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The rat insulin-degrading enzyme*

Molecular cloning and characterization of tissue-specific transcripts

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The primary structure of the rat insulin-degrading enzyme (IDE) was determined by cDNA analysis. Rat IDE, as well as the previously characterized homologs from human and *Drosophila*, contain the carboxyl-terminal consensus sequence A/S-K-L for peroxisome targeting. A stretch of 43 bp surrounding an alternatively used polyadenylation site is highly conserved between rat and human, suggesting that it may contain important regulatory information. Northern blot analysis revealed two IDE transcripts of 3.7 and 5.5 kb in various tissues. Testis was found to be exceptional in having three different RNAs (3.7, 4.1 and 6.1 kb) at a relatively high abundance. The expression of the IDE gene in testis is correlated with sexual maturation.

Insulin-degrading enzyme; Peroxisome; Polyadenylation site; Testis

1. INTRODUCTION

Insulin-degrading enzyme (IDE; EC 3.4.22.11) is a neutral metalloendoprotease [1] that is thought to be involved in the metabolism of internalized insulin [2,3]. The reported high-affinity binding to, and degradation of, transforming growth factor-α [4] and atrial natriuretic peptide [5,6] suggests that the enzyme is also involved in the processing and/or metabolic clearance of signalling factors other than insulin. The primary structures of human [7,8] and *Drosophila* [9] IDEs indicate that IDE is a polypeptide of 110 kDa, is evolutionarily highly conserved, and does not show extensive sequence similarity to other known proteases except for *Escherichia coli* protease III [7]. A number of questions concerning the subcellular localization and biological role of the enzyme are still open. For example, it is not yet clear how IDE, which appears to be cytosolic [10], comes into contact with insulin subsequent to the receptor-mediated internalization of the hormone. In addition, the structure and tissue distribution of IDE transcripts are poorly characterized.

In this report we describe the isolation and characterization of IDE cDNAs from the rat and show the presence of IDE transcripts of various lengths in a number of tissues, as well as its different expression pattern in testis. We find that a recently identified peroxisome-targeting signal [11] is present in IDE.

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*The sequence of the rat insulin-degrading enzyme is registered under EMBL accession no. X67269.

2. MATERIALS AND METHODS

2.1. *cDNA isolation and sequencing*

The purification of rat IDE and partial amino acid sequences from this have recently been reported [5]. Based on the peptide sequence FIIQSEKPPHYLE [5], the mixed 'best-guess' oligonucleotide probe 5′-TTCATCATCCAG(TC/AG)CGAGAAGCCCCCCCACTACCT-GGA-3′ was synthesized (for serine, two codons, TCC and AGC, were incorporated, because these triplets are utilized with equal frequency in the rat [12]). This probe, labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase, was used to screen a λgt11 rat olfactory bulb cDNA library under hybridization conditions essentially as described by Ullrich et al. [13]. A 1.3 kb cDNA was isolated and found by DNA sequencing to contain a 0.6 kb fragment of IDE cDNA (nucleotides 2,336–2,964, Fig. 1) linked to an IDE-unrelated DNA sequence. A *Pst*I fragment from this cDNA, comprising nucleotides 2,337–2,724 (Fig. 1), was labeled with [α-^{32}P]dATP by random priming and used to screen a rat brain cDNA library constructed in λZapII. Hybridization was performed in 20% (v/v) formamide, 5 × SSC, 5 × Denhardt's solution and 50 mM phosphate buffer (pH 6.8), containing 50 μg/ml sheared salmon sperm DNA, at 42°C overnight. Filters were washed (2 × 30 min) at 68°C in 0.2 × SSC, 0.1% (w/v) SDS. From a screen of 700,000 plaques, 17 positive clones were detected and purified. Restriction site analysis revealed the existence of only two classes of cDNA, exemplified by clones b1 (3.4 kb) and b2 (3.8 kb) (Fig. 1). Two cDNAs each of clones b1 and b2 were rescued as pBluescript plasmids utilizing the excision process of λZAPII. After a limited *Eco*RI digestion, full-length inserts were isolated and subcloned in both orientations into M13mp18.

Deletions were prepared using the DNase I method [14]. Sequencing of the subclones was carried out with dye primers using an Applied Biosystems DNA sequencing machine (model 373A).

The radiolabeled *Pst*I fragment (see above) was also used as a probe to screen a rat testis cDNA library (Clontech, Palo Alto, USA), derived from adult rats, in λgt11. Four positive clones were isolated and characterized. The inserts (1.4 kb) of three of these were found (by

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restriction site and sequence analysis) to be identical and represent the central *Eco*RI fragment of the IDE cDNA (nucleotides 1,601–3,004, Fig. 1). Sequence analysis of the fourth clone, termed t1, revealed that t1 contained, in addition to the central *Eco*RI fragment of the IDE cDNA, a 5′ extension of 494 bp and a 3′ extension of 237 bp. The 5′ region was identical in sequence to the corresponding brain cDNA

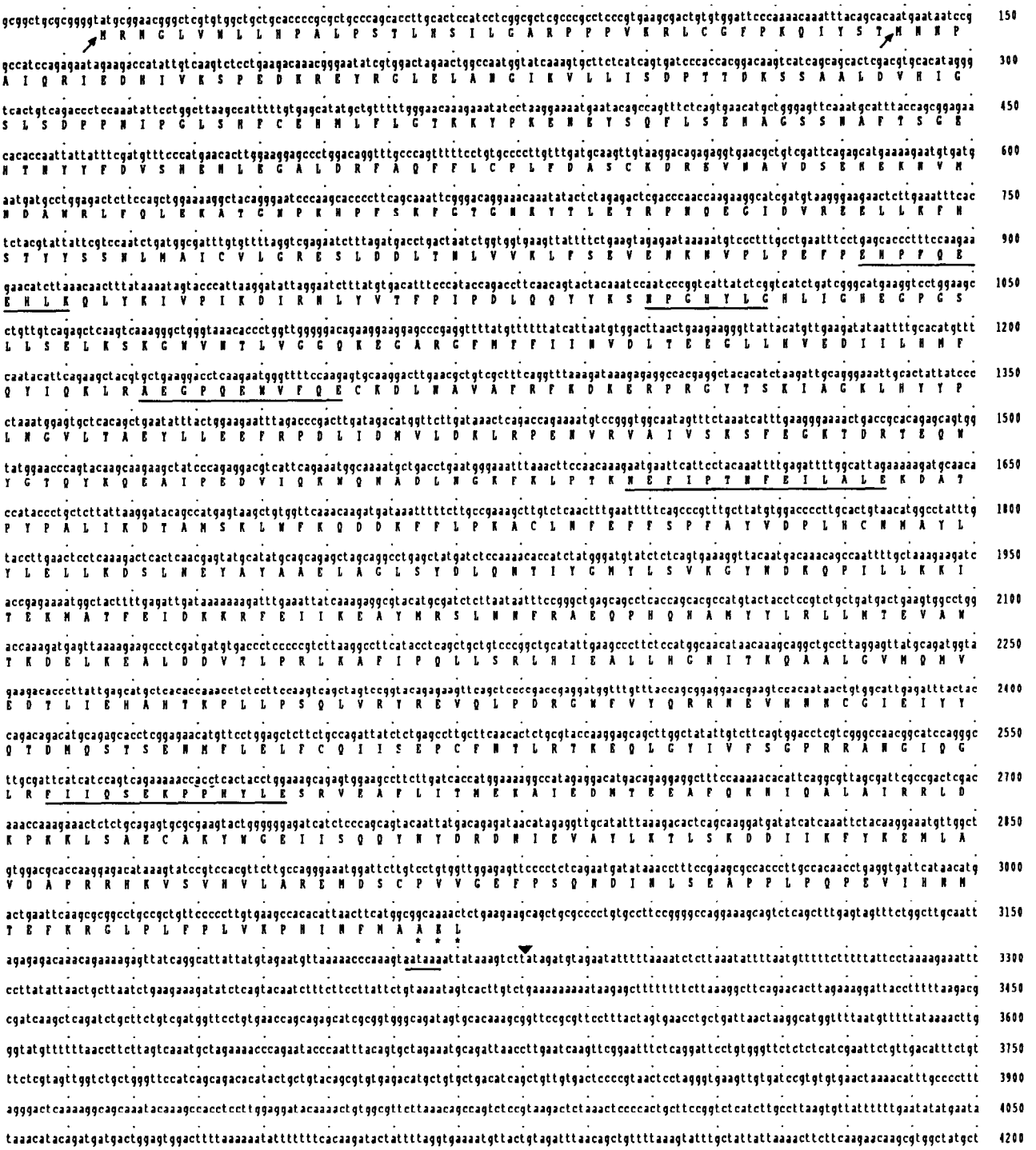

Fig. 1. Nucleotide and deduced amino acid sequence of rat IDE and its cDNA. The nucleotide sequence is derived from the sequences of the overlapping cDNA clones b1 (nucleotides 1–3,377) and b2 (455–4,276). The canonical polyadenylation signal, AATAAA, is underlined and the poly(A) addition site, identified by analysis of clone t1, is marked by an arrowhead. Sequences corresponding to tryptic peptides from the purified rat IDE [5] are underlined. Two possible translation initiation codons at positions 16–18 and 139–141 are indicated by arrows. Asterisks mark the consensus signal, AKL, for peroxisome targeting.

(nucleotides 1,107–1,600, Fig. 1). The 3′ region (downstream of the central *Eco*RI fragment) was identical to the brain cDNA up to nucleotide 3,230 (Fig. 1), but then ended in a stretch of 11 adenosine residues.

2.2. *Northern blot analysis*

Total RNA was extracted from Wistar rat tissues with guanidinium isothiocyanate and purified by CsCl centrifugation [15]. After glyoxylation, the RNA (20 μg) was separated in 1% (w/v) agarose gels and transferred to GeneScreen Plus membranes (Du Pont, Bad Homburg, Germany) according to the manufacturer's instructions. The blots were hybridized with a single-stranded ^{32}P-labeled DNA probe derived from a partial IDE cDNA (comprising nucleotides 2,336–2,964, Fig. 1) in the vector, M13mp18, and created by primed synthesis using the M13 forward ('−20') primer. Hybridization and wash conditions were as previously described [16].

3. RESULTS AND DISCUSSION

Two overlapping IDE cDNAs (named b1 and b2) were isolated from a rat brain cDNA library (see section 2). Clone b1 contains the complete IDE coding region (3,057 bp) flanked by a short (15 bp) 5′- and a 302 bp long 3′ untranslated region (Fig. 1). Clone b2, 3,822 bp in size, differs from cDNA b1 in that it has a 3′ terminal extension of 899 bp but a shorter (454 bp) 5′ end (Fig. 1). Neither cDNA contains a 3′ terminal poly(A) stretch nor a potential polyadenylation signal close to the 3′ end. However, a single consensus polyadenylation motif AATAAA (nucleotides 3,212–3,217, Fig. 1) is present at a distance of 160 bp (cDNA b1) and 1,059 bp (cDNA b2), respectively, from the 3′ termini. Both cDNAs therefore represent transcripts in which the corresponding AAUAAA hexamer was not used for 3′ end formation. They probably result from oligo(dT) priming on A-rich sequences located within the mRNA 3′-untranslated region, such as the $(A)_9$ tract (nucleotides 3,382–3,390) that is present immediately downstream of the 3′ end of the sequence of clone b1. In contrast, a third cDNA (named t1), isolated from a rat testis cDNA library, is characterized by having a 3′-terminal poly(A) tract beginning 13 bp downstream of the noted AATAAA motif; the remaining 5′ sequence of the 2.1 kb long testis cDNA is strictly identical to that of the brain-derived cDNA (nucleotides 1,107–3,230, Fig. 1).

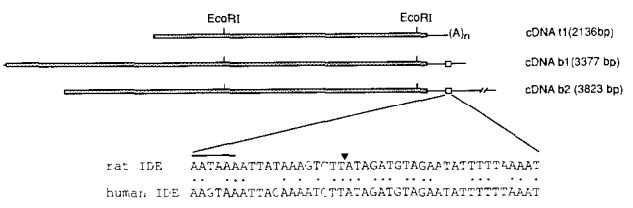

Fig. 2. Conserved sequence element within the IDE cDNA 3′ untranslated region. The diagram illustrates the position of the element in the rat IDE cDNAs and shows an alignment with nucleotides 3,216–3,258 [8] of the human IDE cDNA. Nucleotides that are identical in the two sequences are indicated by dots. The polyadenylation signal is marked by a bar and the poly(A) addition site, identified in cDNA clone t1, is denoted by an arrowhead.

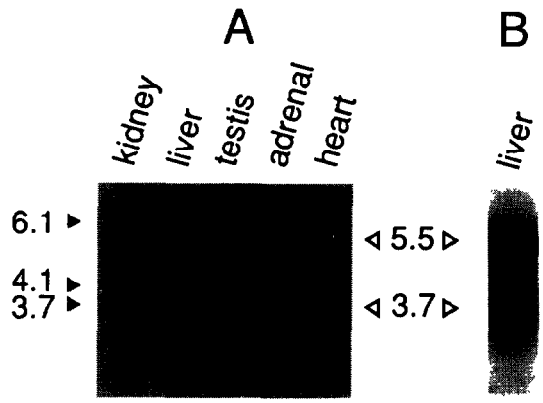

Fig. 3. Northern blot analysis of IDE transcripts in various tissues. (A) Total RNA (20 μg), isolated from different rat tissues, was size fractionated and blotted. The blot was probed with a ^{32}P-labeled single-stranded DNA (0.5×10^9 cpm/μg) the sequence of which is common to all three rat IDE cDNA clones. (B) Probing of a blot containing liver RNA with a DNA fragment of higher specific activity (5.5×10^9 cpm/μg). Sizes (in kb) were determined relative to an RNA ladder, run in parallel.

These findings predict an IDE mRNA heterogeneity due to the utilization of different polyadenylation sites.

The deduced rat IDE consists of 1,019 amino acids (calculated molecular weight of 118 kDa) and contains stretches of amino acids that completely match those of the five peptide fragments obtained after tryptic digestion of the purified enzyme ([5], Fig. 1, underlined sequences). The first putative start codon has been assigned at nucleotide position 16–18, however, its flanking sequence poorly meets the consensus requirement proposed by Kozak [17]. Initiation of translation may alternatively occur at the second ATG codon, located 123 nucleotides further downstream, the surrounding sequence of which is more favourable for initiation [9].

The rat IDE is highly similar (95% amino acid identity) to the cDNA-deduced human sequence [7]; it also exhibits 47% identity to the *Drosophila* homologue [9]. A 12 amino acid motif (GLS/AHFCEHMLFL), which has recently been suggested to represent a metal-binding site [9], is present in rat IDE (residues 105–116), and this sequence is identical to that of the corresponding motif in the human enzyme. Remarkably, the rat, as well as the human and *Drosophila* proteins, contain, at their carboxyl termini, a sequence motif (S/A-K-L) which has recently been characterized to function as a topogenic signal in the translocation of proteins into peroxisomes [11]. Thus, the enzyme contains a well-conserved motif that has been proven to be functional in protein import into a membrane-enclosed organelle. Future studies should address the possible role of IDE in peroxisomes and examine whether the carboxyl terminal signal sequence is responsible for the observed IDE activity in endosomes [18].

The 5′ end of the rat IDE cDNA sequence, defined by a short (15 bp) non-coding region, is rich in G and

C bases (13 GC bp) which may imply a highly structured RNA upstream of the first AUG codon.

Sequence comparisons of the rat, human and *Drosophila* IDE cDNAs shows that the 3′-untranslated region has diverged during evolution at a much greater rate than the remaining sequence. However, a 43 bp segment within the 3′ non-coding region of the rat sequence (nucleotides 3,212–3,254, Fig. 1) exhibits a strong (88%) identity with a segment of the corresponding region of the human IDE cDNA (Fig. 2). This element is as highly conserved as the nucleotide sequences of the coding regions (88% identity) of the rat and human IDE cDNAs. In contrast, the rest of the 3′ untranslated region exhibits only 43% (upstream of the conserved motif) and 34% (downstream of the conserved motif) sequence identity, respectively. The conserved stretch is located 140 bp (in the rat IDE cDNA) and 159 bp (in the human IDE cDNA) downstream of the end of the coding sequence. As shown in Fig. 2, the polyadenylation signal, AATAAA, marks the 5′ terminus; the poly(A) addition site revealed by analysis of cDNA clone t1 is located in the middle of this sequence. The strong evolutionary conservation of this segment of the 3′ untranslated region suggests a functional role possibly in the polyadenylation process. This motif is not present in the *Drosophila* IDE cDNA sequence. However, the *Drosophila* 3′ untranslated region is shorter (136 bp) than that of the rat and human cDNAs and, thus, the reported *Drosophila* sequence [9] simply may not extend sufficiently 3′ to reveal the corresponding segment. Computer-assisted comparison did not reveal significant similarities to cDNA sequences of other proteins, suggesting that the motif is specific for IDE or for only a few proteins.

To analyze IDE gene transcription, total RNA from various rat tissues was examined on Northern blots. Two transcripts of approximately 3.7 and 5.5 kb, both present at very low levels, were identified in kidney, liver, adrenal and heart (Fig. 3A), as well as in brain, spleen and lung (not shown). Compared with these tissues, much stronger signals were observed in testis RNA. In addition to the higher abundance of the transcripts, testis is unusual in that the 5.5 kb transcript appears to be replaced by a longer RNA of approximately 6.1 kb (Fig. 3A). More remarkably, the strongest signal in testis results from an additional RNA of 4.1 kb. Even after a prolonged exposure to X-ray film (not shown) or after hybridization with a probe of an 11-fold higher specific activity, an RNA of this size could not be detected in liver (Fig. 3B) or in any of the other tissues tested (not shown). Thus, within the limit of the tissues examined, the 4.1 kb transcript seems to be testis specific.

To examine whether the occurrence of IDE transcripts in testis is related to the functional maturation of this tissue, RNA from different developmental stages (postnatal days 1, 7, 14, and 28, and months 2 and 6) was analyzed by RNA blotting. As shown in Fig. 4, a significant increase in the levels of the IDE mRNAs occurs between day 14 and day 28, although the 3.7 kb transcript is present, albeit at very low levels, at earlier stages. Thus, the presence of the 4.1 kb and 6.1 kb transcripts, at least, which were exclusively detected in testis, clearly correlates with the onset of testis-specific gene activation and sperm cell differentiation which commences between days 14 and 28 [19]. The relatively high levels of IDE transcripts in testis and the developmentally regulated expression of the gene therein may indicate a particular function for IDE in this tissue.

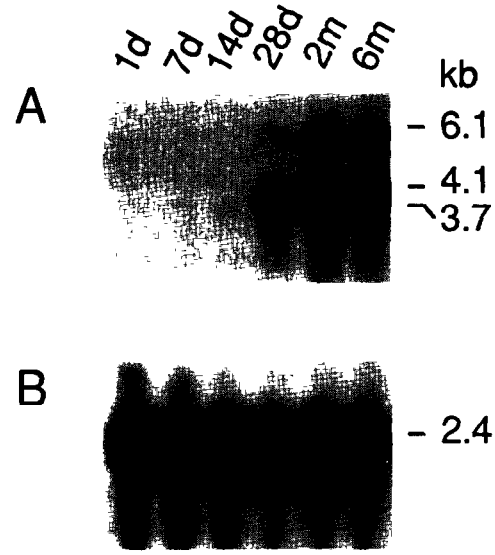

Fig. 4. IDE transcripts in rat testis during development. 20 μg of total RNA from different postnatal stages (day 1 to month 6) were separated, blotted and hybridized to a single-stranded DNA probe derived from the rat IDE cDNA (A). Subsequent probing of the blot with a chicken β-actin cDNA probe (B) confirmed that all lanes contained similar amounts of intact RNA.

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