cDNA cloning of turtle prion protein

Tatjana Simonic*, Stefano Duga, Bice Strumbo, Rosanna Asselta, Fabrizio Ceciliani, Severino Ronchi

Istituto di Fisiologia Veterinaria e Biochimica, Università di Milano, Via Celoria 10, 20133 Milan, Italy

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Abstract Cloning of the cDNA coding for the 270-residue turtle prion protein is reported. It represents the most remote example thus far described. The entire coding region is comprised in a single exon, while a large intron interrupts the 5′ UTR. The common structural features of the known prion proteins are all conserved in turtle PrP, whose identity degree to mammalian and avian proteins is about 40 and 58%, respectively. The most intriguing feature, unique to the turtle prion, is the presence of an EF-hand Ca^{2+} binding motif in the C-terminal half of the protein.

Key words: Reptile prion protein; cDNA cloning; Gene organization; EF-hand Ca^{2+} binding motif

1. Introduction

Prion defines a proteinaceous infectious transmissible pathogen, responsible for several neurodegenerative diseases in humans and animals [1–3]. A large amount of evidence supports the 'protein-only hypothesis', i.e. that prions are essentially composed of the scrapie isoform (PrP^{Sc}) of a normal protein named cellular prion protein (PrP^{c}) [2]. PrP^{c} is converted into a pathologic isoform (PrP^{Sc}) when it adopts an abnormal conformation, by increasing its β-sheet content [2]. This structural transition is accompanied by both an increased protease-resistance and a tendency to polymerize into insoluble amyloid fibrils [2].

Cellular prion proteins of a number of mammalian species have been cloned and the tertiary structure of two of them was determined [4–6]. The identity level within mammalian prion protein sequences is very high (around 90%) [7]. Recently, a gene coding for a mammalian PrP-like protein called doppel (Dpl) has been discovered [8].

The possible presence of PrP in all organisms with a nervous system, including invertebrates, was suggested more than ten years ago [9,10], but no protein with structural features corresponding to the mammalian PrP has been thus far reported, except for avian PrPs [7,11,12].

The first non-mammalian PrP was extracted and its cDNA cloned from chicken brain. Although the deduced primary structure showed less than 35% identity to the mouse protein, all of the mammalian structural domains were conserved [11,12]. Recently, several other avian PrP cDNAs have been isolated, showing a very high degree of amino acid sequence identity to the chicken protein (about 90%) [6]. Moreover, a preliminary structural analysis, carried out on the recombinant chicken PrP, suggested that its N-terminus adopts a different structure than does the mammalian corresponding region [13]. Besides its importance from a structural point of view, this result may be of interest for functional studies. Actually, in spite of many relevant research efforts, the physiological role of PrP remains unknown. The availability of PrP sequences from different animal species and, especially, from lower vertebrate classes, might help to identify the most conserved structural motifs and provide further insight into functional aspects.

The identification and characterization of the sequence coding for the prion protein of a reptile is reported here for the first time. The deduced amino acid sequence allowed confirmation of the presence of the structural features conserved between mammals and birds, but also allowed a unique EF-hand Ca^{2+} binding motif to be found in its C-terminal region.

2. Materials and methods

2.1. RT-PCR assays

Total RNA was extracted from the liver of the red-eared slider turtle *Trachemys scripta* using the RNAwiz kit (Ambion, USA). Five micrograms of total RNA were reverse transcribed using the enhanced avian RT-PCR kit (Sigma, USA) and different primers (Table 1), depending on the purpose of the experiment. PCRs were carried out in a total volume of 50 μl containing 1×PCR buffer (10 mM Tris–HCl, pH 8.3; 50 mM KCl; 1.1 mM $MgCl_2$; 0.01% gelatin), 200 μM dNTPs, 0.4 μM of each primer (4 μM for degenerate primers) and 1.5 U of RED *Taq* DNA polymerase (Sigma, USA). PCRs with degenerate primers were performed using touch-down protocols with an annealing temperature ranging from 5°C above to 5°C below the minimum melting temperature. All other PCRs were performed following standard protocols. Both the 5′ and 3′ ends of cDNA were isolated by RACE as described previously [14].

PCR products were cloned into *Sma*I linearized pUC9, modified as a T-vector [15].

2.2. PCR on genomic DNA preparations

Turtle genomic DNA was extracted from liver tissue using Nucleon BACCI kit (Amersham Pharmacia Biotech, UK). Aliquots of genomic DNA were separately digested by different blunt-end cutting restriction enzymes and then ligated to adaptors, using the Universal Walker kit (Clontech, USA). These DNA preparations were the templates for subsequent PCR assays, performed as described above, on 200 ng of template. Long-distance PCRs were carried out using the Expand 20 kb plus PCR system (Roche Diagnostics, Italy).

2.3. DNA sequence analysis

Sequence analysis of the DNA fragments was performed on both strands, either on plasmids or directly on purified PCR products by the *Taq* dye-deoxy terminator method, using an automated 370A DNA sequencer (PE-Applied Biosystems, USA).

The cDNA sequence of turtle PrP has been deposited in the EMBL database under accession number AJ245488.

*Corresponding author. Fax: (39)-2-2666301.
E-mail: tatjana.simonic@unimi.it

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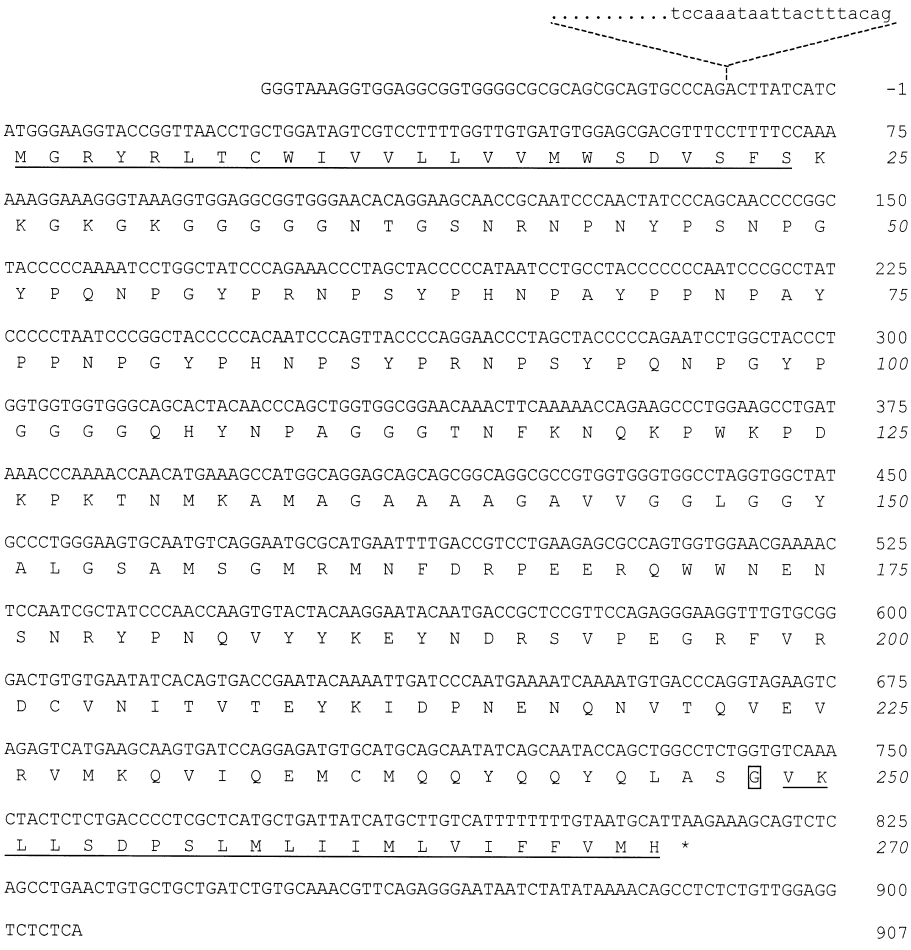

Fig. 1. Nucleotide and deduced amino acid sequences of the cDNA coding for turtle prion protein. The first base of the translation start codon is designated +1 and the stop codon is indicated by an asterisk. The sequence inserted within 5′ UTR and reported in lower-case letters corresponds to the last 25 nt of an intron. The underlined amino acid sequences correspond to the putative N-terminal signal peptide and to the C-terminal peptide, which is cleaved upon addition of a GPI anchor to the preceding Gly^{248} (boxed).

2.4. Northern blot analysis

A Northern blot of total RNA (15 μg) was prepared following standard procedures [16] and hybridized to a 846-bp DNA probe labelled with [α-^{32}P] dCTP, using the Rediprime II labelling kit (Amersham Pharmacia Biotech, UK). The hybridization was performed at 42°C in ULTRAhyb (Ambion, USA). The RNA size marker (RNA millennium) was from Ambion.

Table 1
Oligonucleotides used as primers in amplification assays

Primer	Sequence
F1	^{538}CCIAAYMRIGTNTAYTA^{554}
TF1	^{555}CAAGGAATACAATGACCGCT^{574}
TF2	^{580}CCAGAGGGAAGGTTTGTGC^{598}
TF3	$^{-10}$GACTTATCATCATGGGAAGG^{9}
R1	^{620}AYIGTIATRTTIANRCARTC^{601}
TR1	^{682}TGACTCTGACTTCTACCTGG^{663}
TR2	^{606}ACAGTCCCGCACAAACCTT^{588}
TR3	^{130}TGGGATTGCGGTTGCTTC^{113}
TR4	^{105}CCCACCGCCTCCACCTTTACCC^{84}
TR5	^{65}GAAACGTCGCTCCACATCACAACC^{42}
TR6	^{836}CAGTTCAGGCTGAGACTGC^{818}
5A	GAATTCGTCGACAAGCTTGTCA
FR	TGACTAAGCTTGTCGACG

Numbers preceding and following the sequences correspond to the turtle cDNA reported in Fig. 1.
T, turtle; F, forward; R, reverse; A, anchor.

2.5. Computer-assisted analyses

Amino acid sequence alignments were performed using the PALIGN software (PC-GENE, release 15.0) with an open gap cost of ten and a unit gap cost of five.

Secondary and tertiary structure predictions were performed using the program ProModII at the SWISS-MODEL Automated Protein Modelling Server (http://www.expasy.ch/swissmod/SWISS-MODEL.html).

3. Results

3.1. Isolation and characterization of the sequence coding for turtle PrP

A set of completely degenerated PCR primers was designed on the basis of the most conserved amino acid stretches of known PrPs. A number of products were amplified by RT-PCR with different primer couples. Those showing the expected size were cloned into pUC9 and sequenced. Only with the primer couple F1 and R1 (Table 1) was a product, maintaining the correct translation frame between the two primers, obtained. Although the deduced amino acid sequence was rather different from the known PrPs, due to the pattern of variation/conservation of the residues, it was supposed to encode the reptile version of the same protein. This fragment spanned 83 bp and corresponds to positions 538–620 of the

nucleotide sequence reported in Fig. 1. Starting from this short cDNA region, the coding sequence of turtle PrP was completed using different strategies, as described below.

The 3′ end of the cDNA was isolated by 3′ RACE [14]. Two turtle-specific oligonucleotides (TF1 and TF2; Table 1) were coupled in turn with the same 3′ primer (FR) annealing to the anchor sequence. As demonstrated by sequence analysis, the PCR product comprised the 3′ end of the coding region and 94 bp of 3′ UTR. This fragment spanned 327 bp and corresponds to positions 580–907 (Fig. 1). Further assays by the same procedure, using forward primers located near the 3′ end of the isolated fragment, did not extend to the 3′ UTR.

Several attempts were carried out to amplify the 5′ region of the turtle sequence from differently primed (oligo-dT, random primers, turtle-specific primers) reverse transcripts and using normal or thermostable reverse transcriptases. No results were obtained, neither by 5′-RACE nor by the use of degenerate 5′ primers, located near the translation start codon, coupled with turtle-specific downstream primers. For this reason adaptor-ligated genomic DNA fragments (see Section 2) were used. These 'libraries' were submitted to PCR amplification with a 5′ primer annealing to the anchor sequence and TR1 turtle-specific 3′ primer. A second nested PCR

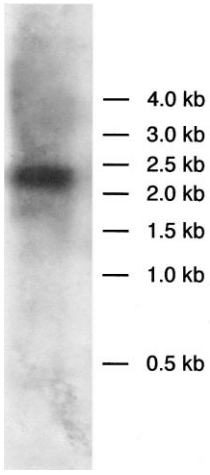

Fig. 2. Northern blot analysis of turtle PrP mRNA. Total RNA (15 μg) from turtle liver was hybridized to a 846-bp homologous cDNA fragment. Positions of RNA size marker bands are indicated on the right side.

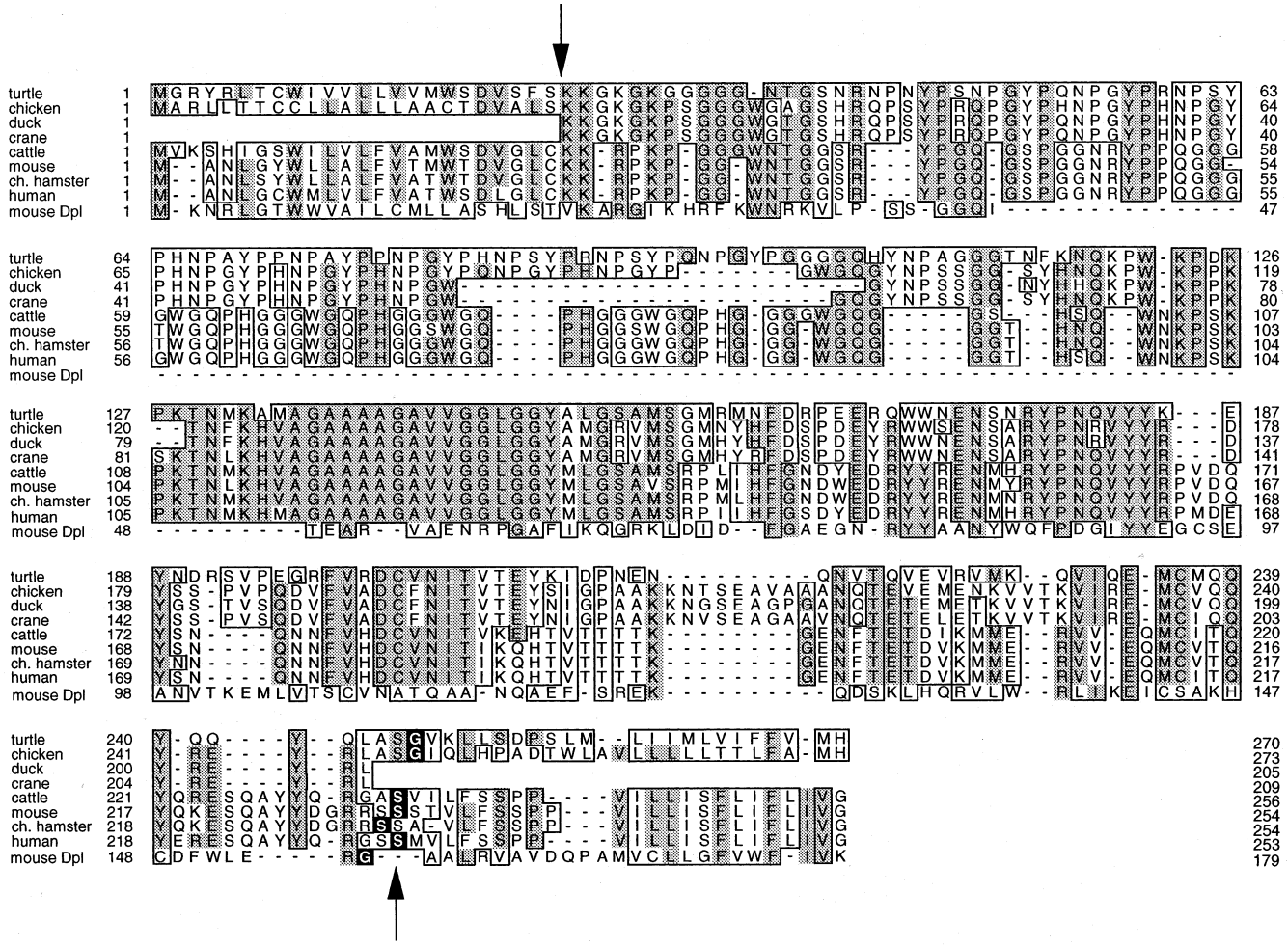

Fig. 3. Comparison of the turtle prion amino acid sequence with selected avian and mammalian prion proteins as well as mouse doppel protein. Conserved amino acids are boxed; grey denotes identical residues. The top arrow indicates cleavage sites of the N-terminal signal peptides; the bottom arrow points to black boxed residues, representing the targets of GPI addition.

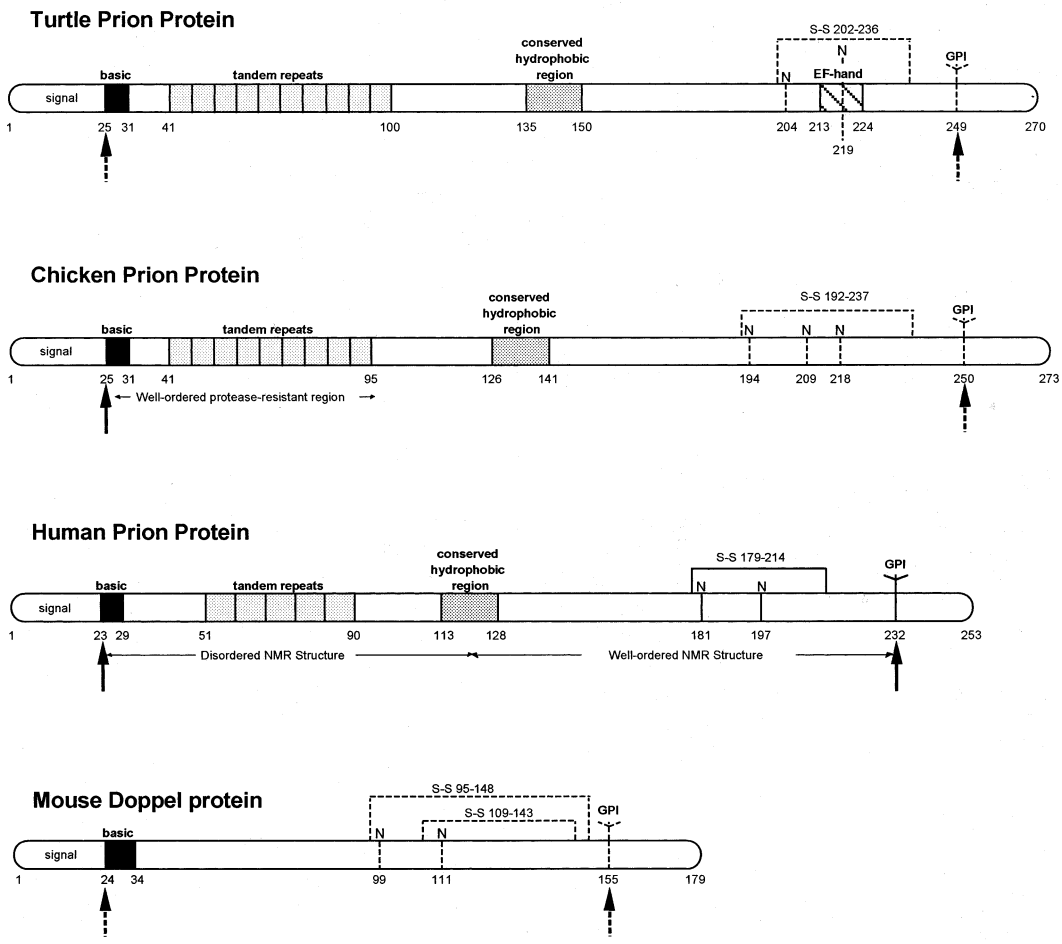

Fig. 4. Diagram showing relevant prion and doppel protein features. Numbers refer to positions in the amino acid sequences. Boldface arrows indicate sites of proteolytic cleavage; disulfide bridges are marked by a line connecting the two involved Cys. N indicates Asn residues which represent glycosylation sites; GPI indicates glycosyl phosphatidylinositol anchor. Dotted lines indicate putative sites.

step with an inner anchor 5′ primer and TR2 led to a product only from *Dra*I-cut DNA preparation. Sequence analysis demonstrated that this DNA fragment (641 bp) covered the whole 5′ coding sequence preceded by 35 bp of UTR.

To verify if this last sequence belonged to a transcribed region, RT-PCRs using different turtle-specific upstream primers and the above TR1 and TR2 were carried out. No product was obtained, likely due to the presence of an intervening GC-rich region and/or to the absence of this sequence in the mature mRNA. The 5′ end of this cDNA was finally isolated by 5′-RACE using the single strand ligation to ssDNA (SLIC) method [17]. Reverse transcription primed by TR3, located upstream of the GC-rich region, was performed and, after single-strand ligation to the oligonucleotide 5A, a double amplification (the second semi-nested) of the anchored material was performed using the anchor 5′ primer (5A) in duplicate with TR4 and then TR5. The longest (117 bp) of the resulting DNA fragments contained 65 bp of the coding sequence as well as 52 bp of the 5′ UTR (positions −52 to +65; Fig. 1).

The 5′ end of the 641-bp DNA fragment, obtained from genomic DNA, resulted as being different from the cDNA sequence in its first 25 residues, actually belonging to the 3′ end of an intron and carrying the canonical consensus of 3′ splicing sites. The 641-bp genomic fragment begins with 25 bp of an intron, followed by 10 bp of the 5′ UTR and the coding region, covering positions −10 to +606 of the cDNA (Fig. 1).

The whole cDNA sequence was obtained from composition of the previously described overlapping DNA fragments. This 959-nt sequence, reported in Fig. 1, includes a 5′ UTR of 52 nt, a coding region with a single open reading frame of 813 nt and a 94-nt 3′ UTR. The encoded protein comprises 270 amino acids, corresponding to a calculated molecular mass of 30134 Da.

3.2. PrP gene structure and expression

The above described 641-bp genomic fragment demonstrated that an intron is present 10 bp before the translation start codon (Fig. 1). This intron would be large, as we were not able to amplify it even using long-range PCR protocols. A further PCR assay carried out on genomic DNA with primer couple TF3/TR6 (Table 1) confirmed that the entire coding region is contained within the same exon.

Expression of turtle PrP mRNA was verified by Northern blot analysis of total RNA from liver tissue, using the whole coding sequence as a hybridization probe. A main band of approximately 2.4 kb was detected, as shown in Fig. 2.

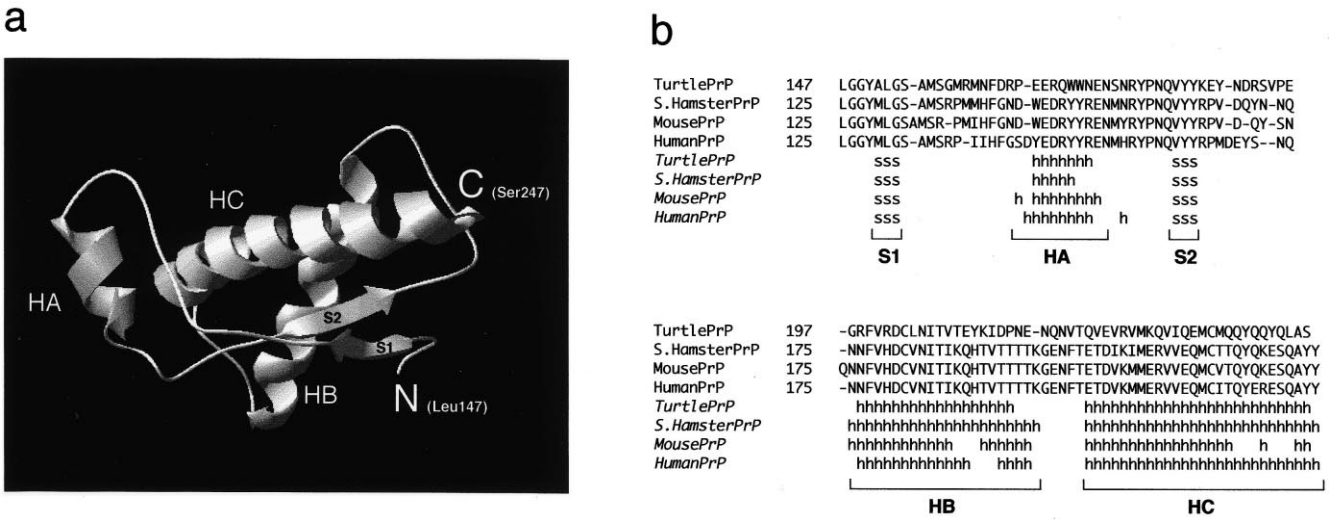

Fig. 5. Predicted turtle PrP secondary and tertiary structures. a: Ribbon diagram of the predicted C-terminal region (residues 147–247) of turtle PrP, indicating the positions of the three α-helices (HA, HB and HC) and the antiparallel two-stranded β-sheet (S1 and S2). This structure was produced using the SWISS-MODEL Automated Protein Modelling Server and is based upon the coordinates of syrian hamster (1B10.pdb), mouse (1AG2.pdb) and human (1QLX.pdb) PrPs. The ribbon diagram was produced using the SwissPDB 3.5 software. b: Alignment of the sequences used to generate turtle PrP 3D structure by comparative modelling. For each amino acid sequence the regions of secondary structure are indicated. h: α-helix; s: β-strand.

4. Discussion

The identification and sequencing of the cDNA coding for a reptile PrP is here reported for the first time. It represents the most remote PrP example thus far known. The major band of 2.4 kb, detected by Northern blot analysis, is in good agreement with the reported sizes for some mammalian (2.0–2.4 kb) [18–20] as well as chicken (2.9 kb) [11] PrP mRNAs. This suggests that UTR(s) are not complete in the turtle cDNA sequence reported here. The presence of a large intron interrupting the 5′ UTR and the lack of intervening sequences in the coding region confirm that PrP gene organization is conserved [12,21].

When compared to mammalian PrPs, turtle PrP amino acid sequence shows an identity level ranging from 38 to 42%. Similar values were obtained aligning the mature proteins. These identity levels are higher than that reported for avian to mammalian PrPs (about 30%) [7], however, the observed difference is likely due to the use of different alignment parameters. In fact, allowing more gaps, the identity levels of avian PrPs to mammalian PrPs increase to 36–38%. Since avian PrP sequences are incomplete, the identity values to the turtle protein were calculated only for mature proteins (55–62%) [7,11,12].

All the PrP structural features present in mammals and birds [7,12] are conserved in turtle (Figs. 3 and 4). The deduced N-terminal sequence of turtle PrP starts with 24 residues, consistent with an endoplasmic reticulum targeting signal peptide. The most peculiar feature of the N-terminal region of the mature protein is the presence of 10 tandem hexarepeats, which correspond to the 6.5–9 hexarepeats of avian [7,12] and to the 5 octarepeats of mammalian PrPs. Turtle and avian hexapeptide repeats share the consensus sequence XNPXYP, except for two Gln for Asn substitutions in birds. Only mammalian PrP octarepeats, which are part of an unstructured, highly flexible N-terminus [4–6], are able to bind Cu^{2+} in vivo. Cu^{2+} binding suggests a possible physiological role of PrP in copper metabolism [22,23]. This should not be the case for avian PrP, since chicken mature protein, which exhibits an N-terminal region structured as a stable, protease-resistant domain, does not bind Cu^{2+} [13]. Due to the remarkable similarity between turtle and avian hexarepeats, the presence of a structured domain and the inability in copper binding should be expected also for turtle PrP, thus supporting the hypothesis that copper binding could not be the primary function of the prion protein [13].

The hydrophobic stretch at positions 135–150 of the turtle protein is perfectly conserved among the three vertebrate classes and is considered a PrP signature (Figs. 3 and 4). This sequence and the seven preceding residues (positions 106–128 of human PrP) is considered a possible site for conformational transitions. In fact N-terminal deletion is able to prevent conversion to PrP^{Sc} only if it includes this hydrophobic region [24]. Interestingly, turtle PrP contains the non-conservative substitution of Ala^{133} for His^{111} of human protein, reported to be crucial to the conformational changes [25]. It has been shown that a peptide carrying such a substitution loses the tendency to adopt different secondary structures in different environments [25]. These findings raise the question if non-mammalian PrPs can undergo conformational transitions.

A prion-like protein (Dpl) has been recently reported in mice as well as in rats and humans [8]. Its identity level to turtle PrP (20%) is similar to that reported for all previously known PrPs (about 25%). The main difference from PrP is the smaller size (179 amino acids in mice), due to the lack of both the repeats and the conserved hydrophobic stretch, while all the other PrP features are conserved (Figs. 3 and 4). When Dpl is upregulated in the central nervous system, it is able to cause ataxia in PrP-deficient mice [8]. It is interesting to note that the expression of a homologous N-terminally truncated PrP, lacking the hydrophobic region, also causes ataxia in PrP

knockout mice. An intriguing interpretation of these data is that the N-terminally truncated PrP could assume a doppel-like conformation, which is neurotoxic [26].

In the C-terminal region two Cys residues, which in mammals form an intramolecular disulfide bond [27], are conserved also in birds and turtle (positions 202 and 236; Fig. 4) and are surrounded by very similar contexts. They encompass a region exhibiting putative Asn-linked glycosylation sites, found at positions 204 and 219 in turtle and conserved in mammals and birds, where an extra site (Asn^{209} in chicken) is present. The last common feature regards the C-terminal sequence of 23–25 amino acids, which is usually cleaved upon addition of a glycosyl phosphatidylinositol (GPI) anchor [11,28]. The turtle and chicken sequences exhibit the same dipeptide Ser-Gly at positions 247–248 and 248–249, respectively; chicken Gly^{249} was supposed to be the target for the anchor molecule [12] (Fig. 3).

The predicted three-dimensional structure of the turtle PrP C-terminal region (Fig. 5) was computed by a comparative protein modelling software [29]. The secondary structure motifs (three α-helices and two antiparallel β-strands), resolved by NMR in some mammalian species [4–6], appear to be well conserved in turtle PrP, suggesting that this structured region should generate the same fold as in mammals.

The most peculiar feature of turtle PrP is the presence of an EF-hand Ca^{2+} binding motif in the C-terminal half of the protein. A canonical 12-residues segment containing the Ca^{2+} binding ligands [30] was found at positions 213 to 224. The flanking sequences correspond to the second and third α-helical tracts present in mammalian PrPs [4–6] and may be consistent with such secondary structure, the only difference concerning an early break of the first helix (the putative E-helix) by the Pro^{214}. This perfectly agrees with the canonical start of the inter-helical loop of an EF-hand motif. This same putative loop comprises Asn^{219}, the second potential glycosylation site and the α-helical flanking regions might be stabilized by a disulphide bond between Cys^{202} and Cys^{236}. The presence of a similar disulphide bridge has been reported in the first EF-hand motif found in an extracellular protein, the matrix protein BM40 [31–32], and this is of particular interest as PrPs are extracellularly bound proteins. Usually at least two Ca^{2+} binding sites are present in a same polypeptide chain, however a single and functional site has been recently reported for *Escherichia coli* lytic transglycosylase Slt35 [33].

It would be interesting to verify if turtle PrP is able to fold into an EF-hand-like structure, to bind Ca^{2+} and if this binding plays some functional role. The results reported here support the presence of PrPs in lower vertebrates and suggest that their primary structures could contain unexpected features.

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