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One of the retinoic acid-inducible cDNA clones in mouse embryonal carcinoma F9 cells encodes a novel isoenzyme of fructose 1,6-bisphosphatase

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Abstract

Rae-30, one of the retinoic acid (RA)-inducible cDNA clones in mouse embryonal carcinoma F9 cells, was sequenced and the deduced RAE-30 protein showed about a 70% homology to mammalian fructose 1,6-bisphosphatase (EC 3.1.3.11) (FBPase), in comparison to over 85% homology observed among the previously documented rat liver, pig kidney and human leukemic HL-60 cell FBPases. The Rae-30 mRNAs were not detected in various tissues of adult mice, including the liver and kidney, but were detected in a placenta and predominantly in the intestine of adult mice. These findings indicate that the Rae-30 cDNA encodes a novel isoenzyme of FBPase, which is likely to be involved in early differentiation in mammalian cells.

Key words: Differentiation; Gluconeogenesis; Intestine; Evolution

1. Introduction

Mouse embryonal carcinoma F9 cells closely resemble pluripotent embryonic stem cells in terms of morphology, biochemical characteristics and growth properties [1,2]. F9 cells differentiate into parietal endoderm-like cells in response to retinoic acid (RA) [2,3]. This system provides a pertinent model for analyses of early differentiation in mammalian cells [1].

To elucidate molecular regulatory mechanisms involved in early mammalian development, we isolated a series of cDNA clones corresponding to those genes, the expression of which increases during RA-induced F9 cell differentiation [4]. We reported that one of the RA-inducible cDNA clones, named Rae-30 (retinoic acid early inducible cDNA clone-30), encodes a protein highly homologous to human fructose-1,6-bisphosphatase (D-fructose-1,6-bisphosphate 1-phosphohydrolase; EC 3.1.3.11) (FBPase) [4], an essential enzyme in gluconeogenesis [5].

We have now characterized the expression patterns of Rae-30 mRNAs and determined the entire nucleotide sequence of Rae-30 cDNA. We found that the Rae-30 cDNA encodes a novel isoenzyme of FBPase, probably an intestinal FBPase. The Rae-30 cDNA should be useful not only to examine the relationship between structure and function of the important metabolic isoenzymes, but also to examine regulatory mechanisms functioning in early differentiation in mammalian cells.

2. Materials and methods

2.1. Cells

Mouse embryonal carcinoma F9 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 15% fetal calf serum, and were induced to differentiate by treatment with 10^{-6} M retinoic acid (RA) (Sigma) [2].

2.2. RNA preparation and RNA analysis

Total cellular RNA was extracted from F9 cells, intestines, 14-day embryos and placentas of BALB/c mice by the acid guanidinium thiocyanate–phenol–chloroform method [6]. Poly(A)$^+$ RNA was prepared on an oligo(dT)-cellulose column. For RNA blot analysis, 10 μg of total cellular RNA was denatured with glyoxal, fractionated by 1% agarose gel electrophoresis and transferred to a nylon membrane [7]. RNA blots were hybridized with ^{32}P-labeled Rae-30 cDNA probe prepared by a multiprime DNA labeling system purchased from Amersham [8].

2.3. Cloning and DNA sequence analysis

Isolation of Rae-30 cDNA clone was as described previously [4]. pBluescript plasmids containing the Rae-30 cDNAs were excised from λZAPII cDNA clones by co-infection with helper phage (Stratagene) and were used for DNA sequencing reactions, after subcloning the restriction endonuclease fragments into pBluescript plasmid. The DNA sequence was determined by the dideoxy chain termination method adapted for denatured plasmid templates, using as primers T3 and T7 oligonucleotides [9].

A DNA homology search was done using the FASTA and BLASTN programs and the non-redundant nucleic acid database, and the deduced protein homology search was done using the FASTA and BLASTX programs and the non-redundant protein database, respectively, at the Human Genome Center, Tokyo, Japan [10].

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3. Results

3.1. Expression of Rae-30 mRNA

In our previous paper [4], we described that Rae-30 cDNA encodes a protein highly homologous to a human FBPase, a regulatory enzyme in gluconeogenesis [5], and described the following observations: (1) Rae-30 mRNAs are not detected in undifferentiated F9 cells; (2) they are induced at about 12 h after RA treatment, and are increased until 72 h; and (3) they are not detected in various tissues of adult mice, including the liver, kidney, and skeletal muscle.

We confirmed that the Rae-30 mRNAs are not detected in undifferentiated F9 cells, but are expressed at high levels at 72 h of RA treatment (Fig. 1). Interestingly, we found that the Rae-30 mRNAs are not present in a 14-day embryo but are present in the placenta and predominantly in the intestine of adult mice (Fig. 1). To determine whether gluconeogenetic pathways are activated during RA-induced F9 cell differentiation, we analyzed the patterns of expression of phosphoenolpyruvate carboxykinase (PEPCK), another enzyme essential for gluconeogenesis [11]. Although PEPCK expression has been reported to be induced in rat hepatoma cells by RA treatment [11], we found no evidence for PEPCK mRNA either in undifferentiated or in RA-treated F9 cells (data not shown). These results suggest that gluconeogenesis is not activated during the F9 cell differentiation.

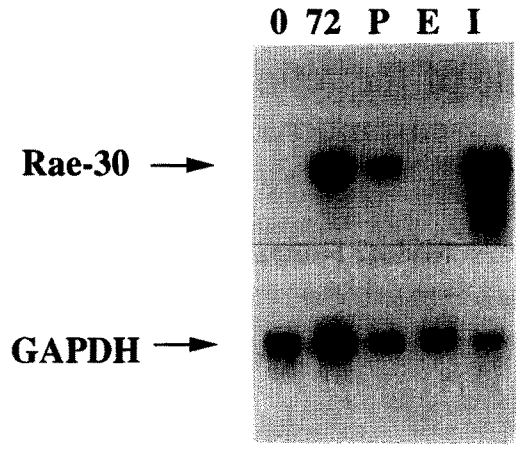


Fig. 1. Northern blot analysis of Rae-30 mRNAs. Total cellular RNAs were extracted from F9 cells after treatment with RA for 0 and 72 h, and from placenta, embryo and intestine of adult mice. Ten μg each of these RNAs was analyzed by RNA blotting using ^{32}P-labeled Rae-30 cDNA as a probe. The intensity of GAPDH mRNA is shown in the lower part of the figure, as a control for the amount of RNA loaded in each lane. Symbols are as follows: 0 = control F9 cells; 72 = F9 cells treated with RA for 72 h; P = 14-day placentas; E = 14-day embryos; I = intestines of adult mice.

3.2. Sequence analysis of Rae-30 cDNA

The entire nucleotide sequence of Rae-30 cDNA was determined and the amino acid sequence of RAE-30 protein was deduced (Fig. 2). The Rae-30 cDNA contained an open reading frame of 354 amino acids with a predicted molecular weight of about 39 kDa. The reading frame was preceded by a typical Kozak initiation sequence (CACAATGA) [12] and was terminated by a TGA stop codon. In the 3′-noncoding region, one putative polyadenylation signal sequence AATAAA was present. These findings indicate that the Rae-30 cDNA probably corresponds to the almost full-length Rae-30 mRNA (Fig. 2).

3.3. Homologies between the mammalian FBPase and the deduced mouse RAE-30 protein

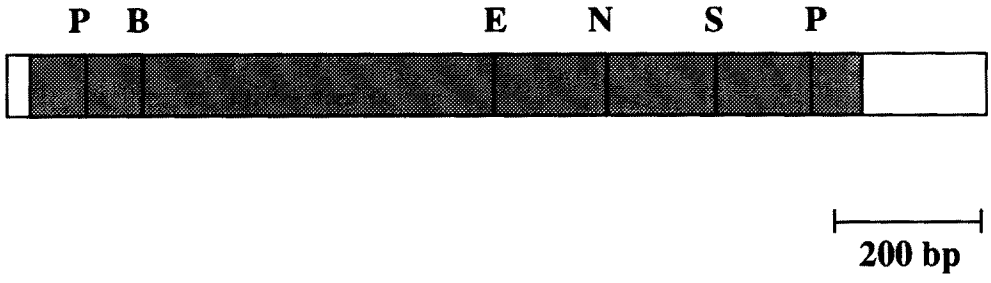
Homology search showed that the deduced RAE-30 protein is highly homologous to mammalian FBPase (Fig. 3), which catalyzes the hydrolysis of fructose 1,6-bisphosphate to fructose 6-phosphate and inorganic phosphate [5]. This enzyme is involved in many different metabolic pathways and is present in most organisms [5]. The amino acid sequences of FBPases from rat liver, pig kidney and human leukemic HL-60 cells were determined directly or were deduced from corresponding DNA sequence [13,14,15]. The deduced amino acid sequence of RAE-30 protein was aligned with those of mammalian FBPases (Fig. 3). The alignment revealed a high sequence homology throughout the entire sequences, except for the C-terminal region consisting of about 40 amino acids. This region is poorly conserved among the 4 proteins.

The activity of mammalian FBPase is controlled by the action of two inhibitors, fructose 2,6-bisphosphate and AMP [5]. In X-ray crystallographic studies, 18 amino acid residues have been located at or near the binding site of fructose 2,6-bisphosphate (see Fig. 3, residues marked with △) [16]. These 18 residues probably participate in active site interactions, and lysine-275 has been reported to be involved in catalytic mechanisms [16]. All these 18 residues and the region around the lysine-275, from residues 274 to 284, G-K-L-R-L-L-Y-E-C-N-P, is completely conserved among these four different proteins (Fig. 3). Twelve amino acid residues have been reported to interact with AMP (see Fig. 3, residues marked with *) [17], and 10 of the 12 residues were conserved between the mammalian FBPase and RAE-30 protein (Fig. 3). All these features indicate that the RAE-30 protein is an isoenzyme of mammalian FBPase.

3.4. Evolution of the mouse RAE-30 gene

The sequence homology among the mammalian FBPases was over 85% in comparison to about 70% homology between the mammalian FBPase and the RAE-30 protein (Fig. 4A). We constructed a phylogenetic tree to elucidate an evolutionary relationship between mouse RAE-30 protein and mammalian FBPase (Fig. 4B). The tree indicated that the gene for mouse

(A)



(B)

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                      M  T  D  R  S  P  F  E  T  D  M  L  T  L   14
   1 TTGGGATCTCATTTGCACAATGACGGACAGAAGCCCCTTTGAGACAGACATGCTGACCCT
     T  R  Y  V  M  E  K  G  R  Q  A  K  G  T  G  E  L  T  Q  L   34
  61 GACCCGTTACGTTATGGAAAAGGGGCGACAGGCCAAAGGGACCGGAGAACTCACCCAGCT
     L  N  S  M  L  T  A  I  K  A  I  S  S  A  V  R  K  A  G  L   54
 121 GCTCAACTCGATGCTGACTGCCATCAAAGCCATCTCTTCCGCAGTGCGCAAGGCCGGCCT
     A  N  L  Y  G  I  S  G  S  V  N  V  T  G  D  E  V  K  K  L   74
 181 GGCCAACCTGTATGGGATTTCGGGGAGCGTGAATGTGACAGGAGATGAGGTGAAGAAACT
     D  V  L  S  N  S  L  V  I  N  M  L  Q  S  S  Y  S  T  C  V   94
 241 GGACGTGCTGTCCAACTCCCTGGTCATCAACATGCTTCAGTCCTCCTACAGCACCTGTGT
     L  V  S  E  E  N  K  E  A  V  I  T  A  Q  E  R  R  G  K  Y  114
 301 GCTCGTCTCCGAAGAGAATAAAGAGGCGGTGATCACAGCCCAGGAGAGGAGGGGGAAATA
     V  V  C  F  D  P  L  D  G  S  S  N  I  D  C  L  A  S  I  G  134
 361 TGTGGTTTGCTTTGACCCTCTGGATGGATCTTCAAACATTGACTGCCTGGCCTCCATCGG
     T  I  F  A  I  Y  R  K  T  T  E  D  E  P  S  E  K  D  A  L  154
 421 AACTATATTTGCTATTTACAGAAAGACCACGGAGGACGAGCCTTCTGAGAAGGATGCCTT
     Q  P  G  R  N  I  V  A  A  G  Y  A  L  Y  G  S  R  T  L  V  174
 481 GCAGCCTGGCCGCAACATCGTGGCTGCGGGTTATGCACTGTATGGTAGTCGAACCCTGGT
     A  L  S  T  G  Q  G  V  D  L  F  M  L  D  P  A  L  G  E  F  194
 541 TGCTCTTTCCACAGGACAAGGAGTGGATCTGTTCATGCTGGACCCGGCTCTTGGAGAATT
     V  L  V  E  K  D  V  R  I  K  K  K  G  K  I  F  S  L  N  E  214
 601 CGTGCTAGTGGAAAAAGATGTCCGGATTAAGAAGAAAGGGAAAATTTTTAGCCTCAACGA
     G  Y  A  K  Y  F  D  A  A  T  A  E  Y  V  Q  K  K  K  F  P  234
 661 GGGCTATGCCAAGTATTTTGATGCTGCTACTGCTGAGTATGTACAGAAAAAGAAATTCCC
     E  D  G  S  E  P  Y  G  A  R  Y  V  G  S  M  V  A  D  V  H  254
 721 CGAGGATGGCAGTGAGCCTTATGGAGCCAGGTACGTGGGTTCCATGGTGGCTGATGTGCA
     R  T  L  V  Y  G  G  I  F  M  Y  P  A  N  Q  K  S  P  N  G  274
 781 TCGCACCTTGGTCTATGGAGGAATCTTCATGTACCCAGCCAACCAGAAGAGTCCTAATGG
     K  L  R  L  L  Y  E  C  N  P  V  A  Y  I  I  E  Q  A  G  G  294
 841 CAAGCTCCGGCTCCTGTATGAATGCAATCCTGTGGCCTATATCATCGAGCAAGCAGGAGG
     M  A  T  T  G  T  Q  P  V  L  D  V  K  P  E  S  I  H  Q  R  314
 901 TATGGCAACCACAGGCACCCAGCCAGTACTGGATGTGAAACCTGAGAGTATTCACCAGCG
     V  P  L  I  L  G  P  L  R  M  C  K  S  I  S  A  V  C  R  E  334
 961 AGTCCCCCTCATTCTGGGTCCCCTGAGGATGTGCAAGAGTATCTCAGCTGTGTGCAGAGA
     T  R  Q  A  G  S  E  P  E  P  M  S  P  L  P  F  V  F  V  N  354
1021 AACCAGGCAGGCAGGTAGTGAGCCTGAACCCATGAGCCCGCTTCCCTTTGTCTTTGTCAA
     TER
1081 TTGAAAAACTAGATGAATGAGCTATGGAGATGGGAGGAAAGGCAAAGAAGTCAAGTGACA

1141 CAGGTCACGGTCAGAACAGCGCCCTGCTGCTAAGGACAGGGTTAGAAGCCAGGGGTAAAG

1201 AAAGATACAGTCTTTGGACTAAAATAAAAATATGAATCTGAA
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Fig. 2. The nucleotide and amino acid sequence structures of Rae-30 cDNA. (A) A cleavage map of the Rae-30 cDNA. The restriction sites: P = *Pvu*II; B = *Bal*I; E = *Eco*RI; N = *Nco*I; S = *Sca*I. The putative coding regions are shaded. (B) The nucleotide and deduced amino acid sequences of Rae-30 cDNA. A putative initiation ATG codon in the 5′-noncoding region, a termination codon and a polyadenylation signal sequence in the 3′-noncoding regions are double-underlined. The predicted amino acid sequence is shown above the nucleotide sequence. The numbers at the left and right sides denote that of nucleotides and amino acids, respectively.

RAE-30 protein diverged from an ancestoral gene of mammalian FBPase 174 million years ago if we take a divergence time between human and rodents as 75 million years ago [18]. This suggests that the counterpart of

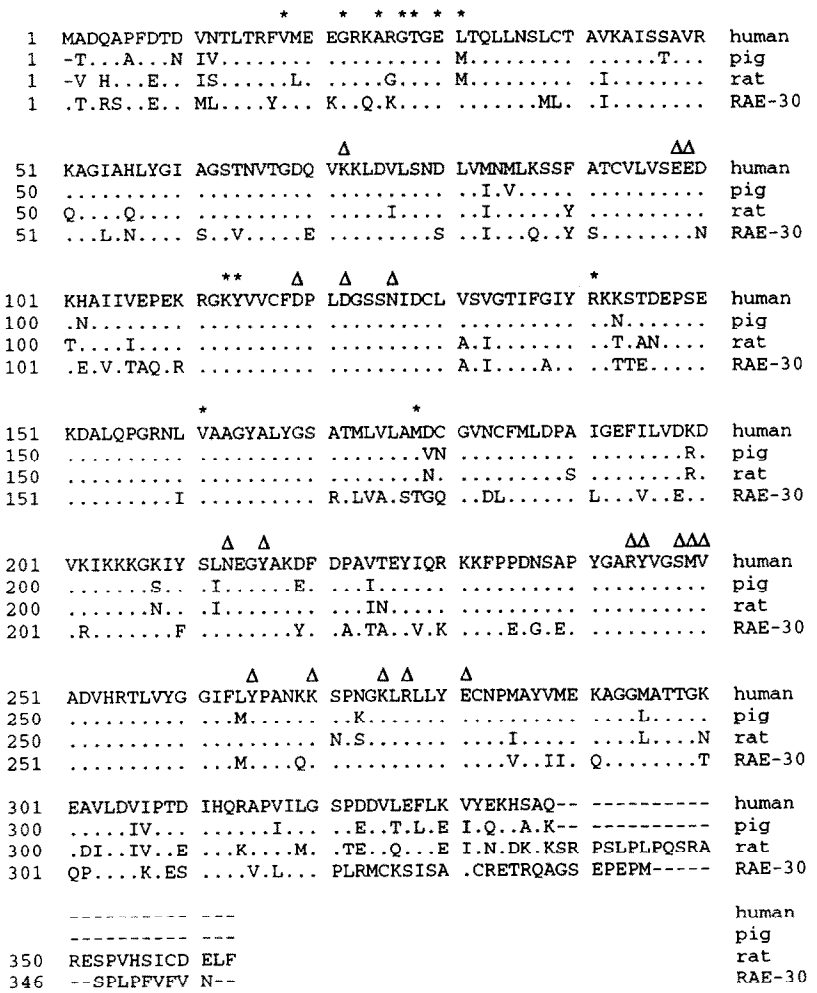


Fig. 3. Alignments of the amino acid sequences among mammalian FBPase and RAE-30 protein. Complete deduced amino acid sequence of RAE-30 protein is shown in lines labeled with RAE-30 at the right. The amino acid sequences of human FBPase, pig FBPase, rat FBPases and RAE-30 protein are shown in lines labeled with human, pig, rat and RAE-30 at the right, respectively. Dots indicate identical amino acid residues and dashes, insertions made during alignment. The amino acid residues marked with △ have been located at or near the binding site of fructose 2,6-bisphosphate [16], and those marked with * interact with AMP [17]. The numbers at the left denote amino acid residue numbers.

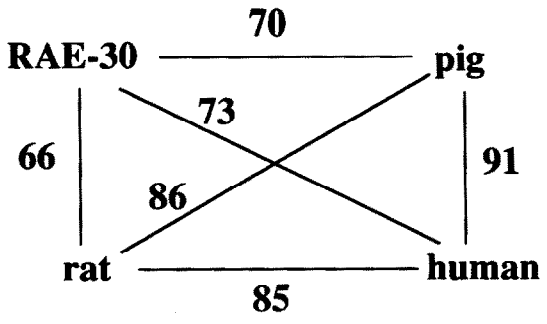
mouse RAE-30 protein exists in every mammalian species.

4. Discussion

We have characterized Rae-30, one of the RA-inducible cDNA clones in F9 cells, and found that although Rae-30 mRNAs are not detected in various tissues of adult mice, including the liver, kidney and skeletal muscle [4], they are present in the placenta and predominantly in the intestine of adult mice (Fig. 1). In response to RA, the Rae-30 mRNAs are induced in F9 cells (Fig. 1) [4], and F9 cells are induced to differentiate into parietal endoderm-like cells, one of the major cell lineages of the placenta [2,3]. These observations indicate that the pattern of expression of Rae-30 mRNAs represents an event occurring in mammalian cells during early differentiation, and indicate that the Rae-30 cDNA provides one model system to examine the developmental stage-specific and tissue-specific transcriptional regulation.

The deduced RAE-30 protein showed about a 70% homology with three different mammalian FBPases (Fig. 4). Mammalian FBPase activities were predominantly present in liver, kidney and skeletal muscle [19]. Significant activity was also observed in the small intestinal mucosa of adult mice [19]. As the intestinal FBPase was 10-fold more sensitive to AMP inhibition than the liver enzyme, they were thought to represent different isoenzymes [19]. However, the amino acid sequence of the intestinal FBPase has not been reported, and the cDNA and genomic DNA structures have not been elucidated. The alignments of amino acid sequences of the deduced RAE-30 protein and the three different mammalian FBPases indicated that the Rae-30 cDNA encodes a novel isoenzyme of FBPase (Fig. 3). We speculate that

(A)



(B)

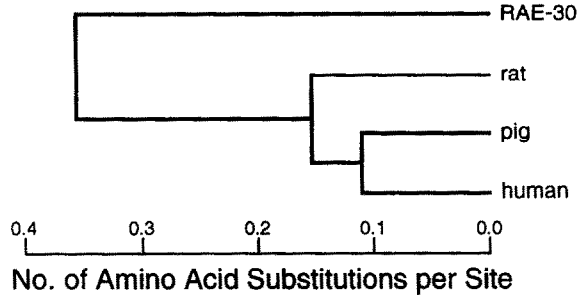


Fig. 4. (A) A scheme showing the degree of sequence homology among the RAE-30 protein and mammalian FBPases. Symbols: human, pig and rat indicate FBPases from human leukemic HL-60 cells, pig kidney and rat liver, respectively; RAE-30 indicates the deduced RAE-30 protein of the mouse. The numbers indicate the amino acid sequence homology in %, calculated from the sequence data summarized in Fig. 3. (B) Phylogenetic relationship of mouse RAE-30 protein and mammalian FBPases. Amino acid sequence difference (d) for each pair of RAE-30, human FBPase, pig FBPase and rat FBPase was calculated and corrected for multiple hits to get a number of amino acid substitutions per site (k) by the equation, $k = -\log(1-d)$ [22]. For this calculation, amino acids from #2 to #338 of human FBPase and RAE-30 protein and those from #1 to #337 of pig and rat FBPases in Fig. 3 were used. Phylogentic tree was constructed by the unweighted pair-group clustering method [23].

this isoenzyme corresponds to intestinal FBPase [19], because the Rae-30 mRNAs are detected at high levels in the intestine (Fig. 1), but not in the liver, kidney and skeletal muscle of adult mice [4].

A striking deficiency of hepatic FBPase activity was demonstrated in a child with hypoglycemia and metabolic acidosis on fasting [20]. A sibling who died with similar features suggested that this is an inherited defect [20]. However, the roles of the RAE-30 protein as well as those of the intestinal FBPase remain to be elucidated. To construct the RAE-30 protein-deficient mice by gene targeting [21] will shed light not only on regulatory mechanisms of gluconeogenesis, but also on the regulatory mechanisms of early differentiation in mammalian cells.

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