Retinoic acid induces gene expression of fibroblast growth factor-9 during induction of neuronal differentiation of mouse embryonal carcinoma P19 cells

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Abstract **We have found that the gene expression of the ninth member of the fibroblast growth factor (FGF) family, FGF9 was induced during retinoic acid(RA)-induced neuronal differentiation of murine embryonal carcinoma P19 cells. We have reported here the nucleotide sequence of the mouse FGF9 cDNA. The murine cDNA showed 92.4% nucleotide sequence homology to the human FGF9 cDNA and 98.2% homology to that of rats. This mouse FGF9 cDNA encoded a polypeptide consisting of 208 amino acids with amino acid sequence identical to that of rats. Only one amino acid was replaced compared to the human homolog. The highly conserved sequence homology of FGF9 suggests its functional importance. FGF9 was originally isolated from a culture medium of a human glioma cell line as a growth-promoting factor for glial cells [5]. Upon induction of neuronal differentiation by forming cell aggregates with 10^{-6} M RA, the gene expression of FGF9 was increased biphasically during the first 96 hours when cells were aggregating and from 168 hours to 192 hours followed by plating onto a tissue culture dish as glia-like cells proliferated. Neither undifferentiated P19 cells nor the cells aggregated without RA remaining undifferentiated expressed FGF9. This indicates that RA regulates the gene expression of FGF9 that may play an important role in neuronal differentiation in both early and late developmental process.**

Key words: FGF9; Neuronal differentiation; Retinoic acid; Embryonal carcinoma cell

1. Introduction

Mouse P19 embryonal carcinoma (EC) cells are pluripotent and can be induced to differentiate into different cell types with forming cell aggregates and upon treatment with varying concentrations of retinoic acid(RA) [7]. The differentiated cell types are dependent on the concentration of RA. P19 cells exposed to lower concentrations of RA (10^{-8} M to 10^{-9} M) are characterized by an abundance of cardiac muscle cells and skeletal muscle cells, while neurons and astroglia appear at higher concentrations (10^{-7} M to 10^{-6} M) of RA [7]. These neurons resemble morphologically those in the central nervous system, and many neuron-specific genes were shown to be expressed in those cells [8]. RA is now believed to play a fundamental role in the development of the vertebrate nervous system [19,20]. Thus, P19 EC cells are a good model system for studying what events occur during the critical phase of neuronal differentiation in vitro.

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Members of the fibroblast growth factor (FGF) family are involved in cell proliferation processes, but also in early and late differentiation processes for a variety of mesoderm- and neuroectoderm-derived cells [1]. Among the nine members of this group identified to date, FGF1/acidic FGF and FGF2/basic FGF are expressed in both embryonic and in adult tissues [2], but are most abundant in nervous tissues [2]. FGF1 and FGF2 have neurotrophic activities which support the survival and growth of cultured peripheral neurons, central neurons and glial cells [1]. On the other hand, other FGF family members, such as FGF3/int-2, FGF4/hst-1, FGF5, and FGF6, have been initially identified, as products of oncogenes, being shown to play roles during murine embryonic development [3]. In addition, FGF5 has been found in brains of adult mouse and rat and promotes differentiation of cultured rat septal cholinergic and raphe serotonergic neurons [16]. FGF8 and FGF9 have recently been found and purified from the culture media of a mouse androgen-dependent mammary carcinoma cell line [4] and a human glioblastoma cell line [5], respectively. FGF8 has been suggested to play a role in mouse embryogenesis [17]. However, the role of FGF9 in cell differentiation and developmental process has not been investigated yet. These FGFs bind to cell-surface receptors to give target cells signals determining their fate to proliferate, survive, or differentiate. As such receptors, four members of the FGF receptor (FGFR) family, of which the cytoplasmic domains are tyrosine kinases, have been identified [14]. Thus, FGFs and FGFRs may regulate not only mesoderm induction but neuronal induction during both early and late developments, although less information is available on the expression pattern in the nervous system of members of the FGF family different from FGF1 or FGF2.

In this study, we have found that the gene expression of the ninth member of the FGF family, FGF9, was induced during retinoic acid-induced neural differentiation in P19 cells. We cloned and sequenced mouse FGF9 cDNA expressed in the cells induced by RA.

2. Materials and methods

2.1. Cell culture and differentiation of P19 cells

EC P19 cells were cultured as described previously [9]. Briefly, the cells were seeded at a density of 10^5 cells/ml in α-minimal essential medium (α-MEM) containing 10% fetal bovine serum, and incubated at 37°C in a humidified atmosphere containing 5% CO_2 and passaged every second day. The cells were dispersed with 0.05% trypsin and0.53 mM EDTA and seeded at a density of 10^5 cells/ml in α-MEM containing 10% fetal bovine serum with or without 10^{-6} M RA (all-*trans*; Sigma) in 100 mm bacteriological dishes. After 48 hours, the aggregates were resuspended in a fresh medium. After a total of 96 hours in suspension culture, the aggregates were collected and treated with trypsin-EDTA. The cells were counted and transferred into 100 mm tissue

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culture dishes at a density of 2×10^5 cells/ml in fresh medium without RA. The medium was changed every second day.

2.2. RNA isolation and gel electrophoresis

At various time intervals, total cellular RNA was isolated with the guanidium thiocyanate extraction method [10]. The amount of total RNA was checked by electrophoresis in 1% agarose gels containing 2.2 M formaldehyde and 0.5 μg/ml ethidium bromide.

2.3. Reverse transcription

First strand cDNA was synthesized using MMLV reverse transcriptase (First strand cDNA synthesis kit; Pharmacia). The RNA (5 μg in 8 μl) was heated at 65°C for 10 min and then cooled on ice. Then, 11 μl of first strand cDNA synthesis reaction mixture (135 mM Tris-HCl, pH8.3, 204 mM KCl, 27 mM $MgCl_2$, 5.4 mM each dNTP, 0.24 mg/ml BSA), 1 μl of 0.2 M dithiothreitol, and 1 μl of either *Not*I $d(T)_{18}$ primer (0.2 μg) or random $d(N)_6$ primer (0.2 μg) were added to the RNA solution. After incubation at 37°C for 1 h the mixture was heated to 95°C for 5 min, chilled on ice and stored at −20°C.

2.4. Polymerase chain reaction (PCR) amplification

PCR was performed in a reaction mixture of 50 μl, 5.0 μl of a cDNA synthesis reaction mixture (from tenfold dilutions to one thousand dilutions), 50 mM KCl, 10 mM Tris-HCl, pH 9.0 at 25°C, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 200 μM in each dNTP, 1.25 units of Taq polymerase (Pharmacia), 25 pmol each of forward and reverse sequence-specific primers for 30 cycles of 1 min at 94°C, 2 min at 55°C, 3 min at 72°C, with an extra 7 min final extension at 72°C for the last cycle. Aliquots of PCR products (10 μl) were electrophoresed in 3.5% agarose gels (Nusieve GTG/Seakem) in Tris-acetate-EDTA buffer, pH 8.0, and stained with 0.5 μg/ml ethidium bromide. Linear amplification dependent on the amount of RNA was obtained under the above conditions (from 1.7 to 170 ng RNA). The amounts of mRNA were adjusted in each RT-PCR reaction by checking amplification of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcript.

The forward and reverse primers used for analysis of FGF9 and GAPDH gene expression are summarized in Table 1.

2.5. Sequence of the mouse FGF9 cDNA

The DNA amplified using the forward primer (5′-GAGTTGGATATACCTCGCCT) and the reverse primer (5′-AAGAACCCACCGCATGAAGC) was extracted with phenol/chloroform and precipitated with ethanol. Then, the DNA was ligated into pT7 Blue vector (Novagen). Cells of competent *E. coli* (Nova Blue) were transformed with the ligated vector. The plasmids of positive clones were extracted and the nucleotide sequences of their inserts were determined in both orientations with a BcaBest dideoxy sequencing kit (Takara) using [α-^{33}P]dCTP.

3. Results

3.1. RA-induced differentiation of EC P19 cells

Fig. 1 shows the morphologies of the cell types found in both RA-treated and non-treated cultures of EC P19 cells. When undifferentiated cells (as shown in Fig. 1A) were dispersed with trypsin and plated into bacteriological dishes with or without 10^{-6} M RA, cells aggregated spontaneously (Fig. 1B, C). After 96 h of incubation in aggregated state and plating in tissue culture dishes, the cells were induced to differentiate to neurons and glia-like cells, as described previously by Edwards and McBurney [7,9]. 144 h from the beginning of the incubation, neurons extended their neurites onto glia-like cells (Fig. 1D). After an additional 24 h of incubation, the number of glia-like cells increased (Fig. 1F). On the other hand, when cells were aggregated but not exposed to RA, the morphology of the cells appeared undifferentiated after 144 h of incubation (Fig. 1E). After an additional 24 h of incubation, many of these undifferentiated cells became super-confluent but not differentiated (Fig. 1G).

Table 1
Sequences and positions of oligonucleotides used as primers in RT-PCR

Target cDNA		Oligonucleotide sequence	Locus (region of cDNA amplified)
FGF9	F	5′-TTAAAGGGGATTCTCAGGCG	MFGF9
	R	5′-AAGAACCCACCGCATGAAGC	(346–872)
GAPDH	F	5′-ACCACAGTCCATGCCATCAC	G3PDH*
	R	5′-TCCACCACCCTGTTGCTGTA	(586–1037)

Sequences of the forward (F) and reverse (R) primers, and the Locus (GenBank) of the cDNA amplified are summarized. *The primers for glyceraldehyde 3-phosphate dehydrogenase were purchased from Clontech, Inc.

3.2. RA-induced expression of FGF9 mRNA in EC P19 cells

To determine the changes in levels of gene expression of FGF9 during the induction of neuronal differentiation of P19 cells by 10^{-6} M RA, we employed RT-PCR analysis with primers for the rat FGF9 (Table 1). The amounts of mRNA were checked by performing RT-PCR using GAPDH primers (Table 1). The specificity of each RT-PCR reaction was verified by restriction analysis and sequencing of the PCR products.

In the undifferentiated P19 cells (at 0 h of incubation), FGF9 transcripts were not detected (Fig. 2). In the RA-treated cells, the gene expression of FGF9 was stimulated in the aggregates from 8 h to 48 hr and from 168 hr to 192 hr as the number of glia-like cells increased and neurons extended an increasing number of neurites onto glia-like cells (Fig. 2 +RA). On the other hand, the gene expression of FGF9 was not stimulated in the cells aggregated but not treated with RA (Fig. 2 −RA).

3.3. Sequence of the mouse FGF9 cDNA

The sequence of the mouse FGF9 cDNA is reported here for the first time (Fig. 3). The forward and reverse primer for amplification of mouse FGF9 cDNA were based on the human and rat FGF9 cDNA sequences [5]. The murine cDNA showed 92.4% nucleotide sequence homology to the human FGF9 cDNA and 98.2% homology to that of rats. This mouse FGF9 cDNA encoded a polypeptide consisting of 208 amino acids with amino acid sequence identical to that of rats. The ninth amino acid from the N-terminus in the mouse FGF9 was serine being replaced by asparagine in the human homolog. Sequence similarity to other members of the FGF family was estimated to be around 30%.

4. Discussion

We have analyzed the mouse FGF9 cDNA nucleotide sequence as expressed in RA-treated P19 cells and showed its

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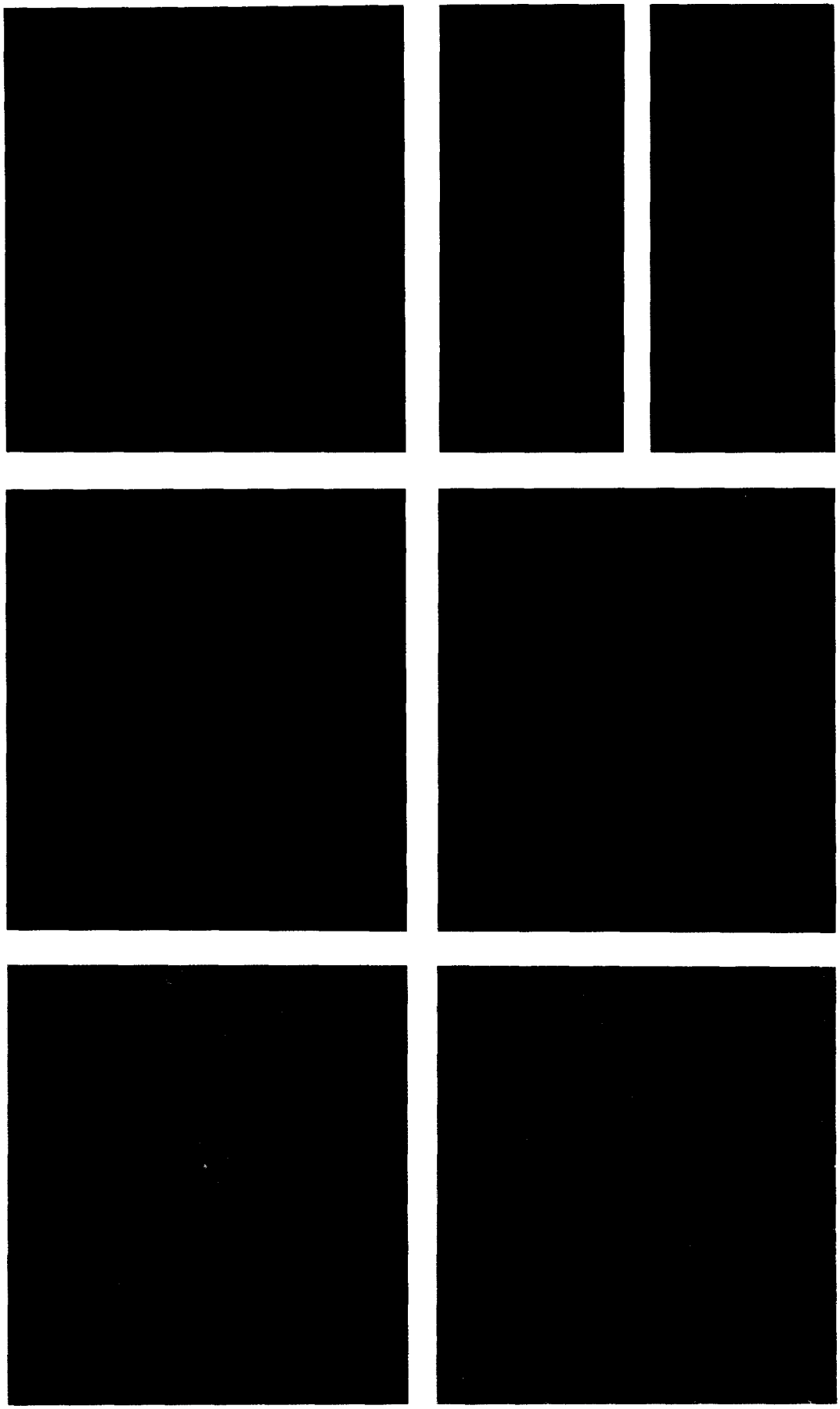
Fig. 1. Phase-contrast photomicroraphs of P19 EC cells. (A) Undifferentiated P19 cells growing on a tissue culture dish. (B) P19 cell aggregates treated with 10^{-6} M RA after 72 h of incubation on a bacteriological dish. (C) P19 cell aggregates without RA after 72 h on bacteriological dishes. (D,F) Differentiated cells plated in a tissue culture dish without RA following aggregation for 96 h and exposure to 10^{-6} M RA. Neurons and glial cells appeared at 144 h (D) or at 168 h (F) from the beginning of the experiments. (E,G) Cells remained undifferentiated at 144 h (E) or at 168 h (G) following plating on tissue culture dishes as described in (C). (Magnification: A, D, E, F, G, ×170, B, C, ×34.)



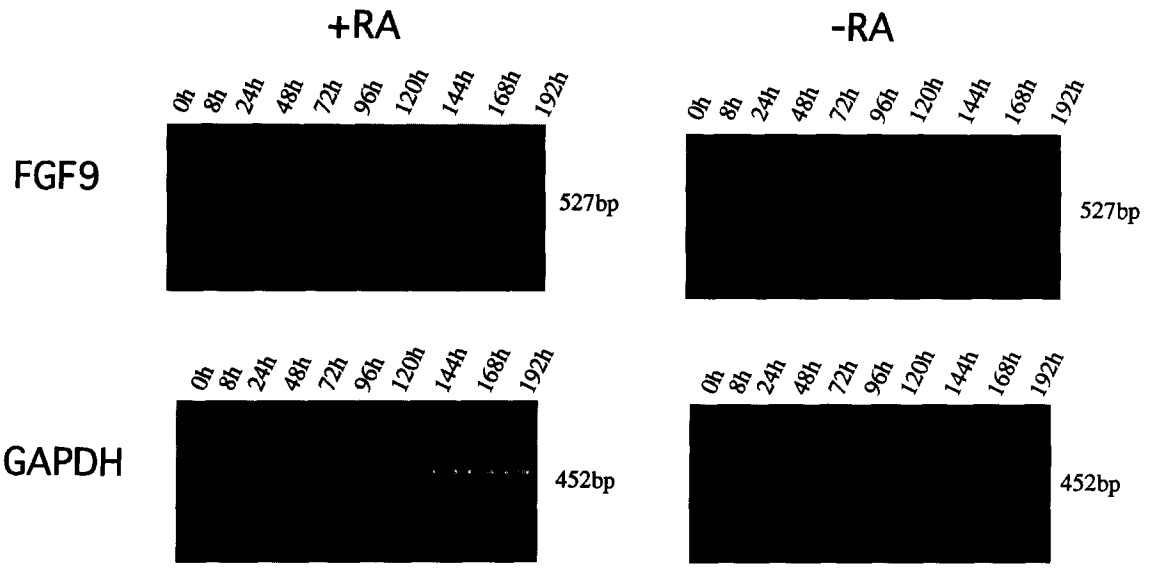


Fig. 2. Expression of FGF9 mRNA in P19 EC cells. Total RNA of P19 cells aggregated in the absence (−RA) or in the presence of 10^{-6} M RA (+RA) and plated onto tissue culture dishes as described in section 2, were analyzed. The RT reaction mixtures containing cDNA were diluted with distilled water, and then 5 μl of the diluted cDNA was used for PCR. The PCR primers for FGF9 and GAPDH are shown in Table 1. The PCR products were electrophoresed in 3.5% agarose gels (Nusieve Siekam) and stained with ethidium bromide.

highly homologous sequence to the FGF9 of humans and rats. The expression of FGF9 correlates with the time when the number of glial cells increases and neurons extends their neurites in tissue culture dishes, while cells aggregated in the absence of RA remained undifferentiatied and did not express FGF9. This suggests its functional importance not only as a glial cell growth stimulating factor, but also as a cue for neurons to extend their neurites onto glial cells. FGF9 may have the characteristics to bind to extracellular proteoglycans following secretion, as FGF1 and FGF2 [3], because FGF9 was originally isolated from a culture medium of a human glioma cell line as a growth-promoting factor for glial cells, using heparin affinity chromatography [5]. Although it has been reported that FGF9 lacks a typical N-terminal signal sequence for secretion, it is constitutively secreted following transfection into COS cells [6]. In an early stage of neural tube development, FGF2 has been reported to be involved in the developmental regulation of adhesive interaction between neuroepithelial cells and the extracellular matrix, thereby controlling their proliferation, migration and differentiation [12]. Thus, FGF9 may play a role in not only stimulating proliferation of glia cells but supporting neurons to differentiate, extend their neurites, and connect neurites and target cell junctions. It remains to be determined if the differentiated neurons or glia-like cells express FGF9.

FGF9 may play a role not only in the late developmental process as described above, but in the early developmental process when the cell fate was determined, because gene expression of FGF9 was induced during the first 48 hours forming cell aggregates with 10^{-6} M RA. FGF9 was found to be secreted from cells after synthesis despite its lack of a typical signal sequence [6]. Thus, it is possible that secreted FGFs such as FGF9 may be the physiological ligands for FGFRs during early nervous system development [15]. While both FGF1 and FGF2 were lacking typical signal sequence and primarily cell-associated proteins, they were expressed during critical phases of development and high in the adult central nervous system [15].

It is also important to know which FGF receptors accept FGF9 as a ligand and transfer the intracellular signals to proliferate or differentiate. Among four members of the FGF receptor family, each member has a different ligand specificity and affinity with some overlapping ligand specificities [14]. They also have different signaling transduction and biological response induction capabilities [18]. In P19 EC cells, FGFR1, R2, and R3 mRNAs were expressed, and all were upregulated after differentiation to derivatives resembling parietal endoderm [11]. FGFR1 and FGFR2 mRNAs were expressed by neurons and oligodendrocytes in the adult central nervous system, respectively [13]. Thus, more detailed analysis of which FGFRs are expressed in each neuronal or glial precursor cell and to which FGFRs FGF9 binds to promote their differentiation remains to be made.

We do not know whether the RA receptor itself directly regulates gene expression of FGF9. Three different RA receptors (RAR-α, β and γ) has been shown to exist in human and mouse. Each RA receptor may play a specific role in RA-triggered cascades of transcriptional regulation of gene expression in development. It has been reported that two isoforms of mouse RA receptor-β are not only preferentially expressed in both adult and fetal brain, but also up-regulated in RA-treated P19 cells [21]. It suggests that they play a specific role in the central nervous system. We are now analyzing the molecular mechanisms by which RA regulates the gene expression of FGF9. Furthermore, it is important to search for the factor(s) that controls the molecular switch to start the differentiation cascade for neuronal differentiation in vivo.

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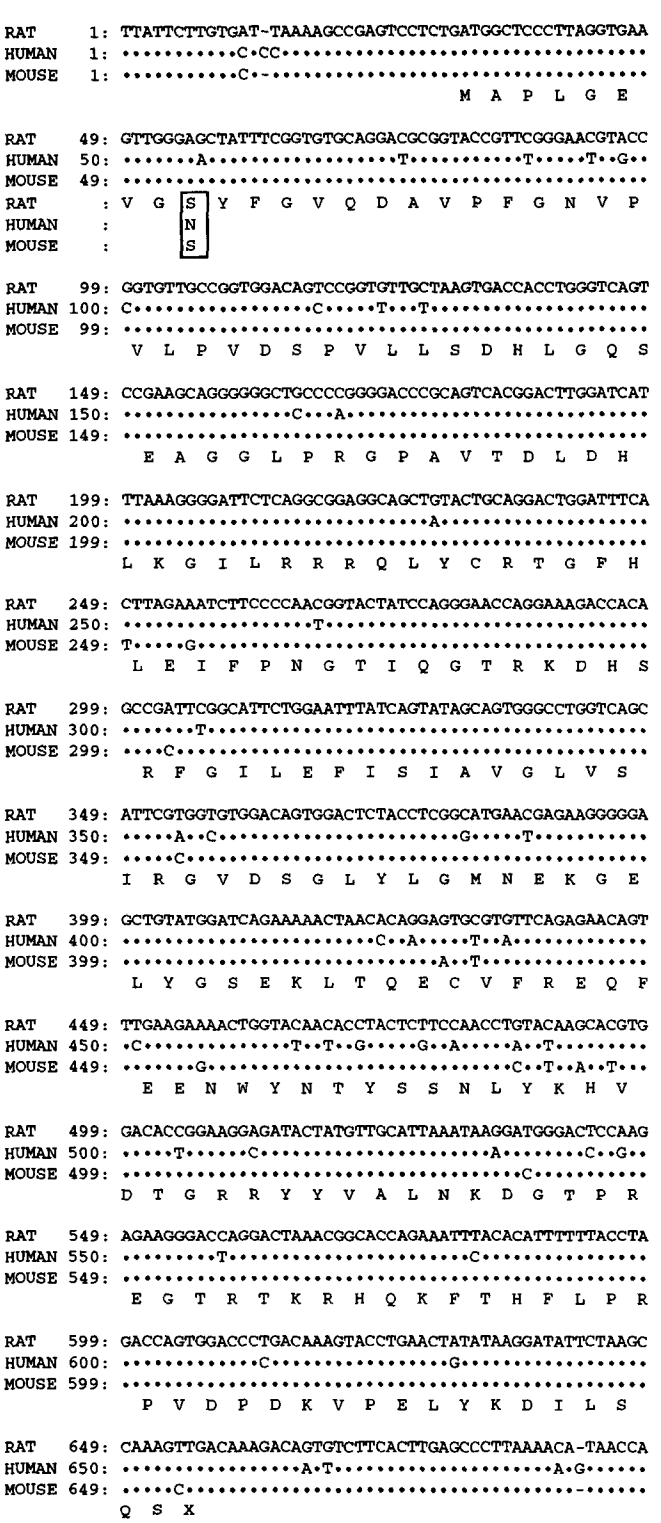

Fig. 3. Sequence of mouse FGF9 cDNA. Differences in the nucleotide and deduced amino acid sequences of the human, rat (5) and mouse FGF9 cDNAs are indicated by closed circles and boxed amino acid residues;rat, human, mouse, serine, aspargine, serine, respectively. The nucleotide sequence data reported in this paper will appear in the GSDG, DDBJ, EMBL, and NCBI nucleotide sequence databases with the following accession number D38258.

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