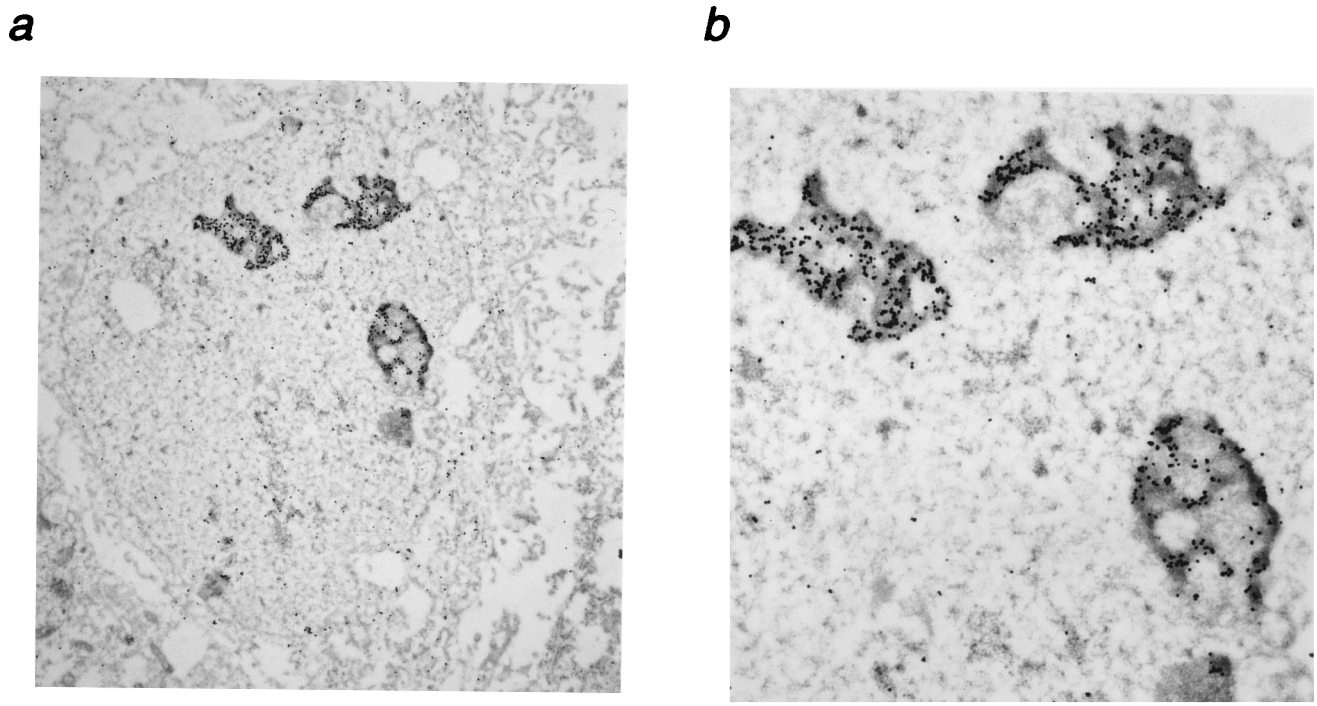


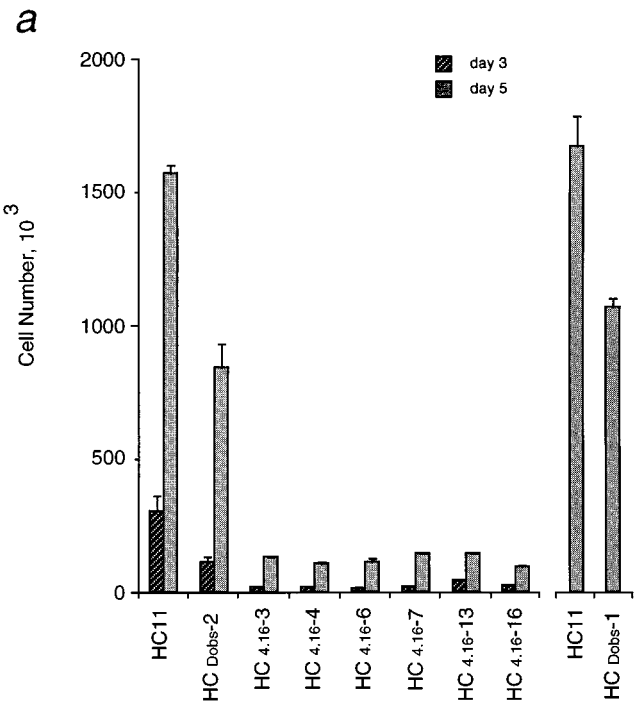
FIG. 6. FGF3 is associated with the DFC of the nucleolus. Following transfection into COS-1 cells, the monolayers were fixed and processed for immunogold electron microscopy as described in the text. Cells transfected with pKC4.12 showed a marked concentration of gold label over the DFCs of the nucleoli. (a) Magnification, ca. ×15,000. (b) Photographic enlargement of the relevant area.

of FGF3 to be sequestered by the secretory pathway (23). We therefore suggest that once a choice to enter the nuclear route has been made, the presence of a second NLS facilitates efficient uptake. In addition, the deletion analyses show that nuclear uptake and nucleolar association are separable processes. The accumulation of mouse FGF3 in the nucleolus is dependent on sequences located in the carboxy terminus of the protein, in particular motif 5. However, motif 4 was also shown to aid this association (Fig. 3). In addition, motif 4 has been shown to act in a concerted manner with the bipartite NLS to transport pyruvate kinase to the nucleus as well as to overcome the effect of the signal peptide on β-galactosidase to establish dual locations, as occurs with native FGF3 (reference 23 and unpublished data). These observations strongly indicate that motif 4 is the second NLS in FGF3.

Motif 5 shows some resemblance to the nucleolar targeting sequences described for the retroviral proteins Rex of human T-cell leukemia virus and Tat and Rev of human immunodeficiency virus (10, 18, 27, 42). Unlike the FGF3 motif, the nucleolar targeting signal of Rex when fused to a heterologous protein directs it to the nucleolus. However, the mode of nuclear uptake and nucleolar accumulation is not clear. Rex has been shown to bind the nucleolar protein B23, which undergoes nucleocytoplasmic shuttling, suggesting that the proposed nucleolar targeting signal may be a binding domain for B23, which transports Rex to the nucleolus (2). In fact, a variety of binding domains has been implicated in the accumulation of cellular proteins with the nucleolus. As this organelle does not appear to possess a membrane barrier, an affinity for a nucleolar component should be sufficient for targeting. For example, the accumulation of nucleolin relies on both RNA-binding domains and a carboxy-terminal glycine-arginine-rich region (11, 19, 30, 40). Similarly, the nucleolar transcription factor upstream binding factor (UBF) has an NLS but requires a large region including the DNA-binding domain and the acidic C-terminal region for its accumulation (28). In the case of FGF3, deletion of motif 5 was sufficient to abolish nucleolar accumulation, but deletion of the amino-terminal region had the same effect. These findings suggest that maintenance of the protein structure as well as the integrity of motif 5 is important for binding to a nucleolar component. This idea is consistent with the inability to confer nucleolar binding to β-galactosidase by fusing it to different regions of FGF3, while addition of motif 5 to the structurally related FGF4 successfully conferred this property (Fig. 5).

Immunogold electron microscopy (Fig. 6) locates FGF3 within the DFC region of the nucleoli, which is regarded by many as the major site of transcription of genes coding for rRNA (for a review, see reference 41). This implies that FGF3 could potentially influence the transcription, splicing, or assembly of ribosomal components. However, there may be other functions of the nucleolus which are yet to be discovered. For example, there is some evidence to implicate this organelle in the posttranscriptional processing and cytoplasmic transport of c-*myc* transcripts, providing a tenuous link with cell growth and division (5). The nucleolar targeting of retroviral proteins such as Rex appears to be necessary for the nuclear export of unspliced or singly spliced viral mRNAs. Thus, it is possible that nuclear FGF3 may affect the splicing of genes such as the FGF receptor genes which are expressed in a variety of spliced isoforms. At present, these possibilities are difficult to reconcile with the observed effect on DNA synthesis.

The nuclear and nucleolar localization of FGFs is not specific to FGF3. FGF2 has been shown to localize to the nucleus by two apparently independent processes. One route follows the exposure of cells to the free ligand. In this process, nucleolar location occurs preferentially during the G_0-to-G_1 transition and is correlated with increased rRNA synthesis (3, 6).



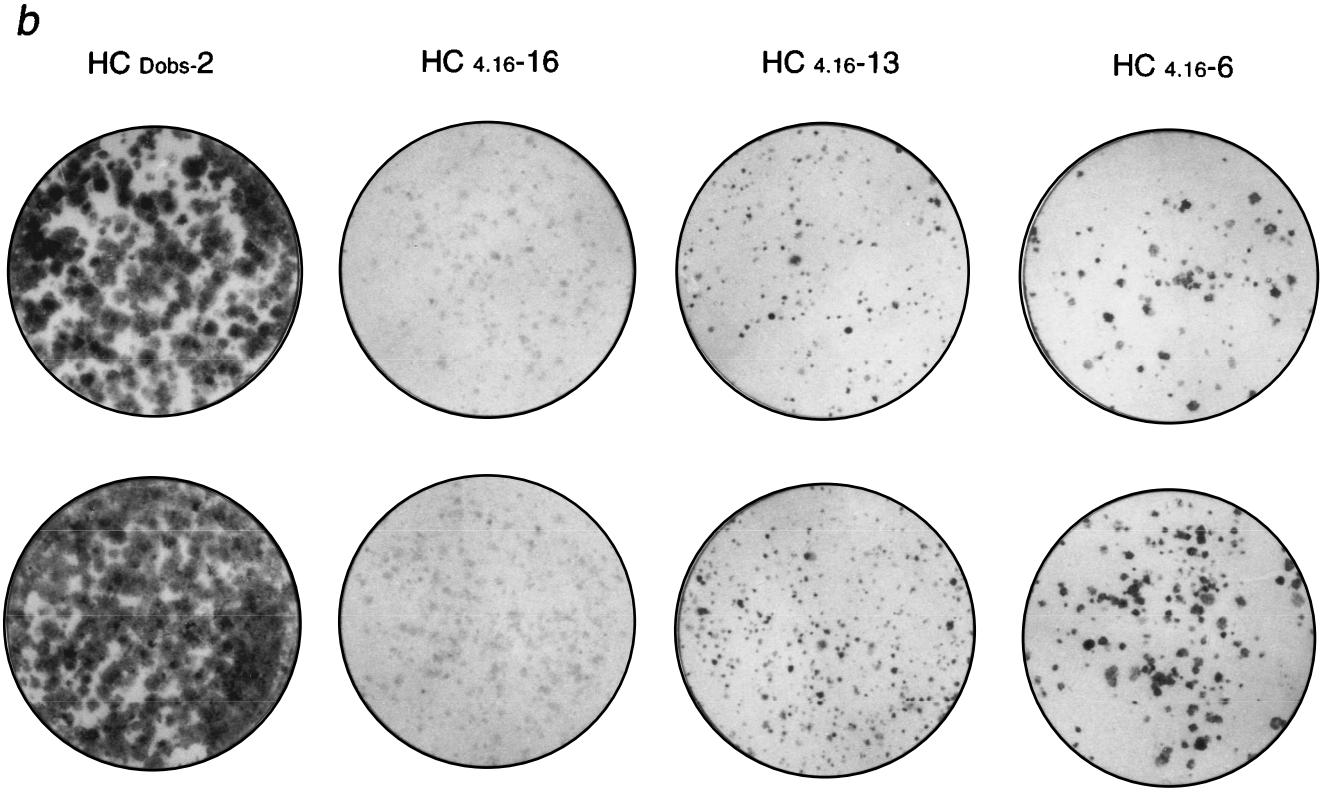


FIG. 7. Growth inhibition by nuclear FGF3. (a) G418-resistant HC11 clones transfected with pDobs4.16 or the pDobs vector were seeded at 10^4 cells per 25-mm-diameter dish and grown for 5 days in Dulbecco's modified Eagle's medium containing 10% FCS, EGF, and insulin. Cells were harvested and counted at days 3 and 5. Counts are plotted as the means of duplicate determinations, with bars indicating standard errors. (b) G418-resistant cell clones were seeded at 2.5×10^3 (upper row) and 5×10^3 (lower row) cells per 25-mm-diameter dish and grown in Dulbecco's modified Eagle's medium containing 2.5% FCS, EGF, and insulin for 14 days before being stained with Giemsa stain.

The second route involves intracellular distribution of amino-terminally extended isoforms initiated at CUG codons (7, 14, 36, 39). However, in contrast to the situation for FGF3, the translocation of FGF2 from cytoplasm to nucleus appears to require glycine-arginine-rich elements encoded in the amino-terminal extensions of FGF2 and does not involve a classical NLS (37). FGF2 also differs from FGF3 in that it is not constitutively secreted but is released from the cell cytoplasm by an unknown process. A nuclear location has also been reported for FGF1 but, as for FGF2, may involve more than one mechanism (9, 15, 20). An NLS has been reported for FGF1, but translocation to the nucleus appears to be inhibited under

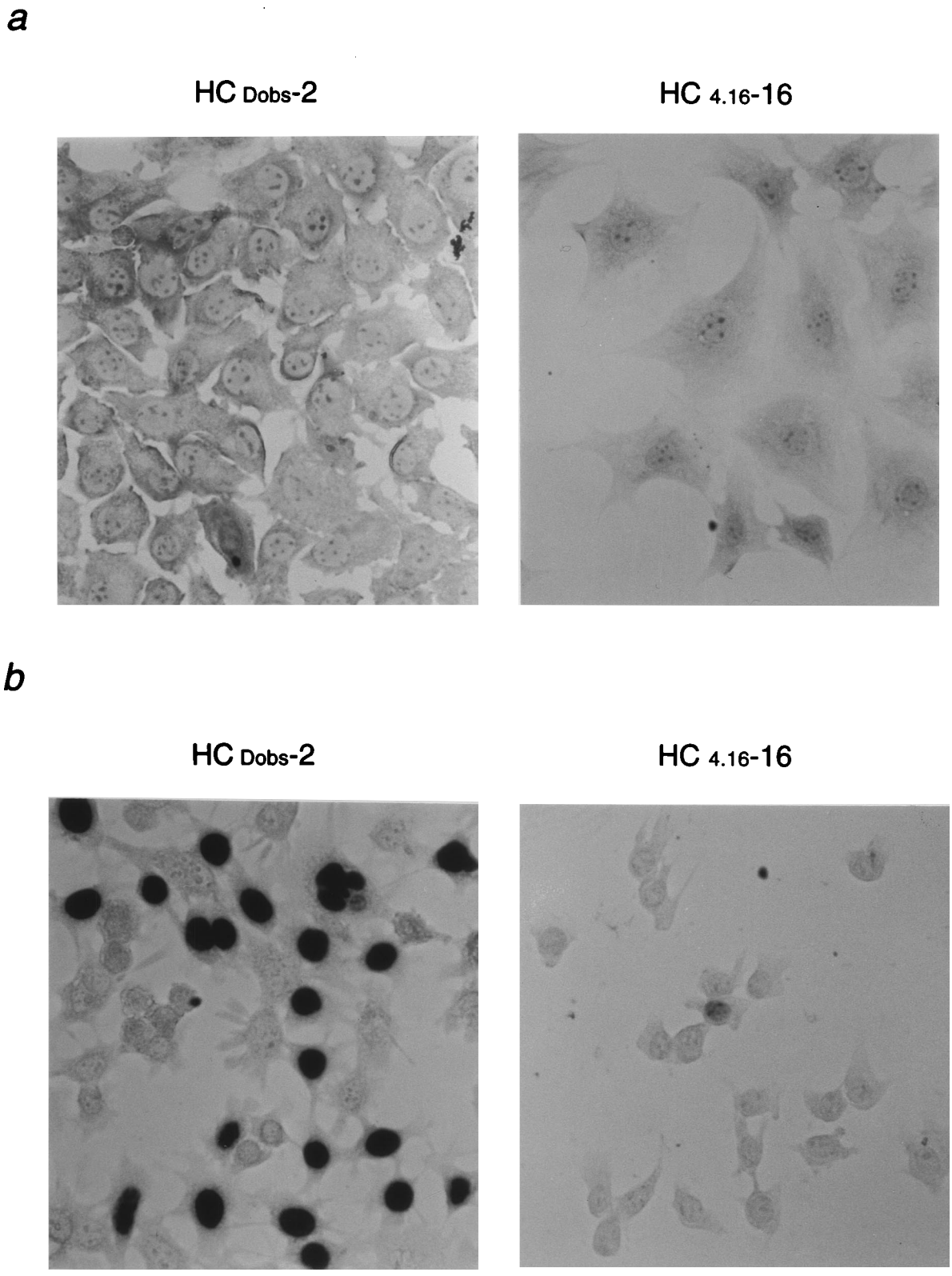


FIG. 8. Cell morphology and inhibition of DNA synthesis following nuclear FGF3 expression in HC11 cells. HC11 cells transfected with pDobs4.16 (HC4.16-16) and control cells (HCDobs-2) were grown under low-serum conditions and photographed under phase-contrast. (b) To monitor DNA synthesis, the same transfected cell clones were assessed for their ability to incorporate BrdU over a 24-h period when grown in complete medium. BrdU incorporation was measured immunocytochemically.

normal conditions (47). In contrast, exogenous FGF1 is translocated into the nucleus by an apparently receptor-dependent process.

To explore the properties of nuclear-nucleolar FGF3, we introduced into HC11 mouse mammary cells a form of FGF3 that was expressed exclusively in the nucleus. We chose to use mouse mammary epithelial cells because they are known to respond to FGF3 in vivo (33, 34, 43). A consequence of expressing the nuclear isoform alone was that the cells grew very slowly. Furthermore, BrdU labelling and fluorescence-activated cell sorting on the basis of DNA content showed that most of these cells were in the G_1 phase of the cell division cycle (Fig. 8 and data not shown). In cells transfected with cDNAs in which the encoded FGF3 lacked the C-terminal motif 5, the growth rates were similar to that of the parental line (data not shown). Thus, the inhibitory effect of nuclear FGF3 appears to require the sequences which have been implicated in nucleolar localization. Interestingly, a low level of expression of CUG-initiated FGF2 in NIH 3T3 cells was similarly shown to be associated with suppression of growth (38). However, for both FGF2 and FGF3, the nuclear target(s) and mechanism(s) of impaired growth remain to be determined, although the lack of a domain corresponding to the FGF3 C terminus makes it unlikely that the targets of the two FGFs are the same.

Secreted FGF3 interacts with cell surface FGF receptors, a process which has been shown to stimulate the mitogen-activated protein kinase pathway and induce DNA synthesis in several cell lines, including HC11 (28a, 29). In vivo, this may occur in an autocrine and/or paracrine manner. Thus, the ability to signal directly to the cell nucleus would provide a cell with the potential to respond differently to an autocrine versus a paracrine receptor-mediated signal. The preliminary evidence presented here suggests that the secreted and nucleolar forms of FGF3 may have opposing effects on cells, leading to the interesting possibility that the autocrine response to this factor may indeed be different from the paracrine response. These considerations could have important implications for the role of FGF3 as an oncogene product and as a signalling molecule during the embryonic and fetal development of vertebrates.

ACKNOWLEDGMENTS

We are extremely grateful to the ICRF electron microscopy department for the immunogold staining shown in Fig. 6. We also thank Gordon Peters and Graham Warren for their useful comments and suggestions on the manuscript and Anna-Maria Florence for technical help in the preparation of plasmid DNAs.

REFERENCES

1. **Acland, P., M. Dixon, G. Peters, and C. Dickson.** 1990. Subcellular fate of the Int-2 oncoprotein is determined by choice of initiation codon. Nature (London) **343:**662–665.
2. **Adachi, Y., T. D. Copeland, M. Hatanaka, and S. Oroszlan.** 1993. Nucleolar targeting signal of Rex protein of human T-cell leukemia virus type I specifically binds to nucleolar shuttle protein B-23. J. Biol. Chem. **268:**13930–13934.
3. **Baldin, V., A.-M. Roman, I. Bosc-Bierne, F. Amalric, and G. Bouche.** 1990. Translocation of bFGF to the nucleus is G-1 phase cell cycle specific in bovine aortic endothelial cells. EMBO J. **9:**1511–1517.
4. **Ball, R., R. Friis, C. Schonenberger, W. Doppler, and B. Groner.** 1988. Prolactin regulation of β-casein gene expression and of a cytosolic 120 kd protein in a cloned mouse mammary epithelial cell line. EMBO J. **7:**2089–2095.
5. **Bond, V., and B. Wold.** 1993. Nucleolar localization of *myc* transcripts. Mol. Cell. Biol. **13:**3221–3230.
6. **Bouche, G., N. Gas, H. Prats, V. Baldwin, J.-P. Tauber, J. Teissie, and R. Amalric.** 1987. Basic fibroblast growth factor enters the nucleolus and stimulates the transcription of ribosomal genes in ABAE cells undergoing G0-G1 transition. Proc. Natl. Acad. Sci. USA **84:**6770–6774.
7. **Bugler, B., F. Amalric, and H. Prats.** 1991. Alternative initiation of translation determines cytoplasmic or nuclear localization of basic fibroblast growth factor. Mol. Cell. Biol. **11:**573–577.
8. **Burgess, W. H., and T. Maciag.** 1989. The heparin binding (fibroblast) growth factor family proteins. Annu. Rev. Biochem. **58:**575–606.
9. **Cao, Y., M. Ekstrom, and R. F. Pettersson.** 1993. Characterization of the nuclear translocation of acidic fibroblast growth-factor. J. Cell Sci. **104:**77–87.
10. **Cochrane, A. W., A. Perkins, and C. A. Rosen.** 1990. Identification of sequences important in the nucleolar localization of human immunodeficiency virus Rev: relevance of nucleolar localization to function. J. Virol. **64:**881–885.
11. **Creancier, L., H. Prats, C. Zanibellato, F. Amalric, and B. Bugler.** 1993. Determination of the functional domains involved in nucleolar targeting of nucleolin. Mol. Biol. Cell **4:**1239–1250.
12. **Dickson, C., R. Deed, M. Dixon, and G. Peters.** 1989. The structure and function of the *int-2* oncogene. Prog. Growth Factor Res. **1:**123–132.
13. **Dixon, M., R. Deed, P. Acland, R. Moore, A. Whyte, G. Peters, and C. Dickson.** 1989. Detection and characterization of the fibroblast growth factor-related oncoprotein INT-2. Mol. Cell. Biol. **9:**4896–4902.
14. **Florkiewicz, R., and A. Sommer.** 1989. Human basic fibroblast growth factor gene encodes four polypeptides: three initiate translation from non-AUG codons. Proc. Natl. Acad. Sci. USA **86:**3978–3981.
15. **Friedman, S., X. Zhan, and T. MacIag.** 1994. Mutagenesis of the nuclear-localization sequence in FGF-1 alters protein stability but not mitogenic activity. Biochem. Biophys. Res. Commun. **198:**1203–1208.
16. **Fuller-Pace, F., G. Peters, and C. Dickson.** 1991. Cell transformation by kFGF requires secretion but not glycosylation. J. Cell Biol. **115:**547–555.
17. **Goldfarb, M.** 1990. The fibroblast growth factor family. Cell Growth Differ. **1:**439–445.
18. **Hatanaka, M.** 1990. Discovery of the nucleolar targeting signal. Bioessays **12:**143–148.
19. **Heine, M., M. Rankin, and P. DiMario.** 1993. The Gly/Arg-rich (GAR) domain of Xenopus nucleolin facilitates in vitro nucleic acid binding and in vivo nucleolar localization. Mol. Biol. Cell **4:**1189–1204.
20. **Imamura, T., K. Engleka, X. Zhan, Y. Tokita, R. Forough, D. Roeder, A. Jackson, J. Maier, T. Hla, and T. Maciag.** 1990. Recovery of mitogenic activity of a growth factor mutant with a nuclear translocation sequence. Science **249:**1567–1570.
21. **Jaye, M., J. Schlessinger, and C. Dionne.** 1992. Fibroblast growth factor receptor tyrosine kinases: molecular analysis and signal transduction. Biochim. Biophys. Acta **1135:**185–199.
22. **Johnson, D., and L. Williams.** 1993. Structural and functional diversity in the FGF receptor multigene family. Adv. Cancer Res. **60:**1–41.
23. **Kiefer, P., P. Acland, D. Pappin, G. Peters, and C. Dickson.** 1994. Competition between nuclear-localization and secretory signals determines the subcellular fate of a single CUG-initiated form of FGF3. EMBO J. **13:**4126–4136.
24. **Kiefer, P., G. Peters, and C. Dickson.** 1993. Retention of fibroblast growth factor 3 in the Golgi complex may regulate its export from cells. Mol. Cell. Biol. **13:**5781–5793.
25. **Klagsbrun, M., and A. Baird.** 1991. A dual receptor system is required for basic fibroblast growth factor activity. Cell **67:**229–231.
26. **Kozak, M.** 1989. The scanning model for translation: an update. J. Cell Biol. **108:**229–241.
27. **Kubota, S., H. Siomi, T. Satoh, S.-I. Endo, M. Maki, and M. Hatanaka.** 1989. Functional similarity of HIV-1 *rev* and HTLV-1 *rex* proteins: identification of a new nucleolar-targeting signal in *rev* protein. Biochem. Biophys. Res. Commun. **162:**963–970.
28. **Maeda, Y., K. Hisatake, T. Kondo, T. Hanada, C.-Z. Song, T. Nishimura, and M. Muramatsu.** 1992. Mouse rRNA gene transcription factor mUBF requires both HMG-box 1 and an acidic tail for nucleolar accumulation: molecular analysis of the nucleolar targeting mechanism. EMBO J. **11:**3695–3704.

28a. **Mathieu, M., E. Chatelain, D. Ornitz, J. Bresnick, I. Mason, P. Kiefer, and C. Dickson.** 1995. Receptor binding and mitogenic properties of mouse fibroblast growth factor 3 (FGF3): modulation of response by heparin. Submitted for publication.

29. **Mathieu, M., P. Kiefer, I. Mason, and C. Dickson.** 1995. Fibroblast growth factor 3 from *Xenopus laevis* (XFGF3) binds with high affinity to FGF-receptor 2. J. Biol. Chem. **270:**6779–6787.
30. **Messmer, B., and C. Dreyer.** 1993. Requirements for nuclear translocation and nucleolar accumulation of nucleolin of Xenopus laevis. J. Cell Biol. **161:**369–382.
31. **Miyamoto, M., K. Naruo, C. Seko, S. Matsumoto, T. Kondo, and T. Kurokawa.** 1993. Molecular cloning of a novel cytokine cDNA encoding the ninth member of the fibroblast growth factor family, which has a unique secretion property. Mol. Cell. Biol. **13:**4251–4259.
32. **Morgenstern, J., and H. Land.** 1990. Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. Nucleic Acids Res. **18:**3587–3596.
33. **Muller, W., F. Lee, C. Dickson, G. Peters, P. Pattengale, and P. Leder.** 1990.

The int-2 gene product acts as an epithelial growth factor in transgenic mice. EMBO J. **9:**907–913.
34. **Ornitz, D., R. Cardiff, A. Kuo, and P. Leder.** 1992. Int-2, an autocrine and/or ultra-short-range effector in transgenic mammary tissue transplants. J. Natl. Cancer Inst. **84:**887–892.
35. **Peters, G.** 1991. Inappropriate expression of growth factor genes in tumors induced by mouse mammary tumor virus. Semin. Virol. **2:**319–328.
36. **Prats, H., M. Kaghad, A. Prats, M. Klagsbrun, J. Lelias, P. Liauzun, P. Chalon, J. Tauber, F. Amalric, J. Smith, and D. Caput.** 1989. High molecular mass forms of basic fibroblast growth factor are initiated by alternative CUG codons. Proc. Natl. Acad. Sci. USA **86:**1836–1840.
37. **Quarto, N., F. Finger, and D. Rifkin.** 1991. The NH2-terminal extension of high molecular weight bFGF is a nuclear targeting signal. J. Cell. Physiol. **147:**311–318.
38. **Quarto, N., D. Talarico, R. Florkiewicz, and D. Rifkin.** 1991. Selective expression of high molecular weight basic fibroblast growth factor confers a unique phenotype to NIH3T3 cells. Cell Reg. **2:**699–708.
39. **Renko, M., N. Quarto, T. Morimoto, and D. Rifkin.** 1990. Nuclear and cytoplasmic localization of different basic fibroblast growth factor species. J. Cell. Physiol. **144:**108–114.
40. **Schmidt-Zachmann, M. S., and E. A. Nigg.** 1993. Protein localization to the nucleolus: a search for targeting domains in nucleolin. J. Cell Sci. **105:**799–806.
41. **Schwarzacher, H., and F. Wachtler.** 1993. The nucleolus. Anat. Embryol. **188:**515–536.
42. **Siomi, H., H. Shida, S. H. Nam, T. Nosaka, M. Maki, and M. Hatanaka.** 1988. Sequence requirements for nucleolar localization of human T cell leukemia virus type I pX protein, which regulates viral RNA processing. Cell **55:**197–209.
43. **Stamp, G., V. Fantl, R. Poulsom, S. Jamieson, R. Smith, G. Peters, and C. Dickson.** 1992. Nonuniform expression of a mouse mammary-tumor virus-driven int-2/fgf-3 transgene in pregnancy-responsive breast-tumors. Cell Growth Differ. **3:**929–938.
44. **Tanaka, A., K. Miyamoto, N. Minamino, M. Takeda, B. Sato, H. Matsuo, and K. Matsumoto.** 1992. Cloning and characterization of an androgen-induced growth factor essential for the androgen-dependent growth of mouse mammary carcinoma cells. Proc. Natl. Acad. Sci. USA **89:**8928–8932.
45. **Wilkinson, D. G., S. Bhatt, and A. P. McMahon.** 1989. Expression pattern of the FGF-related proto-oncogene *int-2* suggests multiple roles in fetal development. Development **105:**131–136.
46. **Wilkinson, D. G., G. Peters, C. Dickson, and A. P. McMahon.** 1988. Expression of the FGF-related proto-oncogene *int-2* during gastrulation and neurulation in the mouse. EMBO J. **7:**691–695.
47. **Zhan, X., X. G. Hu, S. Friedman, and T. MacIag.** 1992. Analysis of endogenous and exogenous nuclear translocation of fibroblast growth factor-1 in NIH 3T3 cells. Biochem. Biophys. Res. Commun. **188:**982–991.