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Primary Structure of Ovine Fibroblast Growth Factor-1 Deduced by Protein and cDNA Analysis

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The amino acid sequence of full-length ovine fibroblast growth factor-1 (FGF-1) was determined by a combination of protein and cDNA sequencing. FGF-1 cDNA analysis indicated that ovine kidney cells express mRNAs encoding both full-length FGF-1 and a truncated FGF-1 variant. An overall comparison of the ovine FGF-1 primary sequence to the eight species studied to date revealed a high degree of conservation, with ovine FGF-1 sharing 90 and 95% sequence identity with human FGF-1 and bovine FGF-1, respectively. Additionally, the FGF-1 proteins from the various species have conserved cysteine residues at positions 30 and 97 and contain acetylated amino-terminal alanine residues. Mass spectrometry analysis confirmed that the blocking group of ovine FGF-1 is also consistent with that of an acetyl-moiety. In contrast to the other FGF-1 proteins, the 154 residue primary sequence of ovine FGF-1 contains three unique amino acid differences: Arg_9, Arg_{44}, and Ile_{123}. Ovine FGF-1, unlike human FGF-1, is a potent mitogenic factor for NIH 3T3 fibroblasts in the absence of heparin. In the presence of exogenous heparin, the mitogenic activity of ovine FGF-1 is potentiated slightly. © 1998 Academic Press

The fibroblast growth factor (FGF) family presently consists of at least 17 structurally related polypeptides (1-8), four of which have been referred to as fibroblast growth factor homologous factors. The members of the FGF family vary in size but share a conserved ~120-amino acid core with ~30-70% amino acid sequence identity. The FGF proteins are functionally diverse and have putative roles in embryogenesis, cellular proliferation and differentiation, chemotaxis, angiogenesis, and wound healing (1,9). The biological effects of FGF proteins are mediated by binding to high-affinity, tyrosine-kinase receptors (10,11) and to low-affinity heparan sulfate proteoglycans (HSPGs) (12-14).

The prototypes of the FGF family, FGF-1 and FGF-2, also known as acidic FGF and basic FGF, respectively, were isolated initially from bovine pituitary extracts (15) and bovine brain (16,17) as mitogens for fibroblasts. They are the most thoroughly characterized members of the FGF family, and their expression has been detected in many tissues and cell lines, both normal and transformed (1). Although they lack a classical signal sequence for secretion, FGF-1 and FGF-2 are found in the extracellular matrix associated with HSPGs. In addition, studies have shown that FGF-1 and FGF-2 bind with a high apparent affinity to immobilized heparin, a property that has been utilized in purifying the proteins to homogeneity (18). Soluble heparin has been shown to protect FGF-1 and FGF-2 from proteolytic degradation, heat denaturation, and acid or base inactivation (19,20). Heparin and HSPGs found on the cell surface act to stabilize the native conformation of FGF (19-21) and may play a role in binding FGF proteins to their tyrosine-kinase receptors (22,23). Heparin has also been shown to potentiate the mitogenic activity of FGF-1 (24-26); however, FGF-1 proteins from different species vary in their degree of heparin dependence for mitogenic activity (27).

The amino acid sequence of FGF-1 is highly con-

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served among different species. A comparison of FGF-1 sequences among mammalian and avian species demonstrates that they share greater than 90% sequence identity (1). However, the amphibian FGF-1 homolog exhibits between 79% and 83% sequence identity with the other FGF-1 members (28). Human FGF-1 (29) and bovine FGF-1 (30) have been sequenced at the protein level and contain blocked amino termini due to acetylation of their amino-terminal alanine residues. The other species' amino acid sequences were deduced from their corresponding cDNA sequences, and their amino-terminal alanine residues are assumed to be acetylated. In all of the species examined to date, the FGF-1 proteins have two conserved cysteine residues; however, there is no evidence that these cysteine residues form intramolecular disulfide bonds in the native proteins (31,32).

As part of a graduate level course that utilizes a laboratory approach to problem-based learning, the characterization of FGF-1 proteins from additional species was initiated. In this paper, the complete primary sequence of ovine FGF-1 as deduced by a combination of protein and cDNA sequencing is described. The ovine amino acid sequence shares a high degree of sequence identity with FGF-1 from other species. Consistent with other FGF-1 proteins, the ovine FGF-1 protein is acetylated at its amino terminus and requires heparin for optimal mitogenic activity.

MATERIALS AND METHODS

FGF-1 purification from ovine brain. Twelve adult sheep brains (Pel Freeze Biochemicals, Roger, AR) were homogenized for 2 min at full speed in a Waring commercial blender in 1.2 volumes (wt/vol) of 50 mM Tris-HCl (pH 7.6), 50 mM EDTA. The homogenate was clarified by centrifugation at 9000 rpm for 60 min, and after straining through cheesecloth, the supernatant was loaded onto a 40 mL heparin-Sepharose (Pharmacia Biotech, Piscataway, NJ) column at 4 °C. The column was washed first with 10 column volumes of homogenization buffer [50 mM Tris-HCl (pH 7.6) 50 mM EDTA], then with 10 column volumes of 0.5 M NaCl in homogenization buffer, and finally with 2 column volumes of 0.65 M NaCl in homogenization buffer. Fractions from each wash were collected. The heparin-Sepharose column was then treated with 3 column volumes of 1.5 M NaCl in homogenization buffer to elute FGF-1.

Electrophoresis and Western blot analysis. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS) was performed essentially as described by Laemmli (33), using a Mighty Small gel apparatus (Hoefer, San Francisco, CA). Proteins were visualized with Coomassie Brilliant Blue (BDH Laboratory Supplies, Poole, England). Proteins were also transferred from the gel to nitrocellulose, and fractions containing FGF-1 were identified using an FGF-1-specific rabbit polyclonal antiserum and ^{125}I-protein A as described previously (34).

Proteolytic digestion and peptide mapping. Purified samples were subjected to SDS-PAGE and then were transferred to nitrocellulose for *in situ* protease digestion using the procedure described by Abersold *et al* (35). Ovine FGF was visualized on the nitrocellulose membrane with Ponceau S (Sigma Chemical Company, St. Louis, MO). The bands were excised from the membrane and were digested at 37 °C for 20 h with sequencing grade endoproteinase Lys-C (Boehringer Mannheim, Indianapolis, IN) at a ratio of 1:20 (enzyme:substrate). Lys-C-derived peptides were isolated using an Applied Biosystems (Foster City, CA) Model 130 reversed-phase, microbore HPLC. Peptides were eluted using 0.1% trifluoroacetic acid (Solvent A) and 0.09% trifluoroacetic acid in 70% acetonitrile (Solvent B) with a linear gradient of 5% to 70% Solvent B. Limited acid hydrolysis was performed on the amino-terminal, Lys-C-derived peptide fraction. Briefly, the amino-terminal fraction was dried in a Speed Vac (Savant Instruments, Farmingdale, NY) and resuspended in 6 N HCl. The reaction proceeded at room temperature for 6 h. The resulting peptides were fractionated using the reversed-phase, microbore HPLC as described above.

Amino acid sequencing. The peptides were subjected to automated Edman degradation using an Applied Biosystems (Foster City, CA) Model 477A protein sequencer equipped with a Model 120A microbore HPLC system for on-line analysis of phenylthiohydantoin (PTH) amino acids.

Amino acid analysis. Amino acid analysis was performed as outlined by Waters Associates (Milford, MA). Briefly, samples were hydrolyzed *in vacuo* in 6 N HCl/0.1% phenol (Pierce, Rockford, IL) at 150 °C for 1 h. Amino acid compositions were determined based on reversed-phase separation of the phenylthiocarbamyl derivatives using a PICO · TAG amino acid analysis system (Waters Associates, Milford, MA).

Cell culture. Ovine MDOK kidney cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD) and cultured in Eagle's minimum essential medium with Earle's balanced salt solution (Mediatech, Herndon, VA), 10% fetal bovine serum (Hyclone, Logan, UT), 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, and a 1:100 dilution of a penicillin-streptomycin-fungizone solution (Mediatech, Herndon, VA).

RNA isolation and RT-PCR analysis. Total cytoplasmic RNA was isolated from ~5×10^6 MDOK cells using RNA STAT-60 (Tel-Test "B" Inc., Friendswood, TX) according to the manufacturer's instructions. The RNA concentration was calculated by measuring UV light absorbance at 260 nm. RNA (1 μg) was converted to cDNA using random hexamer (pd(N)6) primers (Boehringer Mannheim, Indianapolis, IN). PCR was performed with an FGF-1 sense primer corresponding to the bovine cDNA sequence: 5'-GCCACAACCAGCAGCT-GCTGAGCC-3' (nucleotides -24 to -1 from ref. 36) and an internal, antisense primer corresponding to the human FGF-1 sequence: 5'-CTTTCTGGCCATAGTGAGTCCGAG-3' (nucleotides 527 to 550 from ref. 37). The resultant cDNA products were analyzed on a 1.2% agarose gel and visualized by ethidium bromide staining.

Cloning and sequence analysis. PCR fragments were isolated by the freeze-squeeze method as described previously (38), and directly cloned into the pCRII plasmid vector (Invitrogen Corp., San Diego, CA). Plasmid DNA was purified using a Magic Miniprep kit (Promega Corp., Madison, WI) and both strands of each cDNA fragment were sequenced by the dideoxy chain termination method using a Sequenase 2.0 kit (U.S. Biochemicals, Cleveland, OH) and [^{35}S]dATP (1000 Ci/mmol, Amersham Corp.). Due to potential PCR nucleotide misincorporation errors, 2 clones encoding full-length FGF-1 and 2 clones encoding the shorter, variant form of FGF-1 were sequenced. No differences were found in the respective nucleotide sequences.

Mass spectroscopy. Matrix assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry analysis was carried out on a linear time-of-flight mass spectrometer (TOF 101, Comstock Inc., Oak Ridge, TN). Sample and internal standard (Substance P) were diluted and mixed with 2,5-dihydroxybenzoic acid (DHB) matrix [2/1/10] (Sigma Chemical Company, St. Louis, MO) and then dissolved in acetonitrile (Aldrich Chemical Company, Milwaukee, WI). The spectrum was obtained with a 30 kV acceleration voltage and 3 shots of a 337 nm nitrogen laser (VSL-337ND, Laser Science Inc., Newton, MA). Flight time data was obtained with a dual channel plate detector (Galileo Company, Sturbridge, MA) and converted

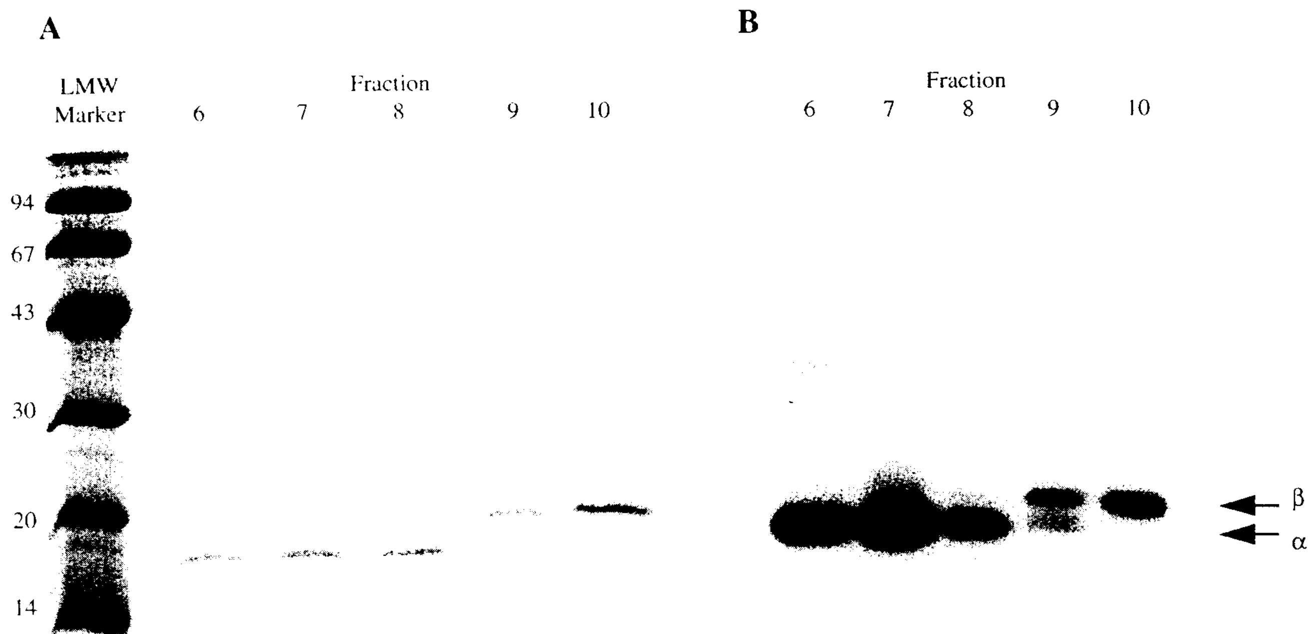

FIG. 1. SDS-PAGE and Western blot analysis of purified ovine FGF-1. (A) Proteins eluted from a heparin-Sepharose column with a 1.5 M NaCl wash were purified to apparent homogeneity by reversed-phase HPLC. HPLC fractions 6-10 were subjected to SDS-PAGE and proteins were visualized by Coomassie blue stain. The samples were loaded as follows: lane 1, low molecular weight markers and lanes 2-6, successive HPLC fractions 6-10. (B) Samples of fractions 6-10 were subjected to SDS-PAGE and Western blot analysis using rabbit polyclonal anti-FGF-1 antiserum and ^{125}I-Protein A.

to molecular weight data using the TOFWARE program (Ilys Software, Pittsburgh, PA).

Mitogenic assay. NIH 3T3 cells (ATCC) were seeded into 48-well plates and grown to ~80% confluence in DMEM (Biofluids, Inc., Rockville, MD) containing 10% calf serum (Biofluids). The cells were serum starved (DMEM, 0.5% calf serum) for 30 h. Varying concentrations of human FGF-1 or ovine FGF-1 in either the absence or the presence of 5.0 U/ml heparin (Upjohn Company, Kalamazoo, MI) were added to the serum starved cells. After 18 h of incubation, the cells were pulsed with 20 μCi/ml of [^{3}H]thymidine (5 Ci/mmol, Amersham Corp.) for 4 h. The cells were fixed with 10% TCA, rinsed with PBS, and then solubilized in 0.5 N NaOH. The amount of [^{3}H]-thymidine incorporated into acid-insoluble material was determined by scintillation counting.

RESULTS

Purification of Ovine FGF-1

FGF-1 was isolated from ovine brain by heparin-Sepharose affinity-based chromatography. Proteins retained by the immobilized heparin column after a 0.65 M NaCl wash were eluted with 1.5 M NaCl and were purified to apparent homogeneity by reversed-phase HPLC. The HPLC fractions were analyzed by SDS-PAGE, and the proteins were visualized by Coomassie blue staining. As shown in Figure 1A, fractions 6 through 9 contained a protein with the apparent molecular weight, ~17 kDa, of the α-form of FGF-1 isolated from other species. Fractions 8, 9, and 10 contained a protein with the apparent molecular weight, ~20 kDa, of the full-length β-form of FGF-1 (Figure 1A). The identities of the Coomassie stained proteins were confirmed by Western blot analysis to be the α- and β-forms of ovine FGF-1 (Figure 1B).

Protein Sequence Analysis

The respective HPLC fractions of ovine αFGF-1 and βFGF-1 were subjected to automated Edman degradation. The sequential release of phenylthiohydantoin (PTH) amino acid derivatives from αFGF-1 was observed for 29 cycles. The first 29 amino acid residues of ovine αFGF-1, which also correspond to residues N_{21}-K_{49} of βFGF-1, are shown in Table 1. The amino acid residues at cycle 10 and cycle 24 could not be determined. When βFGF-1 was subjected to automated Edman degradation, no sequential release of PTH amino acid derivatives was observed, indicating that the amino terminus of ovine βFGF-1 is blocked.

Portions of the amino-terminal sequence of βFGF-1 were determined following proteolytic digestion and

TABLE 1
Amino-Terminal Sequence Analysis of αFGF-1 and βFGF-1

Cycle	Amino acid	α amount (pmoles)	β amount (pmoles)
1	Asn	22.8	n.d.
2	Tyr	35.2	n.d.
3	Lys	44.4	n.d.
4	Lys	48.5	n.d.
5	Pro	35.5	n.d.
6	Lys	28.4	n.d.
7	Leu	45.2	n.d.
8	Leu	62.3	n.d.
9	Tyr	18.5	n.d.
10	n.d.	—	n.d.
11	Ser	8.3	n.d.
12	Asn	11.0	n.d.
13	Gly	26.8	n.d.
14	Gly	35.0	n.d.
15	Tyr	13.8	n.d.
16	Phe	21.8	n.d.
17	Leu	25.6	n.d.
18	Arg	2.8	n.d.
19	Ile	20.7	n.d.
20	Leu	23.0	n.d.
21	Pro	7.7	n.d.
22	Asp	3.7	n.d.
23	Gly	16.4	n.d.
24	n.d.	—	n.d.
25	Val	8.5	n.d.
26	Asp	3.0	n.d.
27	Gly	12.0	n.d.
28	Thr	5.3	n.d.
29	Lys	3.0	n.d.

Note. The sequential release of PTH amino acid derivatives was observed for 29 cycles upon subjecting αFGF-1 to automated Edman degradation. No sequential release of PTH amino acid derivatives was observed for the β-fraction.

Edman degradation. Purified αFGF-1 and βFGF-1 were digested *in situ* with endoproteinase Lys-C and the resulting peptide fragments were purified by reversed-phase HPLC. Two major peptides unique to βFGF-1 were obtained, while the remaining peptides generated were shared by both the αFGF-1 and the βFGF-1 digests (Figure 2). One of the peptides specific to βFGF-1, corresponding to peak 2 in Figure 2, was sequenced and is immediately amino-terminal to and slightly overlapping with the amino-terminal sequence determined for αFGF-1 (Figure 3). The observed sequence was Phe-Asn-Leu-Pro-Leu-Gly-Asn-Tyr-Lys. No sequence was detected when the other Lys-C-derived peptide unique to βFGF-1, represented by peak 1, was subjected to automated Edman degradation. Thus, the peptide was assumed to be the amino-terminal peptide of βFGF-1. The blocked, amino-terminal peptide was sub-digested by limited acid hydrolysis, and the newly generated fragments were purified by reversed-phase HPLC. One acid-derived peptide yielded the sequence Thr-Thr-Phe-Arg-Ala-Leu (Figure 3). Based on its homology to human FGF-1, this peptide was determined to be amino-terminal to the β-specific, peak 2 peptide but did not include the extreme amino-terminal sequence of ovine FGF-1.

In addition, several Lys-C-derived fragments common to both αFGF-1 and βFGF-1 were sequenced, and their positions were established by homology to human FGF-1 (Figure 3). While sequencing the carboxy-terminal peptide, the identity of the last residue was ambiguous. Based on homology with other species, the carboxy-terminal, Lys-C-derived peptide should consist of 12 residues. The identity of the first 11 amino acid residues of this peptide was established by direct amino acid sequencing. Following amino acid analysis of the peptide (data not shown), the resulting composition was consistent with the sequence established by Edman degradation plus an additional residue of aspartic acid. The assignment of the carboxy-terminal residue as aspartic acid is consistent with all other FGF-1 sequences. It must be noted, however, that an asparagine at this position would yield the same result due to the deamidation that occurs during acid hydrolysis.

cDNA Analysis

cDNA analysis of ovine FGF-1 was carried out concurrently in an effort to support the protein data and to obtain sequence data not provided by the enzymatically- and chemically-generated peptides. Ovine FGF-1 cDNA fragments were isolated by RT-PCR using ovine kidney cell RNA and FGF-1 oligonucleotide primers. Two FGF-1 cDNA fragments were detected by RT-PCR analysis. One cDNA fragment was the expected size of ~450 bp, and the other fragment was ~350 bp in size (Figure 4A).

The two cDNA fragments were cloned and sequenced (Figure 3). Analysis of the deduced protein sequences indicated that the longer cDNA, fragment a, encoded amino acid residues 1-134 of full-length FGF-1, while the shorter cDNA, fragment b, encoded amino acid residues 1-56 with four additional residues at the carboxyl terminus (Figure 4B). This variant FGF-1 protein, predicted to be 60 amino acid residues, was not observed during the isolation of FGF-1 from ovine brains for protein sequencing.

The deduced amino acid sequence of the FGF-1 cDNA was used to confirm those residue assignments that were ambiguous during direct protein analysis. The two amino acid residues that could not be deduced initially by N-terminal sequencing were determined to be a cysteine and an arginine at cycles 10 and 24, respectively. Given the inefficiency of detecting cysteine residues by automated Edman degradation without prior modification of their side chains to more stable forms by either alkylation or oxidation, it is not surprising

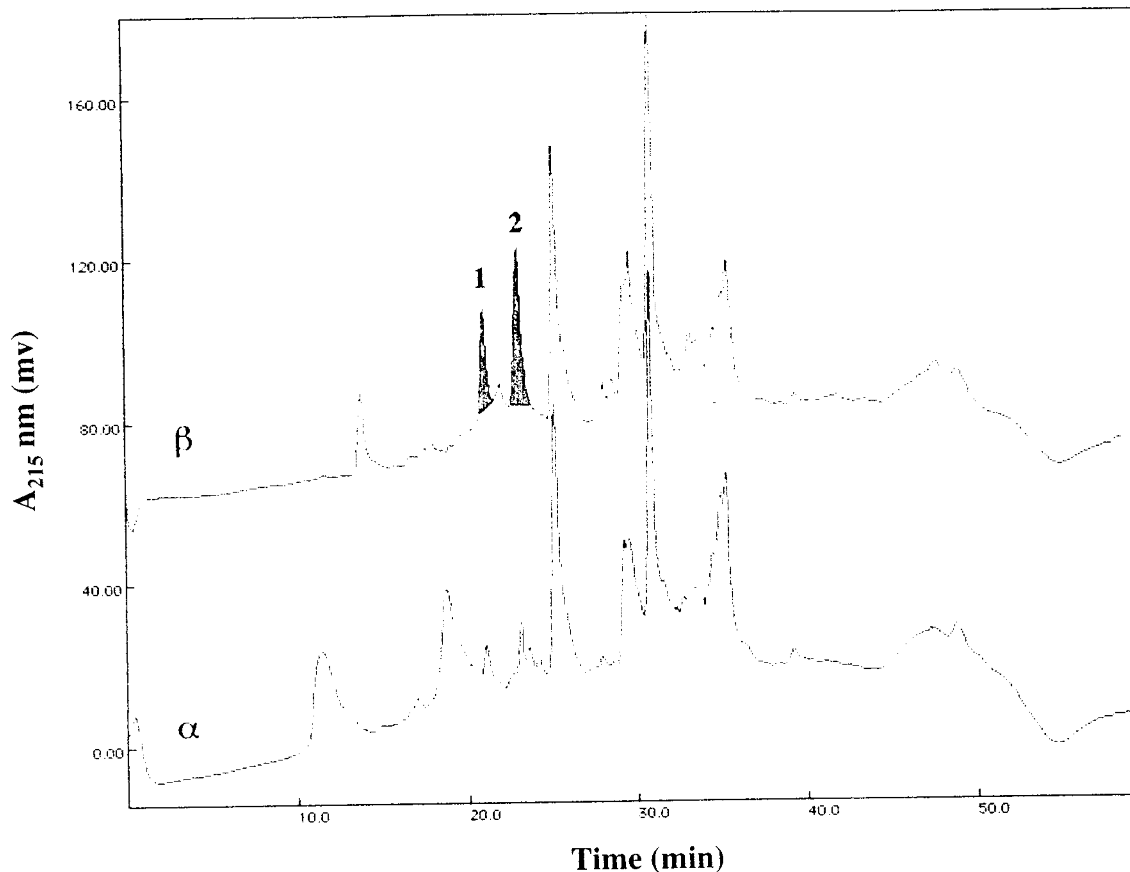

FIG. 2. Reversed-phase HPLC analysis of peptides generated from the digestion of βFGF-1 and αFGF-1 with endoproteinase Lys-C. The peak fractions unique to βFGF-1 are shaded and labeled as 1 and 2. Peak 1 corresponds to the amino-terminal blocked peptide of βFGF-1.

that the cysteine residue at cycle 10 could not be determined. Likewise, after 24 cycles of Edman chemistry, it is not unreasonable to recover low yields of arginine, such that it is difficult to detect. In addition to providing the sequence not yet determined, the deduced protein sequence confirmed the three amino acid substitutions, originally observed during protein sequencing, which are unique to the primary sequence of ovine FGF-1 (Arg_9, Arg_{44}, and Ile_{123}) when compared to all the species sequenced to date (Figure 5).

Mass Spectrometric Analysis of the Blocked Amino-terminus of βFGF-1

MALDI-TOF mass spectrometry analysis was used to identify the amino-terminal blocking group of ovine FGF-1. The Lys-C-derived, amino-terminally blocked peptide from peak 1 of the reversed-phase HPLC analysis was subjected to mass spectrometry. As shown in Figure 6, the peak labeled 1595.53 m/z represents the βFGF-1 fraction. The peak labeled 1347.33 m/z is an internal standard (Substance P), while the remaining peaks are due to the DHB matrix.

The amino acid composition of the βFGF-1 amino-terminal peptide generates a predicted mass of 1553.73, excluding the blocking group. The difference between the observed 1595.53 m/z peak and the predicted 1553.73 mass is 41.8 atomic mass units, consistent with an acetyl moiety in amide linkage with the amino-terminal alanine residue. The predicted mass of 1596.78 for the acetylated, amino-terminal peptide compares favorably with the mass of 1595.53 m/z actually measured by mass spectrometry for the Lys-C-derived peptide.

Mitogenic Properties of Ovine FGF-1

The ability of ovine βFGF-1 to stimulate mitogenesis in NIH 3T3 fibroblasts, as measured by [^{3}H]thymidine incorporation, was compared to that of human βFGF-1. Since heparin has been shown to potentiate the mitogenic activity of FGF-1 isolated from other species (24-27,34,41), a mitogenic assay was conducted over a range of FGF-1 concentrations in either the absence or the presence of exogenous heparin. Human FGF-1 alone exhibits a modest mitogenic activity on NIH 3T3

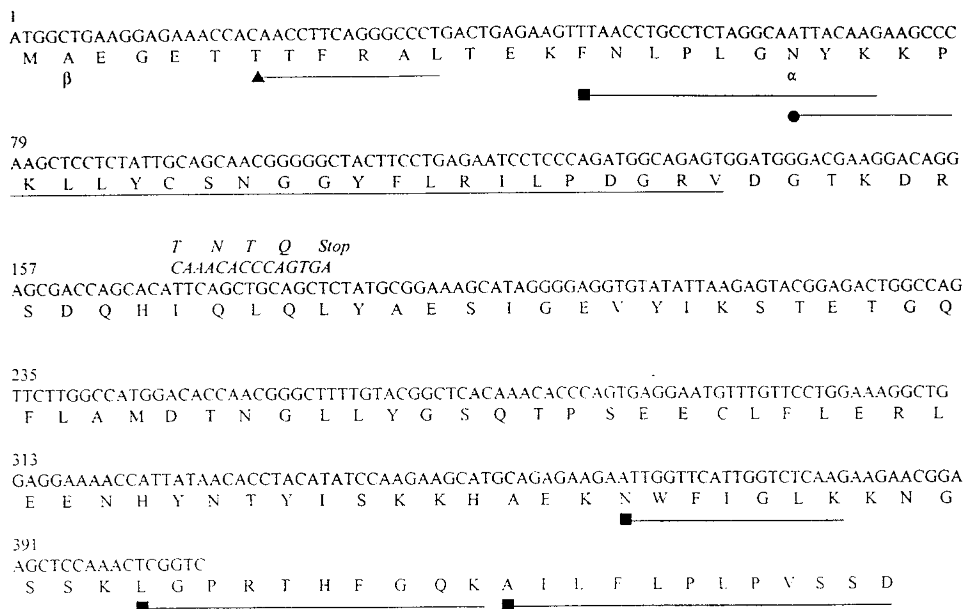

FIG. 3. The cDNA and amino acid sequences of ovine βFGF-1 and αFGF-1. The nucleotide sequence derived from two cDNA clones isolated using an RT-PCR approach was compared to the determined protein sequence. Protein sequence data was obtained from limited acid hydrolysis (▲), endoproteinase Lys-C fragments (■), and N-terminal sequencing of αFGF-1 (●). Due to the use of an internal, antisense primer, the carboxy-terminal sequence was determined solely by protein analysis. The sequence in italics indicates the DNA sequence of the variant FGF-1 cDNA and the amino acid residues it encodes. α and β denote the N-terminus of αFGF-1 and βFGF-1, respectively. B at residue 154 denotes the possibility of D or N at this position.

cells (Figure 7). In contrast, ovine FGF-1 has significant mitogenic activity in the absence of exogenous heparin. Upon the addition of exogenous heparin, the mitogenic activities of both ovine and human FGF-1 are potentiated; however, heparin has a greater stimulatory effect on the activity of the human protein.

DISCUSSION

The primary structures that were initially proposed for bovine and human FGF-1 were incomplete, representing amino-terminal truncations of these proteins (1). It was subsequently shown that the truncated α-form of bovine FGF-1 is likely generated during the purification process (1); however, the exact mechanism by which it is formed is unknown. The full-length, ~20 kDa protein (30), referred to as βFGF-1, is thought to be the primary physiological form. Truncated αFGF-1 results from a cleavage event between Gly_{20} and Asn_{21} of native FGF-1; thus, βFGF-1 contains a 20 residue amino-terminal extension of αFGF-1. Both the α- and the β-forms were identified while isolating FGF-1 from ovine brains.

The primary sequence of ovine FGF-1, as determined by direct protein analysis and cDNA sequencing, shares a significant degree of sequence identity with bovine FGF-1 (95%) and human FGF-1 (90%). The primary structures of FGF-1 proteins are highly conserved among the nine species studied to date (Figure 5). Newt FGF-1, being the most phylogenetically distant, shares ~80% sequence identity with its vertebrate counterparts. In addition, the cysteine residues at positions 30 and 97 are conserved in all of the species presently sequenced. There is no evidence, however, indicating that these conserved cysteine residues are involved in intramolecular disulfide bond formation (31,32). The ovine FGF-1 sequence, when aligned with that of the other species, contains three unique amino acid differences: Arg_9, Arg_{44}, and Ile_{123} (Figure 5). Arg_9 and Arg_{44} introduce positive-charged residues at positions that are otherwise neutral in all the other species with the exception of Lys_{44} found in both avian FGF-1 and newt FGF-1. Isoleucine at position 123 in ovine FGF-1 is a relatively conservative substitution when compared to the valine found at this position in all the other species.

The amino terminus of ovine βFGF-1 is blocked and, therefore, is not susceptible to Edman degradation. The mass spectrometry data, reported herein, indicates that the mass of the ovine FGF-1 blocking group is consistent with that of an acetyl-moiety in amide linkage with the amino-terminal alanine residue. Mass spectrometric analysis was utilized to identify the blocking groups of human FGF-1 (29) and bovine FGF-1 (30) which were also determined to have acetylated, amino-terminal alanine residues.

While conducting the cDNA analysis, a putative splice-variant of ovine FGF-1 was observed. PCR am-

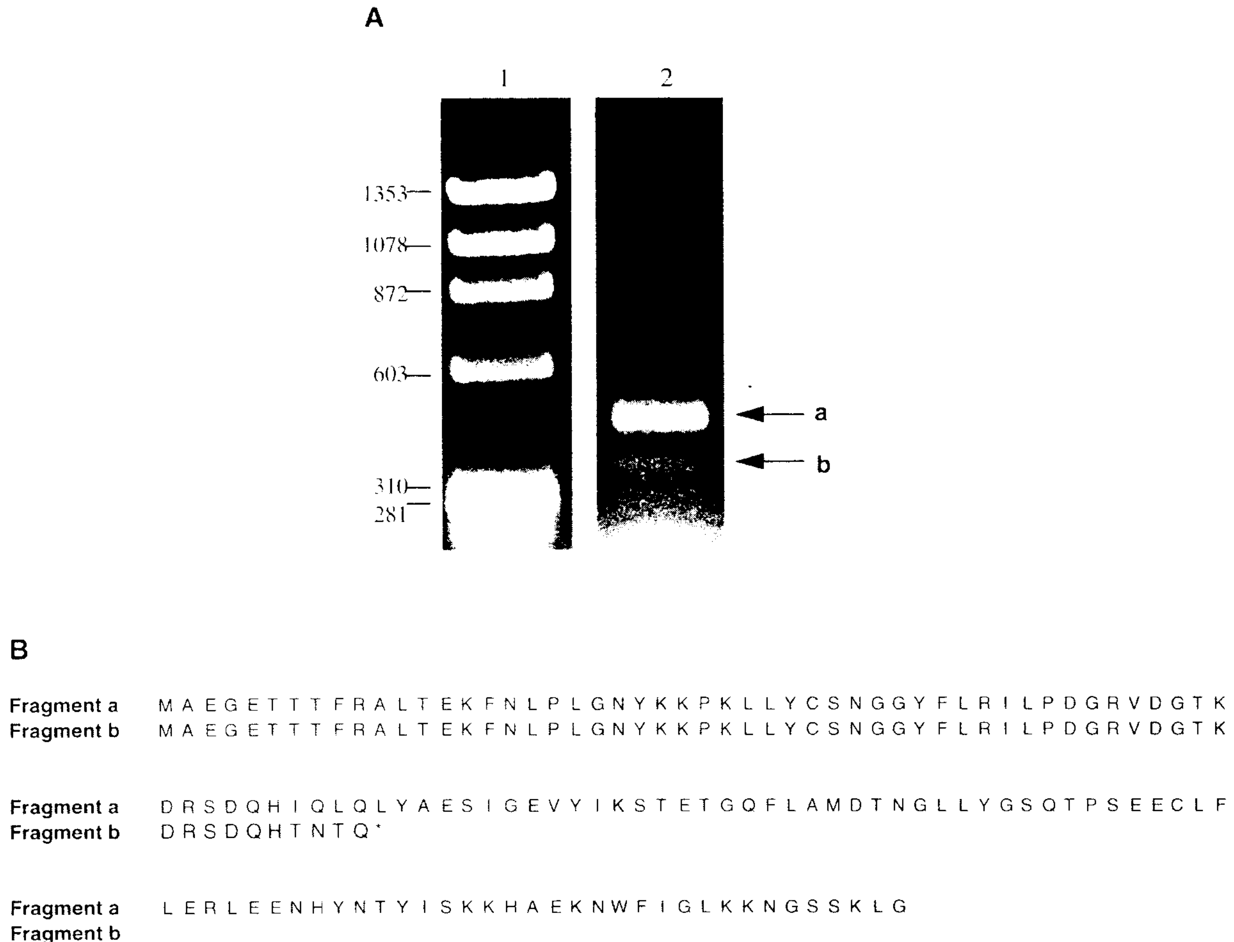

FIG. 4. RT-PCR amplification of ovine FGF-1 cDNA clones. (A) RT-PCR amplified cDNA derived from MDOK kidney cell RNA was separated by agarose gel electrophoresis and visualized by ethidium bromide staining. Lane 1 contains the DNA size markers (in bp) and lane 2 shows the two FGF-1 cDNA fragments, labeled as a and b. (B) The deduced amino acid sequences of the a and b amplification products from (A) were compared. The variant FGF-1 protein has 56 amino acid residues in common with βFGF-1 but has four unique amino acid residues at its carboxy-terminus.

plification of the cDNA derived from ovine kidney cell RNA resulted in the isolation of two distinct cDNA products, the expected FGF-1 fragment (454 bp) and a variant form of FGF-1 (350 bp). Upon cloning and sequencing the two cDNA products, it was determined that the 454 bp fragment encodes amino acid residues 1-134 of βFGF-1. The 350 bp, FGF-1 variant is predicted to encode a protein of 60 amino acid residues with the first 56 residues corresponding to the amino terminus of βFGF-1. This result is consistent with the findings of Yu *et al* (39) who identified the same truncated variant in several human cell lines. They proposed that the human FGF-1 variant, aFGF', is the consequence of an alternate splice of the FGF-1 pre-mRNA which eliminates exon 2 and shifts the open-reading frame, creating a premature stop codon (39). The human aFGF' variant also shares all but the last 4 of it 60 amino acid residues with the amino-terminal region of full-length, human FGF-1. In addition, the variant was also identified by PCR of cDNA prepared from normal ovine kidney tissue (data not shown). Together these data demonstrate that the alternatively-spliced form of FGF-1 is present across species and is not only expressed in cells cultured *in vitro*.

The extensive truncation seen in the FGF-1 variant protein results in the deletion of the reported FGF-1 heparin-binding domain (26,41,42). The ability of FGF-1 to bind heparin-Sepharose with a high apparent affinity has been utilized in purifying the protein from various tissues. Therefore, it is not surprising that the truncated protein, if actively translated, was not detected during the purification and analysis of FGF-1 isolated from ovine brains. Further investigation is required to determine whether the truncated, ovine FGF-1 protein is indeed translated *in vivo* and whether, like human aFGF', it can act as an antagonist of the full-length protein (39).

It has been demonstrated that the addition of exoge-

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sheep FGF-1    Ac- - - - - - T - - - R - - - - - - - - - L - - - - - - - - - - - - - - - Y - - - - - - - - R - - - - K
human FGF-1    Ac- M A E G E I T T F T A L T E K F N L P P G N Y K K P K L L Y C S N G G H F L R I L P D G T V D G T R
bovine FGF-1   Ac- - - - - - T - - - - - - - - - - - - - L - - - - - - - - - - - - - - - Y - - - - - - - - - - - - - K
chicken FGF-1  Ac- - - - - - - - - - - - - - - R - G - - L - - - - - - - - - - - - - - - - - - - - - - - - K - - - - -
rat FGF-1      Ac- - - - - - - - - - A - - - - R - - - - L - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
porcine FGF-1  Ac- - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
mouse FGF-1    Ac- - - - - - - - - - A - - - - R - - - - L - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
hamster FGF-1  Ac- - - - - - - - - - S - - - - R - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
newt FGF-1     Ac- - - - - - - - - - A - - - - - - - - - N - - - Q - - - - - - - - - - - Y - - - - F - - - K - - - - -

sheep FGF-1        - - - - - - - - - - - Y - - - I - - - - - - - - - - - - F - - - - - N - - - - - - - - - S - - - - -
human FGF-1        D R S D Q H I Q L Q L S A E S V G E V Y I K S T E T G Q Y L A M D T D G L L Y G S Q T P N E E C L F
bovine FGF-1       - - - - - - - - - - - C - - - I - - - - - - - - - - - - F - - - - - - - - - - - - - - - - - - - - -
chicken FGF-1      - - - - - - - - - - - - - - D - - - - - - - - A S - - - - - - - - - N - - - - - - - L - G - - - - -
rat FGF-1          - - - - - - - - - - - - - - - A - - - - - - G - - - - - - - - - - - E - - - - - - - - - - - - - - -
porcine FGF-1      - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - S - - - - - - - - - S - - - - -
mouse FGF-1        - - - - - - - - - - - - - - - A - - - - - - G - - - - - - - - - - - E - - - - - - - - - - - - - - -
hamster FGF-1      - - - - - - - - - - - - - - - A - - - - - - G - - - - - - - - - - - - - - - - - - - - - - - - - - -
newt FGF-1         - - - - P Y - - - - F Y - - - - - - - - - - - L - - - - - - - - - S - - Q - - A - - S - S - - - - -

sheep FGF-1        - - - - - - - - - - - - - - - - - - - - - - - I - - - - - - - S - L - - - - - F - - - - - - - - - -
human FGF-1        L E R L E E N H Y N T Y I S K K H A E K N W F V G L K K N G S C K R G P R T H Y G Q K A I L F L P L
bovine FGF-1       - - - - - - - - - - - - - - - - - - - - H - - - - - - - - - R S - L - - - - - F - - - - - - - - - -
chicken FGF-1      - - - - - - - - - - - - - - - - - - D - - - - - - - - - - - N S - L - - - - - - - - - - - - - - - -
rat FGF-1          - - - - - - - - - - - - T - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
porcine FGF-1      - - - - - - - - - - - - T - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
mouse FGF-1        - - - - - - - - - - - - T - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
hamster FGF-1      - - - - - - - - - - - - T - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
newt FGF-1         - - - - - - - N - - - - K - - V - - D - D - - - - I - - - - K T - P - S - - - F - - - - - - - - - -

sheep FGF-1        - - - - -
human FGF-1        P V S S D
bovine FGF-1       - - - - -
chicken FGF-1      - - - A -
rat FGF-1          - - - - -
porcine FGF-1      - - - - -
mouse FGF-1        - - - - -
hamster FGF-1      - - - - -
newt FGF-1         - - - - -
```

FIG. 5. Comparison of the primary sequences of FGF-1 proteins from those species sequenced to date. The complete amino acid sequence (154-residues) of human FGF-1 and those amino acid residues that differ from that of the human sequence in the various species are shown. Ac refers to the acetylation of the N-terminal alanine residues, and the two cysteine residues that are conserved among the species are boxed. Those amino acid residues that are unique to ovine FGF-1 are bolded.

FIG. 6. Matrix assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry analysis of the Lys-C-derived, amino-terminal blocked peptide of βFGF-1. The peak labeled 1595.53 m/z represents the ovinc FGF-1 fraction, while the peak labeled 1347.33 m/z is the Substance P internal standard. The remaining peaks are due to the DHB matrix.

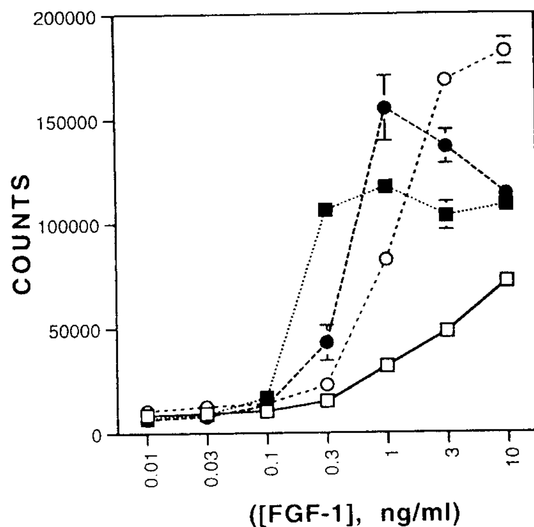

FIG. 7. Stimulation of DNA synthesis in NIH 3T3 cells by human and ovine FGF-1. Serum starved NIH 3T3 cells were treated for 18 h with the indicated concentrations of human FGF-1 (□), human FGF-1 + 5.0 U/ml heparin (■), ovine FGF-1 (○), or ovine FGF-1 + 5.0 U/ml heparin (●). The cells were pulsed with [^{3}H]thymidine and incorporation of radioactivity into DNA was determined by scintillation counting. Values represent the mean ± SE of triplicate wells.

nous heparin potentiates the mitogenic activity of FGF-1 (24-27,34,40). FGF-1 proteins from the different species currently tested vary in their degree of heparin dependence for optimal biological activity. Furthermore, the extent of heparin dependence displayed by a given FGF-1 protein can differ among various cell lines (1). Like avian FGF-1 (27) and bovine FGF-1 (40), ovine FGF-1 has a mitogenic activity that is relatively independent of exogenous heparin, although in the presence of heparin, its mitogenic activity is potentiated moderately. In contrast, human FGF-1 induces a limited mitogenic response in the absence of exogenous heparin and is dependent on heparin for a significant increase in its mitogenic potential. The cause of this variable heparin dependence is unknown. When comparing primary structures, ovine, bovine, and avian FGF-1 sequences are uniformly different from that of human FGF-1 at three positions: $P \rightarrow L_{19}$, $C \rightarrow S_{131}$, and $R \rightarrow L_{133}$. It is unclear whether these amino acid changes impact the heparin dependence. It is unlikely that $P \rightarrow L_{19}$ plays a role in determining heparin dependence as this residue is absent in αFGF-1 which exhibits a heparin requirement similar to that of full-length βFGF-1 (43). Although there is no evidence that the cysteine residues in FGF-1 are involved in intrachain disulfide bond formation, the fact that residues 30 and 97 are evolutionarily conserved and that residue 131 is a serine or cysteine in all the species, except newt, indicates a conservation of function. Ortega *et al* (31) reported that mutagenesis of the cysteine residues in human FGF-1 not only increased the solution stability of the protein but also dramatically decreased its heparin dependence for optimal mitogenic activity. The study presented here adds further support to the hypothesis that the cysteine residues may function as negative regulators of the FGF-1 protein. Consequently, a cysteine at residue 131 may render the protein particularly dependent on exogenous heparin for its biological activity. Furthermore, it is possible that the conserved $R \rightarrow L_{133}$ substitution found in the ovine, bovine, and avian sequences also contributes to the reduced heparin dependence. It should be noted that the remaining species, except newt, are human-like in the region containing amino acid residues 131 and 133. Thus, it will be of interest to determine the degree of heparin dependence for the FGF-1 proteins of the other species.

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