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Expression of synthetic genes encoding bovine and human basic fibroblast growth factors (bFGFs) in *Escherichia coli*

(Recombinant DNA; fusion protein; β-galactosidase; linker; affinity chromatography; angiogenic capacity)

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SUMMARY

Synthetic genes encoding bovine and human basic fibroblast growth factors (bFGFs) were assembled and cloned using established *Escherichia coli* expression plasmids. Transformed *E. coli* cells were able to synthesize either a fusion protein, comprising the first seven amino acids of β-galactosidase, a linker fragment and bovine FGF, or genomic human bFGF. The two growth factors were purified from *E. coli* lysates by cation exchange and heparin-Sepharose affinity chromatography. The purified recombinant proteins were biologically active as monitored by their mitogenic activity for bovine aortic endothelial cells and their angiogenic capacity in the rabbit cornea.

INTRODUCTION

The growth of the new capillaries (angiogenesis) is a complex process which involves both the migration and proliferation of vascular endothelial cells (Folkman, 1985). The vascular endothelium of a normal adult organism rejuvenates only a few times. On the other hand, a rapid turnover can be seen in various pathophysiological responses such as acute inflammation, wound healing, and tumor angiogenesis (Folkman and Klagsbrun, 1987). Basic FGF (bFGF) has been shown to be a very potent mitogen

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Abbreviations: aa, amino acid(s); aFGF, acidic FGF; BAE, bovine aortic endothelial; bFGF, basic FGF; BSA, bovine serum albumin; buffer L, see MATERIALS AND METHODS, section e*l*; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; ED_{50}, half-maximal stimulation of cell proliferation; Elvax, ethylene vinyl acetate; FCS, fetal calf serum; FGF, fibroblast growth factor; HPLC, high-performance liquid chromatography; nt, nucleotide(s); oligo, oligodeoxyribonucleotide; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline: 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4; PolIk, Klenow (large) fragment of *E. coli* DNA polymerase I; SDS, sodium dodecyl sulfate; TIR, translation initiation region; [ ], designates plasmid-carrier state.

and chemoattractant for endothelial cells in vitro (for review, see Gospodarowicz et al., 1986). It stimulates angiogenesis in vivo on the chick chorioallantoic membrane and rabbit cornea (for review, see Folkman and Klagsbrun, 1987).

Bovine bFGF has been purified and characterized from a wide range of tissues including pituitary, brain, hypothalamus, retina, adrenal gland, thymus, corpus luteum, and kidney (Lobb et al., 1986; Gospodarowicz et al., 1986). Although the amino acid and nucleotide sequences of both bovine and human bFGF are known (Abraham et al., 1986), little protein material has up to now been available. It is therefore desirable to produce sufficient quantities of recombinant bFGF to be able to tackle questions concerning the biological role of signals that naturally trigger angiogenesis in vivo or to obtain sufficient growth-factor protein for use as an (essential) component for serum-free cell-culture medium.

Here we report the design, assembly, and expression of synthetic genes encoding bovine and human bFGFs. Since bFGF is a single-chain non glycosylated polypeptide, it seemed reasonable to expect that biologically active material might be produced in *E. coli*. Two different vector systems were used to obtain synthesis of the factors in fused and unfused forms. An initial step toward optimization of the direct expression of human bFGF was taken by manipulating the N-terminal part of the synthetic gene so as to allow efficient translational initiation in the pJLA503 expression vector.

MATERIALS AND METHODS

(a) Reagents and enzymes

Restriction endonucleases and BSA were purchased from Boehringer Mannheim. Bacteriophage T4 DNA ligase, polynucleotide kinase, PolIk, nucleic-acid-grade agarose, heparin-Sepharose, CL-6B; CM-Sephadex C-50, Bio-Gel-P2, and NAP-10 columns were obtained from Pharmacia. Diisopropyl phosphoramidites were obtained from Applied Biosystems, phosphotriester reagents were obtained from Biosearch; [α-^{35}S]dATP and [γ-^{32}P]ATP were obtained from Amersham; DEAE NA45 membranes and nitrocellulose filters were purchased from Schleicher & Schuell; the x-ray films RX were obtained from Fuji; Costar plates were obtained from Costar, Cambridge, MA; PBS, DMEM, and trypsin-EDTA solution were obtained from Gibco; peroxidase-conjugated goat anti-rabbit IgG antibody was purchased from Medac; and Biorad protein assay was obtained from Biorad.

(b) Synthesis and purification of oligodeoxynucleotides

Oligos 1–26 were synthesized using phosphotriester chemistry on the Biosearch model Cyclone oligo synthesizer. Oligos were purified by electrophoresis on 12% to 20% polyacrylamide gel under denaturing conditions. Gel-purified oligos were eluted from the gel in water and desalted on NAP-10 columns.

(c) Phosphorylation and ligation of oligodeoxynucleotides

Oligos used in the assembly of the synthetic *bFGF* gene were phosphorylated as follows: 500 ng of each purified oligo with the exception of numbers 1, 8, 9, 14, 15, 22, 23, and 26 were incubated in 10 μl of 70 mM Tris · HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM DTT, 10 μM ATP, and 0.15 μM [γ-^{32}P]ATP (specific activity 6000 Ci/mmol) containing 10 units of T4 polynucleotide kinase. After 1 h at 37°C, the kinase was inactivated by heating to 95°C for 2 min. After separating labeled DNA from unincorporated [γ-^{32}P]ATP by chromatography through small columns of Bio-Gel-P2, the following subsets of oligos were mixed for ligation to the fragments A, B, C, and C′, respectively: oligos Nos. 1–8, 9–14, 15–22, and 23–26. The indicated subsets were heated to 80°C for 10 min and then slowly cooled to 25°C over 1 h to allow the complementary strands to anneal.

Each solution was adjusted to 70 mM Tris · HCl pH 7.2, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, and 5 mM DTT. Then 0.5 mM ATP T4 DNA ligase was added to 0.8 units per 10 μl and the reaction mixture was incubated overnight at 16°C. Fragments A, B, and C were purified in a 7 M urea/12% polyacrylamide gel run in 90 mM Tris, 90 mM borate, and 2 mM EDTA. The wet gel was autoradiographed overnight and the detected DNA fragments were electroeluted into dialysis bags.

Each gel-purified fragment was incubated in 10 µl of 50 mM Tris · HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine, 0.1 mM EDTA, 0.125 mM ATP, and 10 units of T4 polynucleotide kinase. After 1 h at 37 °C, the kinase was inactivated by heating to 95 °C for 2 min.

The cloning of the kinased fragments took place as follows: fragment A was ligated to *Eco*RI + *Pst*I-digested M13mp18; fragment B to *Pst*I + *Xba*I-digested pGem 4, and fragment C, C′ to *Xba*I + *Hin*dIII-digested M13mp18 using a 10:1 molar ratio of insert : vector. Each double-digested vector DNA was dephosphorylated prior to the insert ligation in 30 µl of 50 mM Tris · HCl, pH 9.5, 50 mM Tris · HCl, pH 9.5, 50 mM NaCl, 0.5 mM DTT, containing 1 µg digested vector DNA and 1 unit of calf intestinal phosphatase. After incubation for 1 h at 50 °C, the enzyme was inactivated by adding 2 µl of 0.5 mM EDTA.

(d) Plasmid construction and DNA cloning

Standard procedures were used for plasmid purification (Birnboim and Doly, 1979), transformation (Mandel and Higa, 1970) and DNA sequencing (Sanger et al., 1977). Large plasmid fragments were separated by agarose gel electrophoresis followed by electroelution on DEAE-NA45 membranes.

(e) Isolation of recombinant basic fibroblast growth factor

(1) Cell lysis

250 ml of an appropriate culture was centrifuged for 15 min at 4000 rev./min. and 0 °C. The cell pellet was resuspended in 4 ml buffer L (50 mM Tris · HCl, pH 8.0, 10 mM EDTA) containing 25% sucrose. After adding 6 mg lysozyme in 1.2 ml buffer L and 2.4 ml of 0.25 M EDTA, 0.25 M Tris · HCl, pH 8.0, the mixture was incubated for 1 h at 0 °C. Then 10 ml of 2% Triton X-100 in buffer L was added and, following sonication at 0 °C, soluble and insoluble material was separated by centrifugation for 17 min at 6000 rev./min. The pellet containing bFGF inclusion bodies was washed twice with 2% Triton X-100 in buffer L and used for purification (see subsection *3*, below).

(2) Purification of soluble FGF

The supernatant remaining after cell lysis (see subsection *1*, above) was applied to a CM Sephadex C-50 column (10 ml bed volume, diameter 4 cm, flow rate 100 ml/h, at room temperature), washed with 0.1 M Na · phosphate, pH 6.5, 0.15 M NaCl, and eluted with 0.1 M Na · phosphate, pH 6.5, 0.6 M NaCl. The eluate was applied to heparin-Sepharose column (2 ml bed volume, diameter 1 cm, flow rate 50 ml/h). The column was washed with 10 mM Tris · HCl, pH 7.0, 0.8 M NaCl and eluted with 10 mM Tris · HCl, pH 7.0, 2.0 M NaCl. The eluate was dialysed against 50 mM ammonium formate and lyophilized.

(3) Preparation of bFGF from inclusion bodies

The washed pellet obtained after cell lysis (see subsection *1*, above) was solubilized in 10 ml buffer L containing 6 M guanidinium · HCl and 0.25 M DTT and incubated for 2 h at 50 °C. Buffer L (30 ml) was then added slowly with vigorous stirring and simultaneous slow cooling to 30 °C. After dialyzing against 10 mM Tris · HCl, pH 7.0, 0.8 M NaCl and subsequent centrifugation, the supernatant was applied directly to a heparin-Sepharose column as described above.

(f) Preparation of rabbit anti-bovine basic fibroblast growth factor

A peptide corresponding to the 19 aa (FPPGHFKDPKRLYCKNGGF) near the N terminus of bovine bFGF (Esch et al., 1985) was synthesized by solid-phase methods using a Biosearch peptide synthesizer. The synthetic peptide was purified by reverse-phase HPLC and then coupled to BSA using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC; Shapira et al., 1984). Rabbits were immunized with 0.5 mg of the BSA-peptide conjugate emulsified with complete Freund's adjuvant. Three weeks later, a booster injection was given using 0.3 mg of the conjugate emulsified in incomplete Freud's adjuvant. Sera were tested by Western blot analysis (see section **h**, below) using bovine brain-derived bFGF.

(g) SDS–PAGE and silver staining

Growth factor fractions from various purification steps were dialyzed against 50 mM ammonium formate overnight, lyophilized, boiled in sample buffer, and analyzed on SDS–15% PAGE (Laemmli, 1970). After electrophoresis the polypeptide bands were visualized by silver staining (Oakley et al., 1980).

(h) Western-blot analysis

For Western immunoblotting analysis, electrophoretically separated polypeptides were transferred onto nitrocellulose at 0.3 mA/cm^2 in 20 mM Tris, pH 7.4, 150 mM glycine, and 20% methanol overnight (Towbin et al., 1979). To block nonspecific binding, the nitrocellulose was incubated with PBS containing 3% BSA for 1 h at room temperature and washed twice with PBS containing 0.1% Tween-20.

The first antibody reaction was carried out by incubating the nitrocellulose with a 1000-fold dilution of the rabbit anti-bovine bFGF antibody in PBS containing 0.1% Tween-20 and 1% BSA for 2 h at room temperature followed by 3 washing steps with PBS containing 0.1% Tween-20. The nitrocellulose was then incubated with a 500-fold dilution of the peroxidase-conjugated goat anti-rabbit IgG antibody for 1 h at room temperature followed by washing.

The immunoreaction was visualized using 0.5 mg/ml diaminobenzidine · HCl in 50 mM Tris · HCl, pH 7.6, 100 mM NaCl, 0.4% nickel sulfate, and 0.01% H_2O_2.

(i) Proliferation assay

Fractions were assayed for growth factor activity on BAE cells as described (Risau, 1986). Briefly, cells were resuspended in DMEM supplemented with 4% FCS, glutamine (0.5 mg/ml), and penicillin/streptomycin (100 units/ml), and plated (10000 cells/0.5 ml per well) on 24-well plates. Unattached cells were removed one day later and 0.5 ml of fresh supplemented DMEM containing growth-factor fractions was added. After three days of incubation, cells were detached from the plates using trypsin/EDTA solution and counted in a coulter counter.

(j) In vivo assay

Heparin-Sepharose-purified recombinant bFGF in Elvax polymers (Langer et al., 1986) was implanted into rabbit corneal pockets as described (Gimbrone et al., 1974; Risau, 1986).

RESULTS AND DISCUSSION

(a) Construction of the bovine and human *bFGF* gene

A synthetic gene encoding the 146 aa of bovine bFGF was designed on the basis of the known amino acid sequence (Esch et al., 1985). The selection of the precise nucleotide sequence for the gene was influenced by the following considerations: (1) The high frequency of a set of statistically 'preferred' codons among highly expressed *E. coli* genes; (2) the obvious convenience of introducing unique restriction endonuclease recognition sites into the sequence; and (3) the need to avoid the presence of excessively stable secondary structures in the encoded mRNA, especially in the early part of the cistron.

Twenty-two oligos ranging in size from 24 to 52 nt were synthesized, purified, and assembled to form the synthetic bovine *bFGF* gene (Fig. 1). To assemble the gene, subsets of oligos were ligated to generate the fragments A, B, and C. These fragments were purified by PAGE under denaturing conditions, phosphorylated, and ligated to appropriately digested, dephosphorylated vectors. The resulting hybrid plasmids were transferred into *E. coli* cells.

Clones containing the predicted DNA inserts as determined by double-digestion of the respective plasmid DNA were selected for the preparation of larger quantities of the A, B, and C fragments, which were subsequently isolated and annealed. The resulting synthetic gene was ligated to *Eco*RI + *Hin*dII-digested pUC8 and used to transform *E. coli* HB101. A clone containing the predicted nucleotide sequence, as determined by Sanger et al. (1977) sequencing, was designated pUC8ABC.

The synthetic gene encoding the 146 aa of the human bFGF was initially constructed by exchanging fragment C of pUC8ABC for a further fragment

C′ (Fig. 1) that was synthesized according to the published sequence of human *bFGF* (Abraham et al., 1986). N-terminally extended forms of the gene were constructed by exchanging the first part of the synthetic sequence with appropriate oligo pairs.

(b) Expression of basic fibroblast growth factor

Plasmid pUC12, an M13mp7-derived system (Vieira and Messing, 1982), was doubly digested with *Sma*I + *Hin*dIII, dephosphorylated, and ligated

Prominent polypeptides with apparent M_rs of

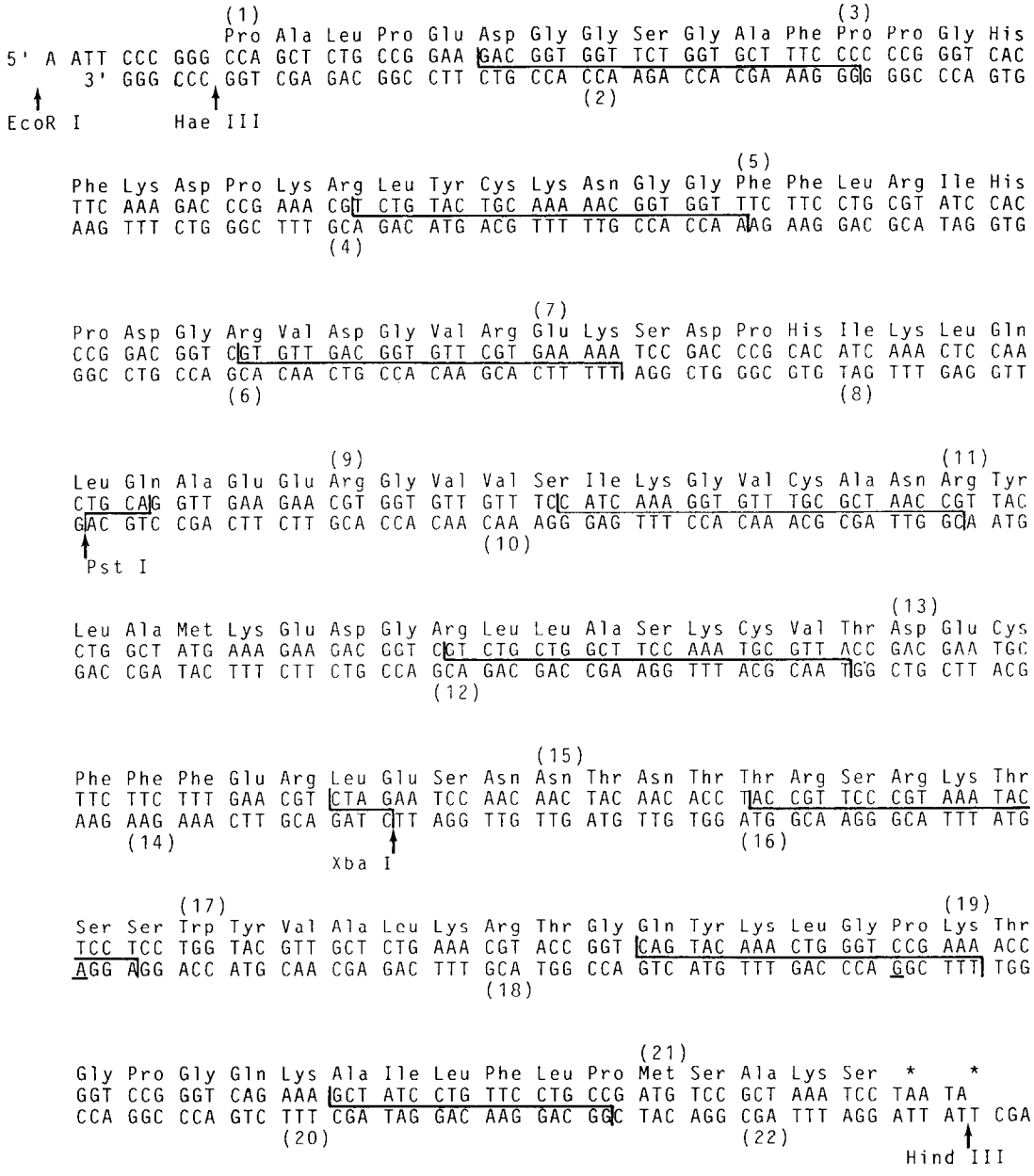

Fig. 1. Nucleotide sequence of the synthetic bovine *bFGF* gene. Numbers in parentheses identify the 22 oligos used in the gene assembly. The lines denote the ends of the oligos and the respective overlapping regions between fragments. Unique restriction sites are marked by upward arrows. Asterisks represent stop codons. The human *bFGF* nucleotide sequence was obtained by exchanging the underlined base pairs T/A of a Ser codon and C/G of a Pro codon into A/T and T/A, respectively, resulting in codons for Thr and Ser.

with the *Hae*III-*Hin*dIII fragment of pUC8ABC containing the synthetic bovine bFGF nucleotide sequence. The resulting recombinant plasmid LD1/2 encodes a 156-aa fusion protein consisting of the first 7 aa of β-galactosidase, 3 aa encoded by part of the multiple cloning site, and the 146 aa of bovine bFGF.

Computer analysis of the translational initiation region of the pUC12-bbFGF encoded mRNA predicted a highly stable (ΔG at 37°C = −14 kcal) hairpin loop structure immediately adjacent to the Shine–Dalgarno region (Fig. 2a). Such a structure would be expected to impinge on translational initiation (see, e.g., McCarthy and Bokelmann, 1988).

In the case of human bFGF a number of constructs differing in the nature of the N-terminal part of the synthetic gene were subjected to analysis. Extra codons, corresponding to part or all of the N-terminal extension to the 146-aa form of bFGF identified by nucleotide sequencing (Abraham et al., 1986), were included in these constructs. One construct encoded the full 155 aa described by Abraham et al. (1986). The extra N-terminal sequence was borne on an *Nde*I-*Hin*dIII DNA fragment, whereby the codon usage was adjusted to prevent the formation of highly stable secondary structure within the translational initiation region formed upon insertion of this new synthetic gene into the expression vector pJLA503 (Fig. 2b). The 155-codon gene, containing the *Nde*I-*Hin*dIII fragment, was initially subcloned into pCB179 (Schneider and Beck, 1987). It was subsequently excised as an *Nde*I-*Sal*I fragment and inserted into appropriately cleaved pJLA503. The latter expression vector is one of a series of plasmids derived from pJLf201 bearing part of the *E. coli atpE* TIR (McCarthy et al., 1986; Schauder et al., 1987). Transcription in these plasmids is initiated by the λ bacteriophage major p_R and p_L promoters (in tandem) and is repressed by the product of the plasmid-borne *c*Its857 gene.

Correct recombinant products, bearing the fused bovine bFGF in pUC12 (LD1/2) and the unfused, fully extended human bFGF in pJLA503 (T36/9), were transferred into *E. coli* cells (strain R1180 : sc122, *lon*$^-$, *htpR*$^-$). Transcription of the human *bFGF* gene was repressed by the λ *c*Its875-coded repressor when cultures of R1180-T36/9 were grown at 30°C. A temperature shift from 30°C to 42°C allowed synthesis of large amounts of human bFGF via inactivation of the λ *c*Its857-coded repressor (Fig. 3). Cultures of R1180[pJLA503] (vector minus DNA insert) grown at 30°C and 42°C served as controls. The bovine bFGF fusion protein encoded by LD1/2 was expressed constitutively in strain R1180.

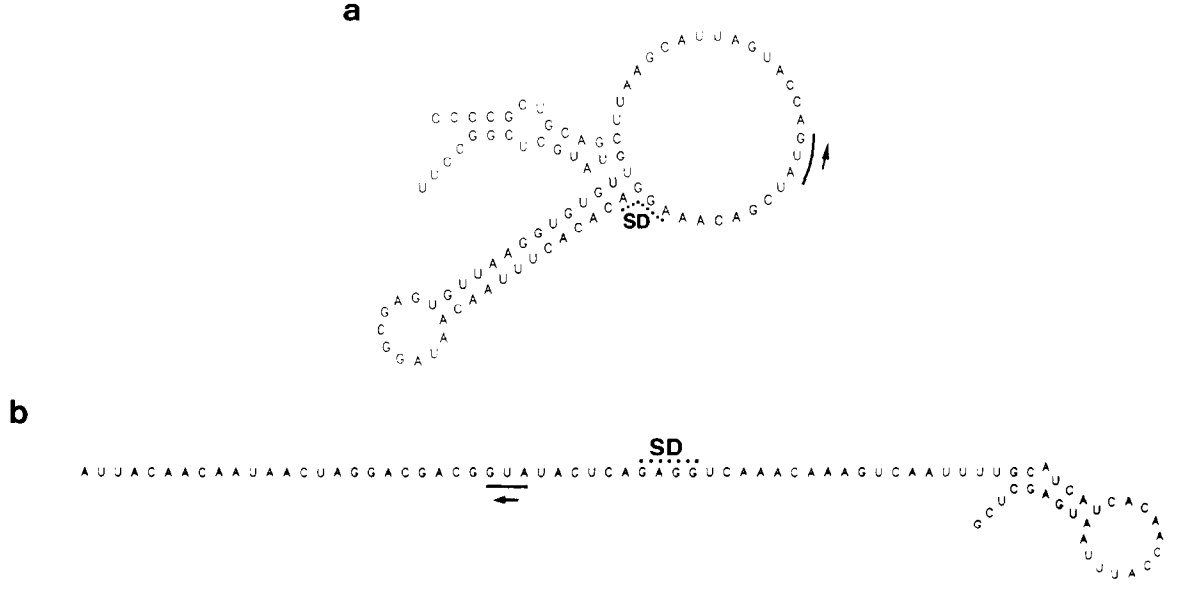

Fig. 2. Secondary structures predicted for the mRNA TIRs encoded by (**a**) pUC12-bbFGF and (**b**) pJLA503-hbFGF (Zuker and Stiegler, 1981). The predicted free energies of base pairing were −14 kcal (pUCI2bbFGF) and + 0.2 kcal (pJLA503-hbFGF) for the regions shown.

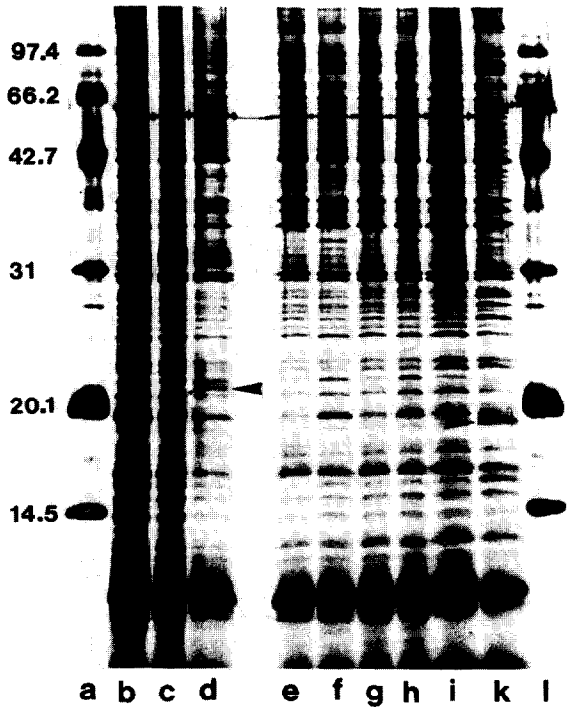

Fig. 3. SDS–15% PAGE analysis of recombinant *bFGF* expression. Cultures of pUC12 (vector only) and LD1/2 (vector + bFGF) were grown overnight at 37°C (final $A_{600\,nm} = 4$); cultures of pJLA503 (vector only) and T36/9 (vector + *bFGF*) *E. coli* R1180 were grown at 30°C (*A* 1), then induced for 3 h at 42°C (*A* 2). Each culture was then diluted to an *A* of 0.5; 40 µl along with 20 µl of loading buffer were boiled for 10 min. Of each sample solution 10 µl were loaded onto the gel. Lanes: **a** and **l**, molecular size protein standards (in kDa, as indicated at the left); **b**, R1180 cells, 37°C; **c**, R1180[pUC12] DNA, 37°C; **d**, R1180[pUC12-bbFGF], 37°C; **e**, R1180 cells, 30°C; **f**, R1180 cells, 42°C; **g**, R1180[pJLA503] DNA, 30°C; **h**, R1180-[pJLA503], 42°C; **i**, R1180[pJLA503-hbFGF], 30°C; **k**, R1180-[pJLA503-hbFGF], 42°C. The arrows indicate the protein encoded by pUC12bbFGF (lane **d**) and by pJLA503-hbFGF (lane **k**), respectively.

18 kDa and 20.5 kDa were present in extracts of induced *E. coli* cultures of R1180[T36/9] and R1180[LD1/2], respectively. These proteins were absent in extracts from control cultures.

Recombinant growth factors were purified from *E. coli* cells using a procedure established for the purification of bFGF from bovine brain (Lobb et al., 1986; Risau et al., 1988). Cells were lysed by a combination of Triton X-100 and mild lysozyme treatment. Following sonication the extracts were clarified by centrifugation.

The supernatant was applied to a CM-Sephadex C-50 cation exchange column. The column was washed and growth factor activity eluted using 0.6 M NaCl. Heparin-Sepharose affinity chromatography was used as the second step in purification. Almost all of the extraneous protein present in the Sephadex eluate was separated from growth factor activity in this second step. The FGF activity itself could be eluted using 2 M NaCl. Sephadex and subsequent heparin-Sepharose-purified growth factor fractions were analyzed by the SDS–PAGE, followed by silver staining or immunoblotting using polyclonal antibodies prepared against a bFGF peptide. Cells transformed by LD1/2 plasmid encoding the bovine bFGF/β-galactosidase fusion protein synthesized a protein of 20.5 kDa (Fig. 4a), which is the expected size calculated from the encoded mRNA.

Recombinant human bFGF encoded by the plasmid T36/9 should have an M_r of 18 000 and a protein of approximately this size could be observed after induction (Fig. 4b). Natural bovine brain bFGF served as a control (Fig. 4a). An immunoreactive peptide of lower M_r, probably a product of proteolytic degradation, was also detected using the antibody. Recombinant bovine bFGF fusion protein appeared as a doublet (Fig. 4a), a phenomenon that has also been described for the brain-derived bovine bFGF (Lobb et al., 1986).

FGF was also present in cytoplasmic inclusion bodies, as is often observed with other recombinant proteins expressed at a high level in *E. coli* (Kleid et al., 1981; Simons et al., 1984; Schoner et al., 1985). Proteins in such inclusion bodies are generally soluble only under denaturing conditions. Thus, the pellet fraction obtained after centrifugation of lysed cells was solubilized in a large volume of guanidinium · HCl together with 0.2 M DTT to cleave possible intermolecular disulfide bonds. For renaturation the treated pellet fraction was carefully diluted and dialysed to prevent aggregation of bFGF. The cleared dialysate was directly applied to a heparin-Sepharose affinity column as described above.

(c) Biological activities of bovine and human recombinant fibroblast growth factor

The purified growth factors were analyzed for mitogenic activity on BAE cells. In this assay, the maximal stimulation observed with brain-derived bovine was similar to that with recombinant human

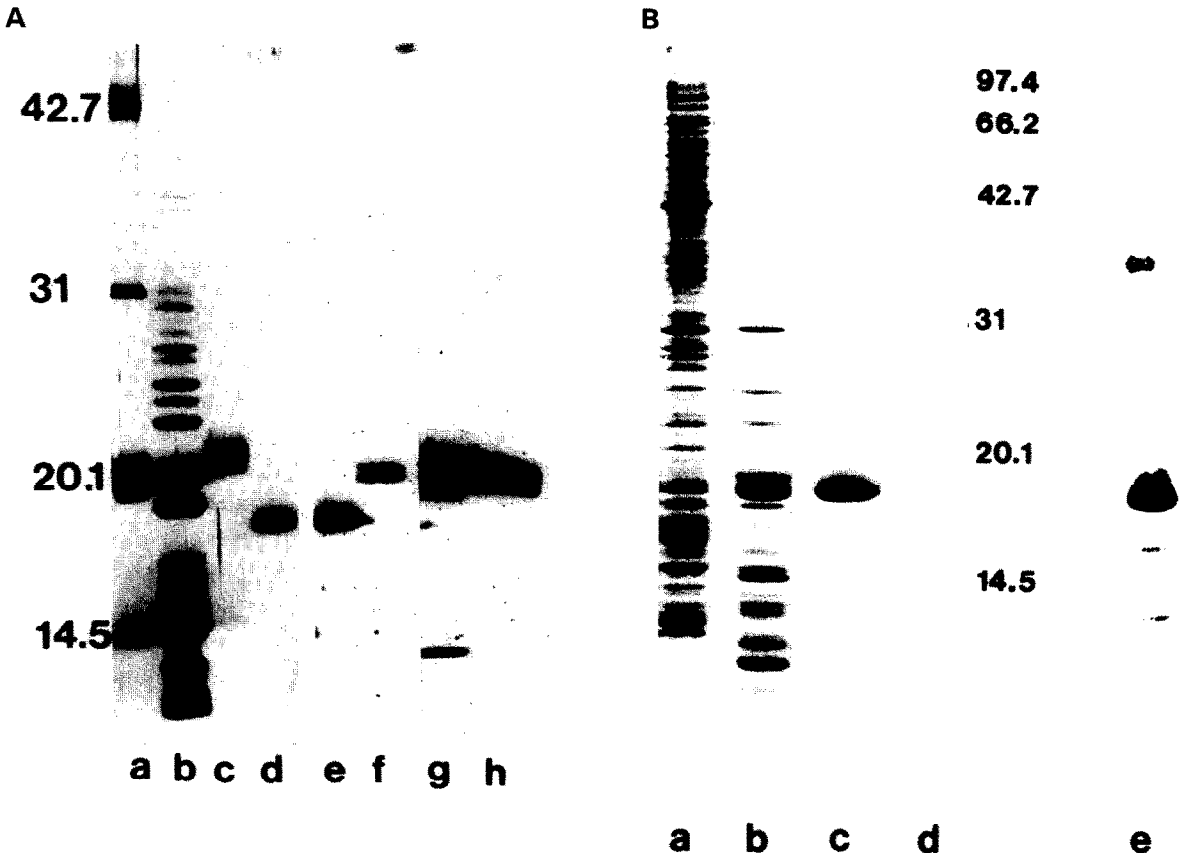

Fig. 4. SDS–PAGE and Western blot analysis of different FGF purification steps. Samples containing about 1 μg protein were separated by SDS–15% PAGE, transferred onto nitrocellulose, incubated with anti-bFGF antibodies, and then reacted with a peroxidase-conjugated goat anti-rabbit IgG antibody. (Panel **A**) Bovine bFGF: lanes **a–d**, silver stained peptides; **e–h**, immunoblot; **a**, molecular size protein standards (kDa as indicated at the left); **b,f**, CM Sephadex C50 eluate; **c,g,h**, heparin-Sepharose eluate; **d,e**, brain-derived bovine bFGF. (Panel **B**) Human bFGF: lanes: **a–d**, silver stained peptides; **a**, cell lysate (R1180[pJLA503-hbFGF], 42°C); **b**, CM Sephadex C50 eluate; **c**, heparin-Sepharose eluate; **d**, molecular size protein standards (kDa as indicated between lanes **d** and **e**); **e**, immunoblot, heparin-Sepharose eluate.

bFGF, as were the concentrations giving half-maximal stimulation [ED_{50} brain-derived bovine bFGF 0.2 ng/ml, ED_{50} recombinant human bFGF (isolated from the supernatant) 0.14 ng/ml] (Fig. 5). The recombinant human bFGF isolated from the pellet fraction showed a somewhat lower ED_{50} of 0.5 ng/ml. The lower specific activity of the human bFGF pellet preparation might be due to the incomplete formation of disulfide bonds that may be important to the correct conformation of bFGF (cf., e.g., aFGF; Thomas et al., 1988). The recombinant bovine bFGF fusion protein showed an ED_{50} of 0.95 ng/ml, probably owing to the presence of additional β-galactosidase amino acids at the N terminus of the molecule.

We also examined the in vivo activity of recombinant bovine bFGF fusion protein and the human bFGF. In rabbit corneas, new sprouts originating from the limbal vascular system penetrated the avascular cornea two days after implantation of Elvax polymers containing 500 ng of recombinant human bFGF and reached the polymer ten days later (Fig. 6). Similarly, recombinant bovine bFGF fusion protein stimulated angiogenesis in the rabbit cornea (not shown). No neovascularization was seen in corneas in which control polymers had been implanted. No inflammation was observed, except for an initial slight cloudiness of some corneas, which disappeared about four days after implantation.

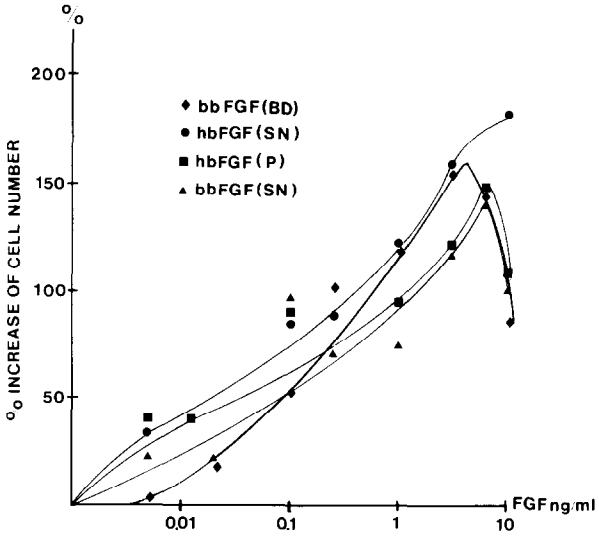

Fig. 5. Proliferation of BAE cells as a function of medium supplement (DMEM supplemented with calf serum and different bFGF preparations). 10^4 cells/20 mm well were seeded in Costar plates as described in MATERIALS AND METHODS, section i. Cells were exposed to medium containing 4% FCS and increasing amounts of growth-factor fractions. ◆, Brain-derived (BD) bovine bFGF, ED_{50}, 0.2 ng/ml; ▲, recombinant bovine bFGF fusion protein, supernatant (SN) ED_{50}, 0.95 ng/ml; ●, recombinant human bFGF, supernatant ED_{50}, 0.14 ng/ml; ■, recombinant human bFGF, pellet (P) ED_{50}, 0.5 ng/ml.

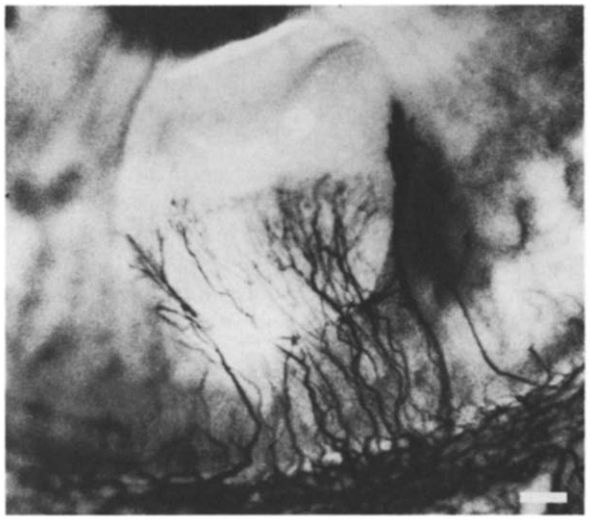

Fig. 6. Angiogenic activity of recombinant human bFGF. Elvax pellets containing 500 ng protein of the heparin-Sepharose purified recombinant bFGF along with rabbit serum albumin to achieve 20% loading of the polymer were implanted into rabbit corneal pockets (four corneas). Capillaries were seen invading the cornea after two days and reached the polymers approx. ten days later. The photograph was taken 18 days after implantation. Bar, 1 mm.

(d) Conclusions

Biologically active recombinant bovine bFGF fusion protein and human bFGF encoded by chemically synthesized genes were isolated in high yield and high purity. They were obtained as a single band on SDS–PAGE (Fig. 4a,b). Quantitation of the expressed growth factors was carried out using the Biorad protein assay.

The described plasmid constructions in the selected *E. coli* strains directed the synthesis of 0.5 mg bovine bFGF protein and 5 mg human bFGF protein per liter culture medium per 1 A_{600nm} isolated from the soluble fraction of the cell extracts.

Three to four times the quantity of biologically active growth factor protein could certainly be obtained from the insoluble material that is also present in the induced cells. This would necessitate the successful renaturation of solubilized inclusion bodies. Large quantities of recombinant bFGF should help us to elucidate the biological function of bFGF in vivo as well as to investigate its potential application under pathological conditions such as wound healing (Sprugel et al., 1988), nerve regeneration (Baird et al., 1988), and cardiac infarction.

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