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Direct effect of basic fibroblast growth factor on gene transcription in a cell-free system

(growth-factor action/transcription regulation)

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ABSTRACT **Recent findings on the translocation of intact fibroblast growth factor (FGF) into the cell nucleus suggest that it functions directly in nuclear events. We examined the effect of human basic FGF (bFGF) on gene transcription in a cell-free system. When mouse genes encoding phosphoglycerate kinases 1 and 2 (*Pgk-1* and *Pgk-2*) were transcribed by using nuclear extracts of Ehrlich ascites tumor cells, FGF affected transcription in different ways: in the presence of bFGF, transcription of the *Pgk-1* gene was inhibited, whereas that of the *Pgk-2* gene was enhanced. When viral genes were tested, transcription of the adenovirus major late DNA was slightly stimulated but that of the adenovirus early *E1A* DNA or the human immunodeficiency virus DNA was not changed by the addition of bFGF. Moreover, the presence of a distinct 5′ upstream region of the *Pgk-2* gene, which includes a negative cis-acting element, was required for transcription stimulation by bFGF. These results suggest that bFGF can regulate transcription directly in the nucleus in a gene-specific manner.**

Growth factors appear to have important roles in the proliferation of mammalian cells. Fibroblast growth factor (FGF) may be involved in angiogenesis and in the induction of mesodermal tissues during the early stage of embryogenesis (1–3). Mitogenic signals provided by growth factors may be transmitted to the cell nucleus by the action of intracellular messengers that are produced through an interaction of growth factors and their specific receptors on the cell membrane (4, 5). However, growth factors could also act directly in the cell nucleus (6, 7). We previously suggested that basic FGF (bFGF)-like growth factor present in the nucleus is responsible for tumor cell growth (8, 9). There is evidence that bFGF is localized in nuclei of a variety of cultured cells and animal tissues (10–14) and that exogenous bFGF is transported into the nucleus (15, 16). More recent studies have shown that only the CUG-initiated human bFGF is transported into the nucleus, while the AUG-initiated form remains in the cytoplasm, suggesting a translational control of nuclear localization of bFGF (17–19). Translocation of acidic FGF (aFGF) into the nucleus, mediated through a nuclear targeting signal present within aFGF, was reported to be a prerequisite for the stimulation of endothelial cell growth (20). In early embryos of *Xenopus*, bFGF has been shown to translocate into the nucleus concomitantly with the induction of mesoderm (21). These results suggest that FGF acts directly on nuclear events such as transcription and DNA replication. In this study we examined the effect of bFGF on gene transcription in a cell-free system and found that bFGF regulates transcription in a promoter-specific manner.

MATERIALS AND METHODS

FGF and Anti-FGF Monoclonal Antibodies. Monoclonal antibodies bFM-1 and bFM-2 raised against bovine bFGF (22, 23) and HA against human epidermal growth factor (24) were prepared as described. Recombinant human bFGF was provided by Kaken Pharmaceutical (Tokyo) and California Biotechnology (Mountain View, CA) (25).

Cell Growth Assay and Radioimmunoassay. The effect of bFGF on the growth of cultured fibroblast cells was determined by measuring the [^{3}H]thymidine incorporation into DNA as described (22). The concentration of bFGF in cell lysates was determined by a radioimmunoassay with bFM-1 as described (26).

DNA Constructs. The following DNA constructs were used as templates in cell-free transcription reactions (Fig. 1): P1HCAT, the region between nucleotide positions −2500 and +50 of the mouse phosphoglycerate kinase 1 gene (*Pgk-1*) (27, 28), relative to the transcription initiation site at +1, was inserted in pBLCAT3, which contains the gene for chloramphenical acetyltransferase (CAT) (29); P1BCAT, the region between −850 and +300 of the mouse *Pgk-1* gene was inserted in pBLCAT3; P2ECAT, the region between −3600 and +30 of the mouse *Pgk-2* gene (28, 30) was inserted in pBLCAT3; P2PCAT, the region between −430 and +30 of the mouse *Pgk-2* gene was inserted in pBLCAT3; P2EBg, about 3.6 kilobase pairs (kbp) of 5′-flanking sequences and the entire coding region of the mouse *Pgk-2* gene were inserted in pUC19; pHIVCAT, the region between −450 and +80 of the human immunodeficiency virus type 1 (HIV-1) DNA was inserted in pSV0CAT (31); pSmaF (32), a *Sma* I F fragment (unit 11.5–18.2) of the adenovirus type 2 (Ad2) DNA was inserted in pBR313; and pE1ACAT, the 500-bp left-most terminal region of the Ad12 DNA containing the 5′-end region of the *E1A* gene was inserted in pBLCAT3.

Nuclear Extracts and Cell-Free Transcription. Nuclear extracts were prepared from Ehrlich ascites tumor cells as described (33, 34). Cell-free transcription was conducted in a 25-μl reaction mixture containing 12 mM Hepes (pH 7.9), 0.12 mM EDTA, 0.3 mM dithiothreitol, 60 mM KCl, 5 mM $MgCl_2$, 0.3 mM phenylmethylsulfonyl fluoride, 12% (vol/vol) glycerol, 0.5 mM each NTP, 5 mM creatine phosphate, ≈0.2 mg of extract protein, and the following template DNAs: covalently closed P1HCAT, P1BCAT, and P2EBg at 8 μg/ml; P2ECAT, P2PCAT, and pE1ACAT at 20 μg/ml; and pHIVCAT at 80 μg/ml, except that pSmaF DNA was used at 20 μg/ml after digestion with *Sma* I. At these concentrations, each DNA template gave a maximum level of the transcript.

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Abbreviations: Ad, adenovirus; Ad2 and Ad12, Ad types 2 and 12; FGF, fibroblast growth factor; aFGF, acidic FGF; bFGF, basic FGF; CAT, chloramphenicol acetyltransferase; Pgk, mouse phosphoglycerate kinase; HIV-1, human immunodeficiency virus 1.
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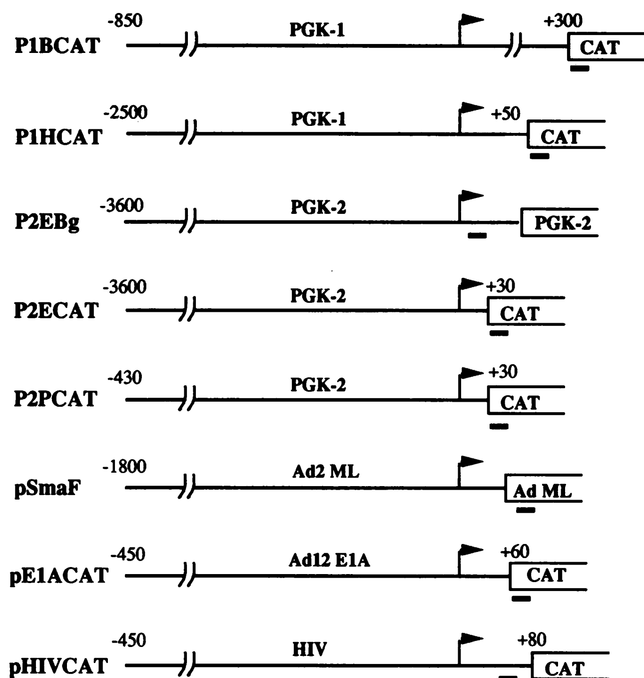

FIG. 1. Structure of DNAs used in cell-free transcription. Partial structure of DNAs used as templates in cell-free transcription reactions is shown. The number shows the nucleotide position relative to the most distal transcription initiation site (shown with the arrowhead) as +1, except that a proximal initiation site is numbered +1 for the adenovirus type 12 (Ad12) *E1A* gene. The thick bar indicates the position of a primer. ML, major late DNA.

In a reaction with mixed DNA templates, 0.2 μg of P1BCAT and 0.5 μg of P2ECAT DNAs were simultaneously transcribed in a 25-μl reaction mixture. All components including bFGF and antibodies were simultaneously combined. After a 1-hr incubation at 25°C, RNA transcripts were extracted and analyzed by primer extension (34) with the following ^{32}P-labeled specific primers: a CAT primer with the sequence 5′-TCTCGCCAAGCTCCTCGA-3′ for transcription of P1HCAT, P1BCAT, P2ECAT, P2PCAT, and pE1ACAT DNAs; a *Pgk-2* primer with the sequence 5′-ATGGTATG-CACAACAGCCTC-3′ for transcription of P2EBg DNA; an HIV primer with the sequence 5′-GAGGCTTAAG-CAGTGGGTTC-3′ for transcription of pHIVCAT DNA; an Ad2 major late primer with the sequence 5′-TAGCGCA-GAAGTCATGCCGT-3′ for transcription of pSmaF DNA. Poly(A)-containing RNA isolated from adult mouse testes (35) was similarly analyzed with the *Pgk-2* primer. Primer-extended products were separated on an 8% or 10% polyacrylamide/7 M urea gel followed by autoradiography.

RESULTS

Cell-Free Transcription of the *Pgk* Genes. bFGF exists in the testis (36, 37), and its role in the function of nonspermatogenic Leydig and Sertoli cells has been suggested (38). Moreover, bFGF has been discovered recently in the nuclei of mouse spermatogenic cells (13). We first tested the effect of bFGF on cell-free transcription of the mouse genes encoding two Pgk isozymes that are involved in a transcriptional switch during spermatogenesis from somatic-type Pgk-1 to sperm-specific Pgk-2 (35). During primer extension analysis of Pgk-2 mRNA in the mouse testis, we found clustered transcription initiation sites in a region more proximal to the coding sequences than those previously identified (28) (Fig. 2). Failure to detect these proximal initiation sites in our previous study was probably due to insufficient

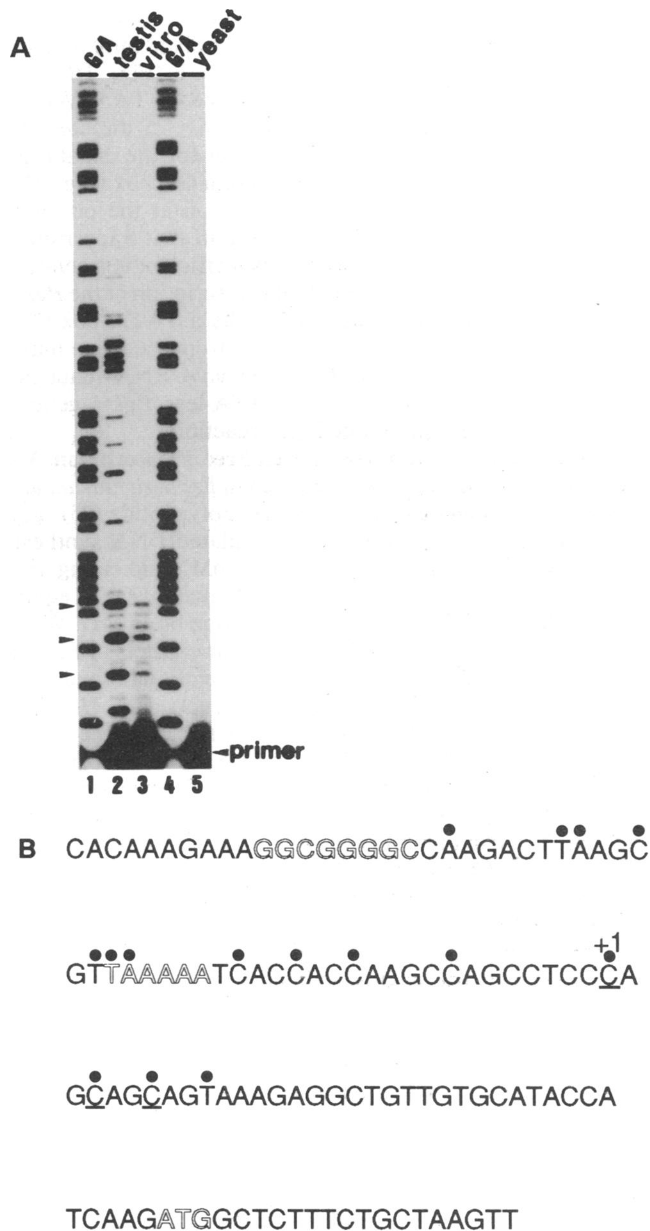

FIG. 2. Determination of transcription initiation sites of the *Pgk-2* gene with *in vivo* and *in vitro* RNAs. (*A*) Primer-extension analysis of RNA transcripts. Adult mouse testis poly(A)-containing RNA, 1 μg (lane 2), transcripts from P2EBg DNA in a cell-free reaction (lane 3), and 1 μg of yeast soluble RNA (lane 5) were analyzed by primer extension with a ^{32}P-labeled *Pgk-2* primer. Primer-extended products were separated on a 10% polyacrylamide/7 M urea gel followed by autoradiography. The positions of three major signals derived from *in vitro* transcripts are indicated with arrowheads. Lanes 1 and 4 contain a G/A sequence ladder (39) as markers. (*B*) Transcription initiation sites of the *Pgk-2* gene. The sequence of the 5′-end region of the *Pgk-2* gene (28) is shown. Transcription initiation sites observed with RNAs *in vivo* and *in vitro* are indicated with dots and underlines, respectively. The most distal initiation site in cell-free transcription was numbered +1. A GC box-like sequence, a TATA box-like sequence, and a translation initiation codon are shown by outlined (blackboard) letters.

resolution of the primer-extended products during electrophoresis, since the signals migrated close to the free primer. Transcription of the *Pgk-2* gene occurred mostly at the proximal sites in a cell-free system (lanes 2 and 3 in Fig. 2*A*), and transcripts from the distal sites were detectable only when the gel was exposed to an x-ray film for an extended period (data not shown). This result was confirmed by an S1

nuclease mapping assay of the cell-free transcripts (data not shown). We tentatively defined the most distal initiation site observed in cell-free transcription as +1 (Fig. 2*B*). We presumed that a TATA box-like sequence, 5′-TAAAAA-3′, in the region around nucleotide position −25 directed the proximal transcription, while a promoter for the distal transcription seemed to be under control of a GC box (Fig. 2*B*). The more frequent transcription initiation at the proximal sites was consistent with a previous report that transcription driven by a TATA-less promoter was inefficient in a cell-free assay (40). However, we found that transcription of the *Pgk-1* gene, whose promoter apparently lacks a TATA box (28), occurred actively in a cell-free system from the same initiation sites as those observed with *in vivo* RNA (data not shown). It is not clear why the TATA-less *Pgk-1* gene is efficiently transcribed in a cell-free reaction.

Differential Effect of bFGF on Cell-Free Transcription. We used human bFGF that was produced in *Escherichia coli* and purified to homogeneity as an 18-kDa polypeptide (25) (Fig. 3*A*). This preparation maximally stimulated DNA synthesis in BALB/c3T3 cells at 90 pg/ml (5 pM), indicating that mitogenic activity of the recombinant human bFGF is equivalent to that of bFGF purified from bovine brain (41). When the *Pgk-1* gene was transcribed in nuclear extracts of Ehrlich ascites tumor cells, exogenous bFGF greatly inhibited transcription from two different DNA constructs (Fig. 3*B*, lanes 1–4). On the contrary, transcription of the *Pgk-2* gene was significantly stimulated in the presence of bFGF (Fig. 3*B*, lanes 5 and 6). The different appearance of primer-extended products from the *Pgk-2* promoter between this experiment and that shown in Fig. 2*A* is due to the use of different primers and gels. Measurement of the transcription signals in five independent experiments, including those shown here, revealed that bFGF (144 μg/ml) stimulates *Pgk-2* gene transcription about 5-fold (4.9 ± 0.9). Transcription of the Ad2 major late DNA was slightly enhanced, but that of the Ad12 early gene *E1A* and the HIV-1 DNA was not affected by the addition of bFGF (Fig. 3*B*, lanes 7–12). These results show that bFGF directly modulates transcription in a cell-free system either positively or negatively depending on the genes used as DNA templates.

We next examined the dose–response of bFGF in a transcription reaction containing both the *Pgk-1* and *Pgk-2* genes (Fig. 4*A*). As the amount of bFGF increased, *Pgk-1* gene transcription gradually decreased, whereas transcription of the *Pgk-2* gene increased. Transcription of the two *Pgk* genes was completely shut off when more than 0.4 mg of bFGF per ml was added to the reaction (data not shown). As much as 40 μg (2.2 μM) of bFGF per ml was required to affect transcription of either of the *Pgk* genes. An anti-bFGF monoclonal antibody was then added to the transcription reaction to see if the effects on *Pgk* gene transcription observed above were actually caused by bFGF. A monoclonal antibody, bFM-1, raised against bovine brain bFGF, which neutralizes the mitogenic action of bFGF (22), almost eliminated the stimulatory effect of bFGF on *Pgk-2* gene transcription, whereas the inhibitory effect on *Pgk-1* gene transcription was unchanged (Fig. 4*B*, lanes 3 and 7). Similar results were obtained when another monoclonal antibody, bFM-2, was used (data not shown). A monoclonal antibody against epidermal growth factor used as a control had no effect (Fig. 4*B*, lanes 4 and 8). These results indicate that stimulation of *Pgk-2* gene transcription was indeed caused by bFGF and suggest that the same molecular domain of bFGF is responsible for the stimulatory function in cell growth and in cell-free transcription. The basis of the inhibitory effect on *Pgk-1* gene transcription remains to be elucidated.

Mechanism of bFGF Action in Cell-Free Transcription. To examine whether transcription stimulation of the *Pgk-2* gene by bFGF requires particular DNA regions, we first examined the effect of deletions in the 5′ upstream region of the *Pgk-2* gene in cell-free transcription (Fig. 5). Deletions expanding to

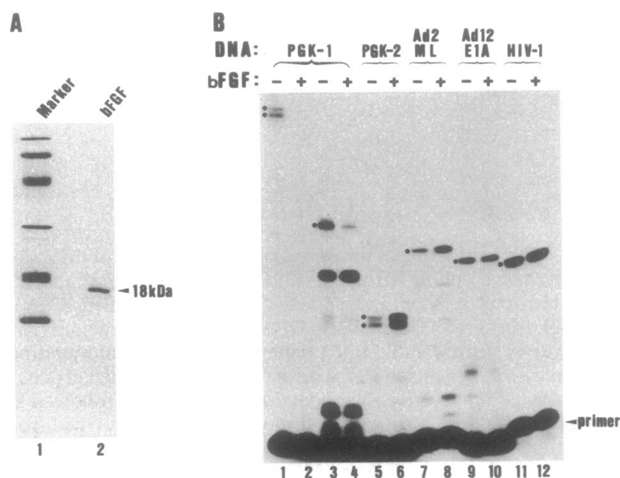

FIG. 3. Effect of bFGF on cell-free transcription of various genes. (*A*) Gel electrophoresis of purified bFGF. Bacterially expressed human bFGF, 0.72 μg, and size markers were separated on a 10% polyacrylamide gel containing sodium dodecyl sulfate and stained with Coomassie brilliant blue R250. (*B*) Primer extension analysis of cell-free transcripts from various DNA templates. P1BCAT (lanes 1 and 2), P1HCAT (lanes 3 and 4), P2ECAT (lanes 5 and 6), pSmaF (lanes 7 and 8), pE1ACAT (lanes 9 and 10), and pHIVCAT (lanes 11 and 12) DNAs were transcribed in cell-free reactions in the presence (lanes +) or absence (lanes −) of bFGF at 144 μg/ml (8 μM). RNA transcripts were analyzed by primer extension with various ^{32}P-labeled primers as described. An autoradiogram of an 8% polyacrylamide/7 M urea gel is shown. Primer-extended products derived from accurate transcripts are marked with dots, and the position of free primers is indicated by an arrowhead.

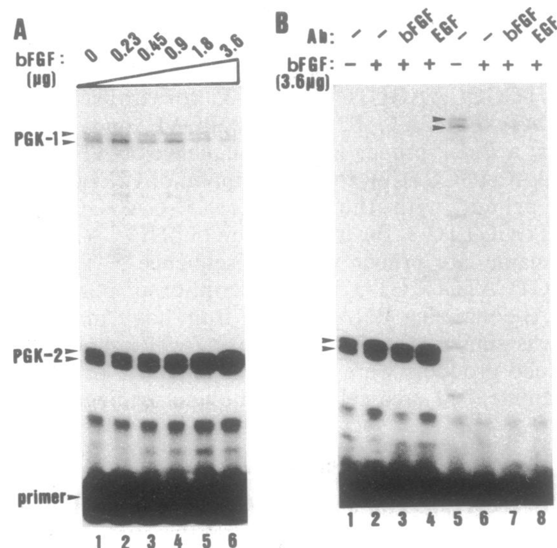

FIG. 4. Dose–response of bFGF and the effect of antibodies. (*A*) Dose–response of bFGF in cell-free transcription of the two *Pgk* genes. P1BCAT and P2ECAT DNAs were simultaneously transcribed with increasing amounts of bFGF (0, 9.2, 18, 36, 72, and 144 μg/ml in lanes 1–6, respectively). Positions of the primer-extended products derived from accurate transcripts are shown by arrowheads. (*B*) Effect of anti-bFGF antibody on bFGF action. P2ECAT (lanes 1–4) and P1BCAT (lanes 5–8) DNAs were transcribed in the presence (lanes 2–4 and 6–8) and absence (lanes 1 and 5) of bFGF at 144 μg/ml. Monoclonal antibodies against bFGF (lanes 3 and 7) and against epidermal growth factor (EGF) (lanes 4 and 8) were added at 0.4 mg/ml to the transcription reaction.

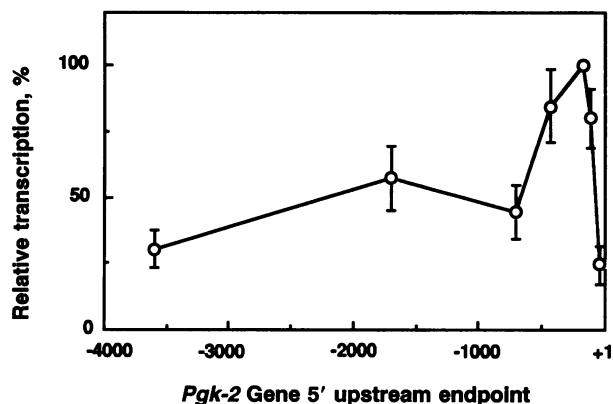

FIG. 5. Effect of the 5′ upstream region on cell-free transcription of the *Pgk-2* gene. DNA fragments containing various 5′-end regions of the *Pgk-2* gene were inserted in pBLCAT3 at the upstream of CAT gene, and resultant DNAs (20 μg/ml) were transcribed in nuclear extracts of Ehrlich ascites tumor cells. Transcripts were analyzed by primer extension with a ^{32}P-labeled CAT gene primer. The amounts of specific primer-extended products were quantified in an image analyzer and normalized relative to the value in the reaction with DNA containing the 5′-end region 170 bp upstream as 100%. The 5′ endpoints of DNA constructs, from left to right, are −3600 (P2ECAT), −1700, −710, −430 (P2PCAT), −170, −110, and −43. Vertical bars show the standard deviations from four independent experiments.

nucleotide position −710 did not cause a significant change in *Pgk-2* gene transcription. However, transcription increased when the deletion continued to nucleotide position −430. A further deletion in the next 280-bp region resulted in the most efficient transcription, but transcription markedly decreased with the loss of adjacent regions near the transcription initiation site (+1). These results suggest that *Pgk-2* gene transcription is regulated by both negative and positive DNA elements present in the regions between nucleotide positions −710 and −170, and −170 and −43, respectively.

We next performed cell-free transcription in the presence and absence of bFGF using P2ECAT and P2PCAT DNAs that contained the 3.6- and 0.43-kbp upstream regions, respectively. Transcription from P2PCAT DNA was not altered by the addition of bFGF (Fig. 6, lanes 4–6), whereas transcription from P2ECAT DNA was stimulated (lanes 1–3). Transcription from a DNA having a 1.7-kbp upstream region

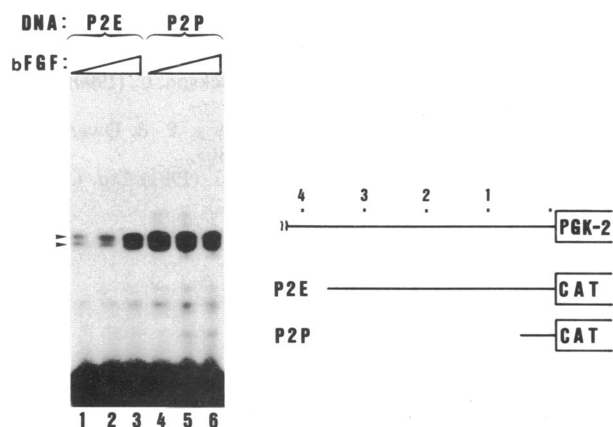

FIG. 6. Requirement of the 5′ upstream region for transcription stimulation by bFGF. Two different DNA constructs of the *Pgk-2* gene were transcribed in the presence of bFGF [none in lanes 1 and 4, 72 μg/ml (4 μM) in lanes 2 and 5, and 144 μg/ml (8 μM) in lanes 3 and 6]. The DNA templates were P2ECAT containing a 3.6-kbp upstream region (lanes 1–3) and P2PCAT containing a 430-bp upstream region (lanes 4–6).

was still enhanced in the presence of bFGF (data not shown). These results indicate that stimulation of *Pgk-2* gene transcription by bFGF depends on the presence of the region between nucleotide positions −1700 and −430, which might include a cis-acting negative element for *Pgk-2* gene transcription (Fig. 5). It is likely that bFGF requires the postulated negative cis element to achieve transcription stimulation of the *Pgk-2* gene in a cell-free reaction.

DISCUSSION

We have demonstrated that bFGF modulates gene transcription in a cell-free system, suggesting an alternative mechanism for the signal transduction pathway provided by FGF. The generally accepted mechanism involves second messengers that transmit growth factor-derived signals to the cell nucleus. Our results suggest that FGF directly regulates transcription of various genes after being transported into the cell nucleus by a nuclear targeting signal (17–20). Nuclear localization of bFGF has been reported in a variety of cultured cells and tissues (10–14). It also has been reported that exogenous bFGF is translocated to and accumulates in the nucleolus of cultured endothelial cells (15, 16) and that bFGF synthesized in cultured cells upon DNA transfection is localized in the nucleus (12, 17, 19). However, it is unclear whether bFGF enters cells through interaction with a specific receptor on the cell membrane (1). Other growth factors such as epidermal growth factor, nerve growth factor, and platelet-derived growth factor are also incorporated into cultured cells, and considerable amounts of those factors are recovered in the chromatin fraction (42). The platelet-derived growth factor A chain (43) and FGF-related Int-2 oncoprotein (44) are localized to the cell nucleus, depending on the presence of particular amino acid residues near the amino terminus, when DNAs encoding those proteins are introduced into cultured cells. Interleukin-1 is transported to the nucleus as a complex with its receptor (45). Therefore, a feasible model of growth factor action could involve translocation of the factors into the cell nucleus and their direct participation in the regulation of transcription, replication, or other nuclear events.

Transcription modulation by bFGF appears to exhibit gene specificity. In addition, transcription stimulation of the *Pgk-2* gene by bFGF requires the presence of a distinct DNA region that includes a negative cis-acting element possessing a silencer-like function (K.M., Y.M., and Y.N., unpublished data). Thus, it is probable that bFGF alters the function of cis elements determining the transcription efficiency of each gene. bFGF probably competes with trans-acting factors for binding to corresponding cis elements, resulting either in stimulation or inhibition of transcription depending on the mode of action of those cis elements. In the case of the *Pgk-2* gene, we presume that transcription stimulation occurs when bFGF occupies the negative cis element, preventing the putative sequence-specific trans-acting factor from binding to it. When bFGF was tested for DNA-binding activity with several DNA probes in gel-shift and DNase I footprint assays, nonspecific DNA-binding was always observed (data not shown). Therefore, it is likely that bFGF does not recognize any distinct DNA regions but rather binds to DNA in a sequence-independent fashion. This could explain why micromolar orders of bFGF were required to affect gene transcription. Moreover, many cultured cell lines and animal tissues contain considerable amounts of bFGF mostly localized in the cell nucleus. For example, the concentration of nuclear bFGF in a cell line A431 was determined to be as much as 54 μg/ml (about 3 μM) by a radioimmunoassay using bFM-1 (26), and the content of nuclear bFGF in transformed fetal bovine aortic endothelial GM7372 cells has been estimated to be 10^5 molecules per nucleus (corresponding to a

concentration of about 1.5 μM) (46). We therefore maintain that the concentration of 140 μg/ml, which was required for bFGF to achieve maximum stimulation of *Pgk-2* gene transcription *in vitro* (Fig. 4), is not far beyond physiological concentrations of bFGF in the cell nucleus.

Inhibition of *Pgk-1* and stimulation of *Pgk-2* gene transcription by bFGF coincide well with the transcription switch of the two *Pgk* genes, which has been suggested to occur in the mouse testis during spermatogenesis (28, 35). Hence, besides the action on nonspermatogenic Leydig and Sertoli cells (38), bFGF might play an important role in the mammalian spermatogenic pathway by regulating the transcription of specific spermatogenic genes such as *Pgk*. Although further studies are required to draw any conclusions as to the role of bFGF in transcription regulation, we believe that our findings provide new insight into growth-factor actions.

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