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Basic fibroblast growth factor enters the nucleolus and stimulates the transcription of ribosomal genes in ABAE cells undergoing $G_0 \rightarrow G_1$ transition

(cell cycle/ribosomal gene expression/nucleolin)

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ABSTRACT **The cellular action of growth factors, among them basic fibroblast growth factor (bFGF), is mediated by their interaction with a cell surface receptor, but the mechanism of transfer of mitogenic (or other) signals to the nucleus has not been identified. In this work, we show that bFGF is translocated to and accumulated in the nucleolus. Furthermore, the nucleolar localization of bFGF is correlated with a stimulation of transcription of ribosomal genes during $G_0 \rightarrow G_1$ transition induced by bFGF alone in adult bovine aortic endothelial cells (ABAE cells). Stimulation of ribosomal gene transcription is preceded by a significant increase of the major nonhistone nucleolar protein, nucleolin. *In vitro*, the growth factor has a direct effect on the enhancement of RNA polymerase I activity in isolated nuclei from quiescent sparse (G_0) ABAE cells. The direct action of bFGF on the level of ribosomal gene transcription could correspond to an additional growth-signaling pathway, mediated by this growth factor.**

The family of fibroblast growth factors (FGFs) includes the factors described as endothelial cell growth factor, chondrosarcoma growth factor, and heparin-binding growth factors (1). Preliminary physical analysis of some of these mitogens has suggested their classification in two groups: acidic fibroblast growth factors (aFGFs) (2) and basic fibroblast growth factors (bFGFs) (3). *In vitro*, aFGFs and bFGFs are potent mitogens for a wide variety of mesoderm- and neuroectoderm-derived cells, including vascular and capillary endothelial cells (4) and, as *in vivo* (5), they induce the angiogenic response (6).

The cellular action of FGFs is exerted through its interaction with specific cell surface receptors (7, 8), but the intervening steps and the mechanism of transfer of mitogenic (or other) signals to the nucleus, leading to the "pleiotropic response" required to bring quiescent cells into full proliferation, are at present unknown.

The proliferation state and ribosome biogenesis, which involve a series of coordinated nucleolar events, among them the transcription of ribosomal genes (rDNA), are closely related. The level of transcription of rDNA is modulated by cell growth conditions, growth-promoting hormones (9), and growth factors (10). A specific nucleolar protein, nucleolin, was shown in different eukaryotic cells to play a direct role in the control of the synthesis of the precursor to ribosomal RNA (pre-rRNA) and assembly of ribosomes (11, 12). Barely detectable in resting cells, nucleolin represents up to 5% of nucleolar proteins in exponentially growing cells. *In vitro*, run-off experiments with rDNA as template have shown that endoproteolytic cleavage of phosphorylated nucleolin controls rDNA transcription (13).

In this report, we have focused on the effects of bFGF on the reinitiation of ribosome biogenesis in cells undergoing the $G_0 \rightarrow G_1$ transition. We show by immunocytochemistry using a monospecific polyclonal anti-bFGF antibody that the reinitiation of pre-rRNA synthesis is preceded by the accumulation of nucleolin and bFGF predominantly in the nucleolus. Furthermore, the transcriptional activity of RNA polymerase I, in nuclei isolated from quiescent sparse adult bovine aortic endothelial (ABAE) cells, is specifically increased *in vitro* by bFGF.

MATERIALS AND METHODS

Cell Culture and Cell Labeling. ABAE cell cultures were established from the aortic arch. The cells were cloned and routinely subcultured according to Darbon *et al.* (14). Quiescent sparse endothelial cells (G_0) were obtained as follows: Endothelial cells were seeded at low density (5×10^4 cells per 10-cm dish) in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum and bFGF (1 ng/ml). After 48 hr, cells were washed twice with serum-free DMEM supplemented with transferrin and cultures were continued in the same medium for 48 hr. $G_0 \rightarrow G_1$ transition was obtained by stimulation of quiescent cells with serum-free DMEM containing only bFGF (50 ng/ml) for 2 hr. Cells undergoing $G_0 \rightarrow G_1$ transition were radioactively labeled with [5,6-^{3}H]-uridine (Amersham; 10 μCi/ml; 1 Ci = 37 GBq) for 15 min. For measurements of DNA synthesis, cells were pulse-labeled for 1 hr with [*methyl*-^{3}H]thymidine (Amersham; 5 μCi/ml) at different times after stimulation by bFGF.

Isolation of bFGF and Preparation of an Antiserum. Basic FGF was purified to homogeneity from bovine pituitaries according to Gospodarowicz *et al.* (15). A rabbit antiserum was generated according to Bugler *et al.* (11). Affinity purification of polyclonal anti-bFGF antibody was performed by the method described by Lapeyre and Amalric (16) with an additional step of purification. Purified bFGF (300 μg) was coupled to AH-Sepharose (Pharmacia) according to the manufacturer's instructions. The IgG fraction of the antiserum was adsorbed to the gel after extensive washing with PBS (PBS = 0.15 M NaCl/0.01 M sodium phosphate buffer, pH 7.5), and anti-bFGF IgGs were eluted with 0.1 M glycine·HCl, pH 2.5. Column fractions were neutralized with 1 M K_2HPO_4 and dialyzed against PBS.

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Abbreviations: FGF, fibroblast growth factor; bFGF, basic FGF; rDNA, ribosomal genes.

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Indirect Immunofluorescence Staining. For these experiments, cells were grown on glass coverslips. Quiescent sparse cells (G_0) were treated with bFGF as above and fixed according to Murthy *et al.* (17). Fixed cells were rinsed three times with ice-cold PBS and incubated at 4°C overnight with affinity-purified anti-bFGF IgG in a humidified atmosphere. After three washes with 0.5% bovine serum albumin in PBS, cells were further incubated for 30 min at 37°C with fluorescein-conjugated sheep anti-rabbit antibody (Nordic Immunological Laboratories, Lausanne, Switzerland). Finally, the coverslips were extensively washed with 0.5% bovine serum albumin/PBS, mounted on glass slides, and examined in a Leitz Ortholux II microscope equipped for epifluorescence, with a 100-W mercury lamp. Because of the low level of fluorescence, in some experiments a video camera with a light intensifier (Lhesa) was used and the preparation was observed on a video monitor (RCA). Micrographs were obtained after an exposure of 2 min. Immunodetection of nucleolin with polyclonal antibody (11) and of tubulin with monoclonal antibody (Amersham) was performed by using the same procedure except that the light intensifier system was not necessary.

Quantification of nuclear fluorescence was obtained by digitalization of micrographs with a video/digital converter (Digivector DS-65) plugged into a microcomputer (Apple IIe) and linked to a video camera (JVC S100). Light-emission histograms were computed from the 40 × 40 matrix that was obtained from the picture. Three different light level bands were selected—low, medium, and high—and integration was operated for each level.

Transcription by Isolated Nuclei and Analysis of RNA. Nuclei were purified for *in vitro* transcription experiments according to Schibler *et al.* (18). The transcription reactions were carried out in the presence or absence of bFGF in a medium (0.1 ml final volume) containing 50 mM Hepes at pH 7.9; 20% (vol/vol) glycerol; 90 mM KCl; 5 mM $MgCl_2$; 0.2 mM dithiothreitol; 0.2 mM EDTA; 0.3 mM each of ATP, GTP, and UTP; 0.03 mM CTP; 10 μCi of [α-^{32}P]CTP (Amersham; 400 Ci/mmol); and about 3×10^5 nuclei. The reaction proceeded at 30°C for 45 min. RNA was extracted with phenol and chloroform, then precipitated with ethanol. Redissolved RNA was treated by RNase-free DNase I (Appligène) and reextracted as above. Hybrid selection of labeled rRNA was carried out according to Gurney *et al.* (19) on nitrocellulose filters to which 10 μg of alkali-denatured DNA plasmids containing mouse rDNA fragments [pMr 974 (20) and pMEB 3 (21)] were bound. The efficiency of hybridization was around 40%.

RESULTS

Nucleolar Localization of bFGF in ABAE Cells. Quiescent sparse ABAE cells (G_0 phase) were obtained after 48 hr of growth in the absence of serum and bFGF. Addition of bFGF alone induces the transition from G_0 to G_1 phase of the cell cycle, and stimulated cells enter the S phase within 3 hr, as shown by incorporation of labeled thymidine (Fig. 1). To characterize the bFGF pathway in the cell, a polyclonal serum was prepared by injection of purified bFGF into a rabbit. The serum is highly specific, and it did not recognize any antigen in a total extract of untreated cells (Fig. 2*G*). Two hours after bFGF addition (50 ng/ml) to the culture medium of quiescent sparse cells, the growth factor is immunodetected predominantly in the nucleolus by affinity-purified anti-bFGF IgG (Fig. 2 *B* and *C*). A low-intensity diffuse staining is also observed in the nucleoplasmic network. Conversely, in control cells (no bFGF treatment), no staining is observed (Fig. 2*A*). Moreover, we could not detect any staining when bFGF-treated cells were exposed to a preimmune rabbit IgG (Fig. 2*D*). In additional controls, MCF_7 cells, a human breast

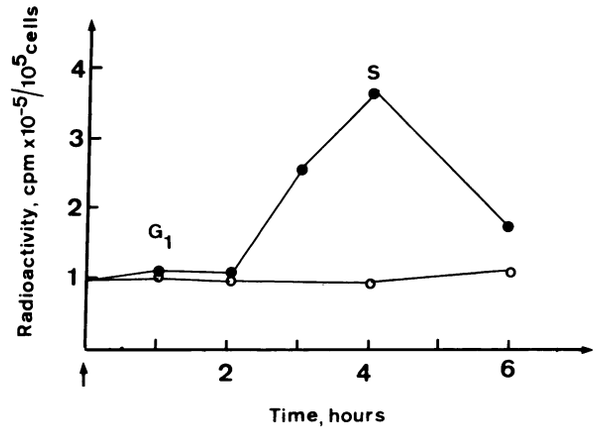


FIG. 1. Reinitiation of DNA synthesis in G_0-arrested ABAE cells. G_0-arrested ABAE cells were stimulated (↑) by bFGF alone (50 ng/ml). Cells were pulse-labeled with [*methyl*-^{3}H]thymidine at several times after bFGF stimulation. The rate of DNA synthesis was measured by cell counting and determination of cpm of [^{3}H]thymidine incorporated into trichloroacetic acid-insoluble material. ○, Control cells; ●, stimulated cells.

cancer cell line whose growth and proliferation are not bFGF dependent, were grown in the presence of bFGF. The growth factor was not detected in these cells even after several hours of treatment (Fig. 2*E*). To eliminate the possibility that a restructuring of the nucleus and nucleolus after bFGF stimulation was responsible for a nonspecific trapping of antibody within these organelles, controls were performed with an unrelated monoclonal antibody (anti-tubulin). Staining detected with this antibody was the same in quiescent and stimulated cells and always excluded the nucleus (Fig. 2 *F* and *F'*).

Kinetics of bFGF Appearance in the Nucleus and Nucleolus. The kinetics of bFGF appearance in the nucleus and nucleolus were studied by quantification of immunofluorescence in the cell nucleus at different times after bFGF stimulation (Fig. 3 *Bottom*). After 15-min treatment a significant amount of bFGF is detected in the nucleolus and in the nonnucleolar part of the nucleus, while a preferential accumulation of the growth factor is observed in the nucleolus after 2 hr of bFGF stimulation. The kinetics of bFGF appearance in the nucleolus are parallel to those of nucleolin import as determined by the same quantification assay (results not shown) and by scanning silver-stained gels (Fig. 3 *Middle*).

Induction of rRNA Synthesis and Nucleolin Accumulation in ABAE Cells Stimulated by bFGF. In unstimulated cells, rRNA synthesis represents only 1–2% of the level observed in exponentially growing cells. To assay the effects of bFGF on rRNA synthesis, quiescent sparse cells were exposed for 15 min to [5,6-^{3}H]uridine at various times after stimulation by bFGF. As shown in Fig. 3 *Middle*, rRNA synthesis was markedly increased 1 hr after bFGF stimulation and reached a maximum by 2 hr, at a level identical to that in exponentially growing cells (data not shown).

The effect of bFGF stimulation on the amount of nucleolin in the nucleus was followed by electrophoretic analysis of nuclear protein from isolated nuclei at different times after bFGF treatment. Quiescent sparse cells contain 0.05 pg of nucleolin per nucleus, while exponentially growing cells contain 0.5 pg per nucleus. In cells stimulated by bFGF alone (50 ng/ml), the nucleolin level rises to 0.5 pg per nucleus after 1 hr of stimulation (Fig. 3 *Middle*). This increase of nucleolin in stimulated cells is also detected by indirect immunofluorescence staining (Fig. 3 *Top*). Nucleoli of quiescent cells show a low level of nucleolin, whereas after bFGF stimulation all nucleoli are intensely fluorescent. It is noteworthy

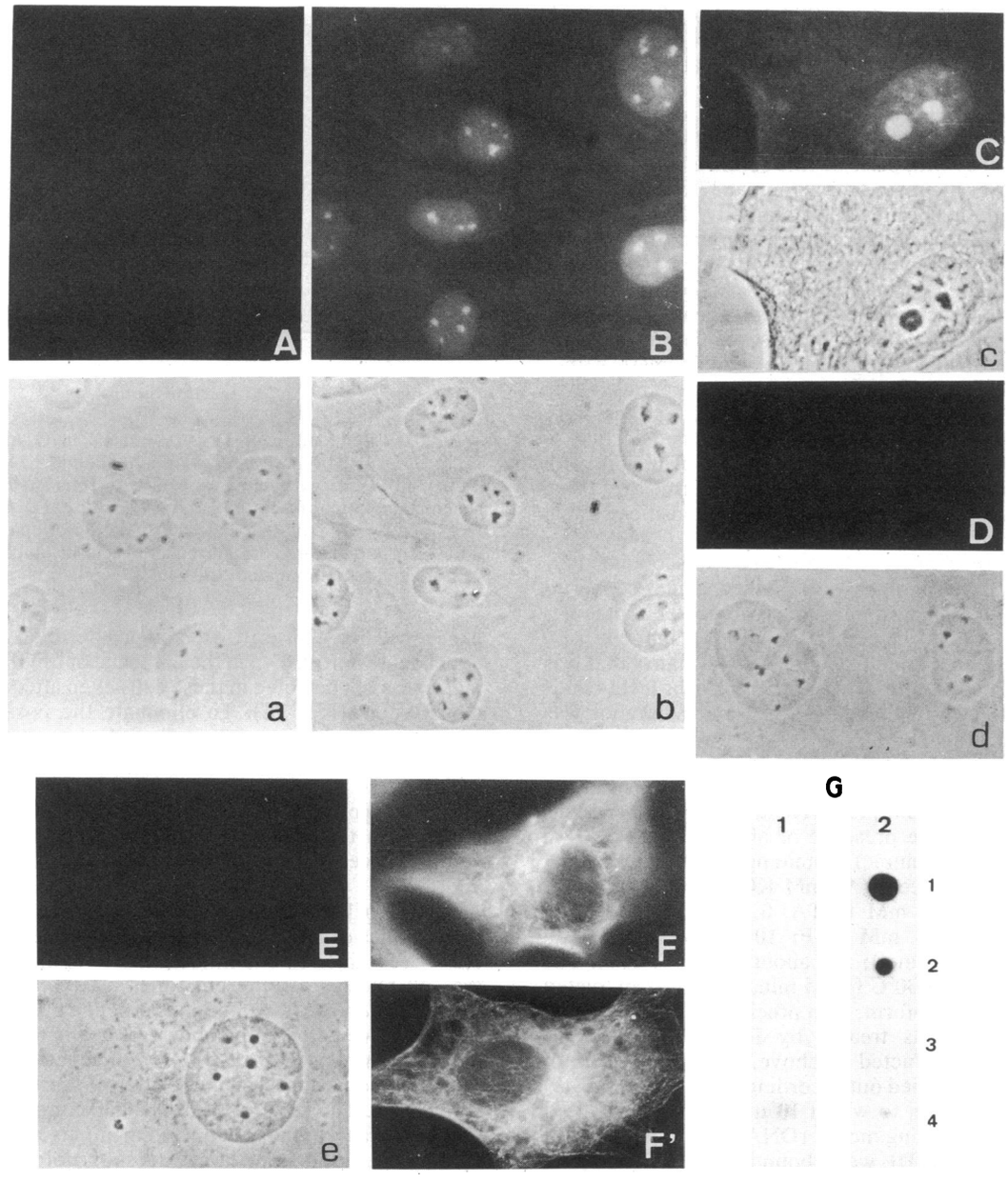


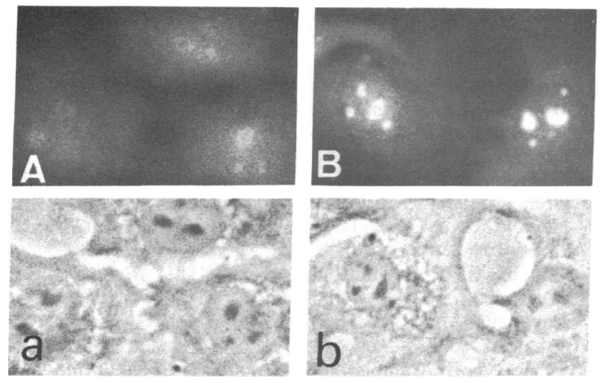
FIG. 2. Indirect immunofluorescence staining of ABAE cells (*A*, *B*, *C*, *D*, and *F*) and MCF_7 cells (*E*) with affinity-purified anti-bFGF IgG (*A*, *B*, *D*, and *E*) and anti-tubulin IgG (*F*). *a–e* are corresponding phase-contrast photos. (*A*) Quiescent cells; (*B* and *C*) bFGF-stimulated cells; (*C*) high-magnification picture; (*D*) control with preimmune rabbit IgG; (*F* and *F'*) control with anti-tubulin monoclonal antibody (Amersham); (*F*) quiescent cells; (*F'*) stimulated cells. (*G*) Antibody specificity depicted by dot blotting: lane 1, preimmune rabbit IgG; lane 2, affinity-purified anti-bFGF IgG; spot 1, 5 ng of bFGF; spot 2, 1 ng of bFGF; spot 3, 5 μg of ABAE cellular proteins; spot 4, 5 μg of purified histones. Immunodetection was performed according to Murthy *et al.* (17). (*A* and *B*, ×220; *C–F*, ×360.)

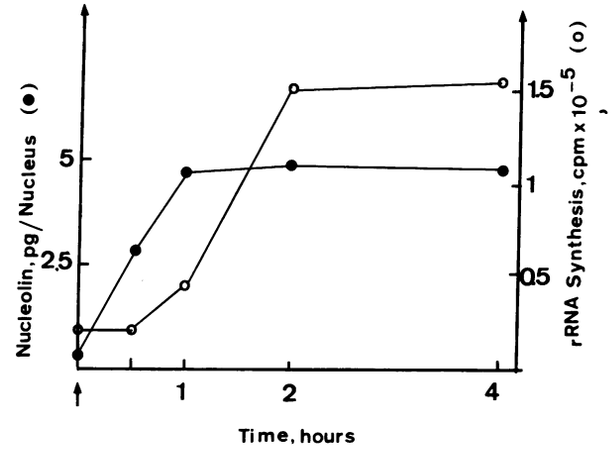
that pre-rRNA synthesis and nucleolin accumulation are not synchronous (Fig. 3 *Middle*). The nucleolin per nucleus is maximal 1 hr after the stimulation, while rRNA synthesis reaches a plateau only after 2 hr of treatment.

Effect of bFGF on the Transcription of rDNA in Isolated Nuclei from Quiescent Sparse Cells. To determine whether bFGF acts directly on rDNA transcription, *in vitro* studies were carried out with isolated nuclei from quiescent sparse ABAE cells. Nuclei were incubated in a reaction mixture containing [α-^{32}P]CTP without (control) or with bFGF at 0.1 or 1.0 nM and RNA was extracted. rRNA synthesized *in vitro* was detected by hybrid selection using cloned rDNA fragments bound to nitrocellulose filters (Table 1). These studies indicate that bFGF directly stimulated the transcription of ribosomal genes. At 1 nM bFGF, total RNA synthesis is increased by a factor of 3, while RNA polymerase I activity is increased by a factor of 5.6. Taking into account the efficiency of hybridization (40%), rRNA represents 74% of RNA synthesized in bFGF-treated nuclei, compared with 38% in control nuclei.

DISCUSSION

Despite considerable advances, a clear picture of the mechanism(s) of action of any single growth factor on the stimulation of a specific gene is not yet available. Information continues to accrue regarding processes and events that are stimulated by the interaction of growth factors with their responsive cells (1, 4, 22–24). However, the transduction system of mitogenic (or other) signals to the nucleus, which





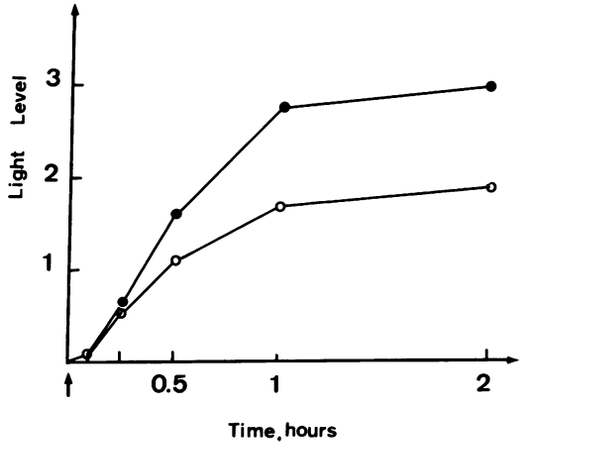


FIG. 3. Accumulation of nucleolin and bFGF in nucleoli of quiescent ABAE cells (G_0). (*Top*) (*A* and *B*) Indirect immunofluorescence staining with polyclonal anti-nucleolin antibody; (*A* and *a*) quiescent sparse cells; (*B* and *b*) 2 hr after bFGF stimulation; (*a* and *b*) phase contrast. (×230.) (*Middle*) Time course of nucleolin accumulation and rRNA synthesis in ABAE cells undergoing $G_0 \rightarrow G_1$ transition after bFGF stimulation. Cells were stimulated by bFGF at time 0 and pulse-labeled several times with [5,6-^{3}H]uridine. rRNA synthesis was determined by hybrid selection (19). Nucleolin synthesis was determined by scanning a silver-stained $NaDodSO_4$/12% polyacrylamide gel. The material deposited in each lane of the gel corresponds to nuclear proteins extracted from 10^5 nuclei. (*Bottom*) Time course of bFGF accumulation in nuclei (minus nucleoli) (○) and in nucleoli (●) in ABAE cells undergoing $G_0 \rightarrow G_1$ transition after bFGF stimulation. Light level determination is described in *Materials and Methods*. Each point represents the mean of five determinations.

Table 1. *In vitro* transcription in isolated nuclei from quiescent sparse ABAE cells: Effect of bFGF on synthesis of total RNA and rRNA

bFGF, nM	RNA synthesized, cpm/3 × 10^5 nuclei	Stimulation factor	rRNA hybrid-selected,* cpm/3 × 10^5 nuclei	Stimulation factor
0	47,000		7,200	
0.1	93,000	2	31,400	4
1.0	141,500	3	40,500	5.6

About 3 × 10^5 nuclei isolated from quiescent sparse ABAE cells were incubated 45 min at 30°C in the presence of [α-^{32}P]CTP with or without bFGF. Total RNA synthesis was determined as follows: Before hybrid selection, an aliquot of purified RNA (1/25th of total RNA) was mixed with 100 μl of 10% trichloroacetic acid containing 100 μg of calf thymus DNA as carrier. Insoluble material was collected by filtration on GF/C filters, washed with 50 ml of cold 5% trichloroacetic acid and then 20 ml of cold 95% (vol/vol) ethanol, and assayed for radioactivity in an Intertechnique SL200 liquid scintillation counter.

*Efficiency of hybridization was 40%.

is related to the pleïotropic response of the stimulation of quiescent cells into full proliferation, has not been identified. Central to this problem remains the internalization of growth factor–receptor complexes by endocytosis and the role, if any, of intracellular receptors, either as sites for further activity or as messengers themselves (for review see ref. 24).

Detection of bFGF in the Nucleolus of ABAE Cells. In this study we have observed an accumulation of immunodetectable bFGF in the nucleoli of stimulated cells. bFGF was detected in the nucleolus as early as 15 min after bFGF stimulation, and accumulation was maximum by 2 hr. The translocation of bFGF from the cell surface and its transport to the nucleus and nucleolus require internalization of a growth factor or a growth factor–receptor complex by endocytosis. We have recently found that after 5-min treatment by bFGF, most of the internalized growth factor is observed in cytoplasmic vesicles. In the presence of chloroquine, previously shown to block the release of hormonal peptides and growth factors into the cell (25), we do not detect any immunofluorescence in the nucleus and nucleolus of cells stimulated by bFGF, and the growth factor remains localized in endosomal and lysosomal structures (unpublished data).

bFGF Acts Directly on the Transcription of Ribosomal Genes. Our studies reveal a correlation between the nucleolar localization of bFGF and the stimulation of ribosomal gene transcription. The kinetics of translocation of the growth factor to the nucleus and nucleolus indicate that bFGF is present in the nucleolus 15 min after stimulation of the cells and before reinitiation of ribosome biogenesis. The import of nucleolin to the nucleolus, where it plays a key role in ribosomal biogenesis (11, 12), has the same kinetics as the translocation of bFGF. One of the early effects of bFGF and other growth factors during the $G_0 \rightarrow G_1$ transition is a large and rapid stimulation of protein synthesis (7). This increase, which appears to be controlled at the level of translation initiation, could be accounted for by the recruitment from the pool of stored nonpolysomal mRNA, including nucleolin mRNA, into actively translating polysomes (26).

The correlation observed *in vivo* between the translocation of bFGF to the nucleolus and the stimulation of ribosomal biogenesis during the first 2 hr of $G_0 \rightarrow G_1$ transition of ABAE cells is corroborated by *in vitro* experiments. We have shown that bFGF acts directly on the transcription of isolated nuclei from quiescent sparse cells and increases RNA polymerase I transcriptional activity by a factor of 5.6. The mechanism of bFGF action, at the molecular level, on the transcription of ribosomal genes requires further investigation. Our prelimi-

nary results suggest that bFGF acts by activating a nucleolar cyclic-AMP-independent protein kinase, NII, whose specific substrate is nucleolin. We have shown previously that endoproteolytic cleavage of phosphorylated nucleolin is the event that triggers ribosomal gene transcription *in vitro* (13).

The direct action of bFGF on the level of ribosomal gene transcription could correspond to a growth-signaling pathway mediated by this growth factor. Effectively, in G_0-arrested Chinese hamster lung fibroblasts (39) bFGF (50 ng/ml) alone is capable of reinitiating DNA synthesis. It has been reported that the bFGF receptor signaling pathway is not coupled to phospholipase C activation and that early mitogenic events and reinitiation of DNA synthesis can be initiated independently of the pathways invoked in the generally accepted models for the action of growth factors (23). These pathways involve binding to cell surface receptors, induction of a breakdown of inositol lipid, and activation of protein kinase C (1). However, it remains to be established whether the activation of this signaling pathway is sufficient or even required to trigger the full mitogenic response.

In conclusion, the results presented here show that during the change from the quiescent to the growing state ($G_0 \rightarrow G_1$ transition) in ABAE cells, bFGF penetrates into the cells and is translocated predominantly to the nucleolus. The specific localization of this growth factor is accompanied by the activation of ribosomal RNA transcription and, *in vitro*, by the direct and specific stimulation of pre-rRNA synthesis, one of the major regulated events in the transition from a quiescent state to full proliferation (10).

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