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Short sequence-paper

Cloning, structural organization and tissue-specific expression of the rabbit transferrin gene

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Abstract

We cloned the rabbit transferrin (*rTf*) cDNA and gene, and quantified the expression of the *rTf* gene at the RNA level in various organs. The tissue-specific pattern of expression of *rTf* gene is different to those in other species, with a high expression in mammary gland and kidney. The exon/intron structure of the *rTf* gene (17 exons/16 introns) is similar to those of transferrins from other species. The sequence of the *rTf* cDNA already published is corrected and lengthened in the 5′ region, and a likely polymorphism is documented.

Keywords: Mammary gland; Kidney; Liver; Iron responsive element; Polymorphism

Transferrin (Tf) is the major iron-binding glycoprotein found in the serum of all vertebrates, and is mainly secreted by the liver. In mammals, another iron-binding glycoprotein related to Tf lactoferrin (Lf) is also produced. The exon/intron structure of both genes is the same (17 exons and 16 introns) [1–6]. Consequently, the *Lf* gene is thought to have arisen from a *Tf* gene duplication during evolution [7]. Despite its name, Lf is not only found in milk but also in a variety of other exocrine secretions [8–10] and in granules of neutrophils [11]. Moreover, organs expressing the *Lf* gene are not the same in all species, and in some like rabbit, rat and dog the milk is devoid of Lf [8]. In the same manner, tissue-specific transferrin expression differs between species, and only mice, rats and rabbits have been shown to express the *Tf* gene in their mammary glands. This mammary-specific *Tf* expression is at least as high as the liver-specific expression in the mouse [12], twofold higher in the rat [13] and threefold higher in the rabbit [14]. The rabbit thus appears to display some unique features in the tissue specificity of the *Tf* gene expression. In order to define the precise pattern of the rabbit *Tf* gene expression, we first cloned the *rTf* cDNA and quantified the expression of the *rTf* gene at the RNA level in various organs. We then cloned the entire *rTf* gene, determined its structure and compared it with those of other *Tf* and *Lf* genes.

Rabbit *Tf* cDNA was cloned by RTPCR following standard procedures and using two oligonucleotides Tf1.1 (5′-CCACCACCCCTGAGCTACAA-3′) and

Abbreviations: bp, base pair(s); cDNA, DNA complementary to RNA; hTf, human Tf; IRE, iron response element; IRP, iron responsive protein; kb, kilobase(s); Lf, lactoferrin; mTf, mouse Tf; PCR, polymerase chain reaction; rTf, rabbit Tf, ; RT, reverse transcriptase; Tf, transferrin; tsp, transcription start point; UTR, untranslated region

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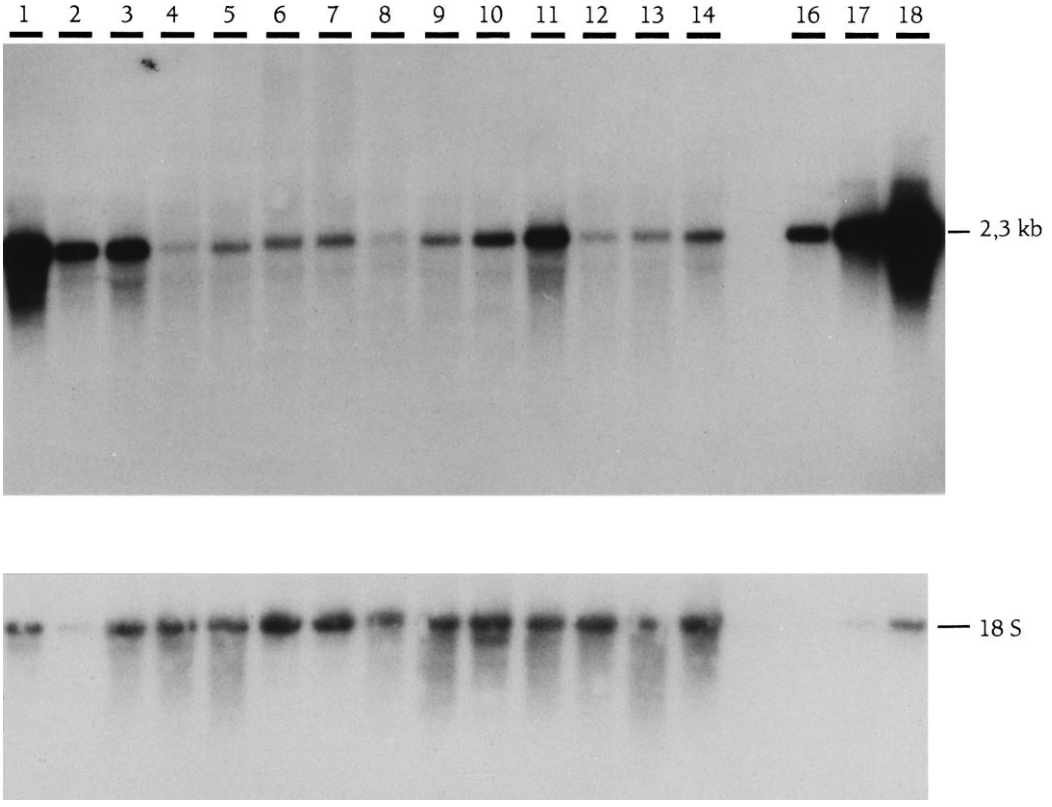

Fig. 1. Northern blot analysis of rabbit transferrin. Except where otherwise stated, 20 μg of total RNA were loaded in each lane. RNA were from liver (lanes 1 and 2 (1 μg)), brain (lane 3), heart (lane 4), intestine (lane 5), muscle (lane 6), ovary (lane 7), pancreas (lane 8), lung (lane 9), spleen (lane 10), kidney (lane 11), adrenal gland (lane 12), testis (lanes 13 and 14) and mammary gland (lanes 16 (200 ng), 17 (1 μg) and 18). The control hybridization with an 18S RNA probe is presented below. Total RNA were isolated using standard methods [28] and separated to 1.5% agarose gels containing formaldehyde, transferred to Biohylon-Z+ membranes (Bioprobe) by the capillary method, and immobilized by UV irradiation. Membranes were hybridized using *rTf* cDNA as a probe following standard procedures.

Tf17.2 (5′-CTGCATTCTACACACTTGGC-3′) deduced from the published *rTf* cDNA sequence [15]. To examine the pattern of *rTf* expression, we performed Northern blot analysis on total RNA isolated from various organs. As shown in Fig. 1, the *rTf* cDNA probe revealed a single transcript of 2.3 kb in all the organs tested. Hybridization signals fixed on the membranes were quantified using an ImageQuant PhosphoImager (Molecular Dynamics). If by convention the value 100 is assigned to the *rTf* expression in the liver, the *rTf* expression was 344 in mammary gland, 22 in brain, 19.7 in kidney, 10 in spleen, 7 in testis and less in other organs. If this pattern is compared to those known in other species, the following points are to be noted. First, in the rabbit the mammary-specific *Tf* gene expression is highest, and exceeds the maximum levels of *Tf* gene expression in any organ in all other species examined. Second, the significant levels of *Tf* RNA found in brain are consistent with the data obtained in rats [16,17], mice [18] and baboons [19]. Third, the relatively high expression in kidney was unexpected as *Tf* gene expression in kidney has been recorded as very low in rats [16,17], mice [18] and baboons [19]. Fourth, rabbit appears to have a lower testicular/liver *Tf* expression ratio (7%) than the baboon [19], but higher than the rat (1.8%) [16]. The high levels of *Tf* expression in mammary gland and in kidney were thus very striking features of the *rTf* gene pattern of expression. Whilst it is easy to propose that Tf might function as an iron transporter in the milk, high expression of the *Tf* gene in rabbit kidney is less easy to explain.

Using an entire *rTf* cDNA probe [15], 40 genomic λ clones were first isolated. Since the exon/intron structure of the *Tf* gene is conserved between human

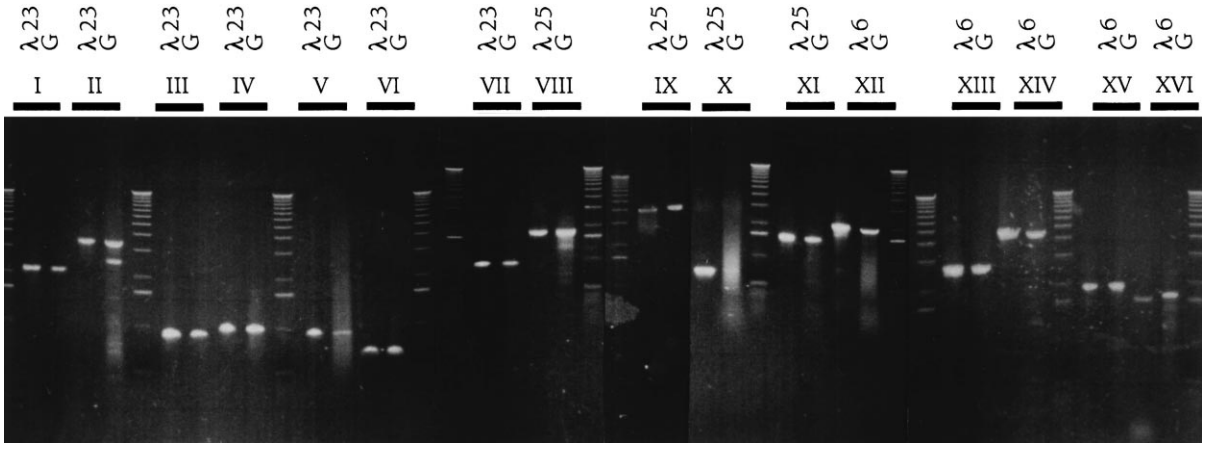

Fig. 2. Agarose gel electrophoresis of PCR products for introns 1–16. Roman numerals correspond to the intron number. The amplification of each intron and its two flanking exons was performed with genomic DNA (G) and with λ DNA. Due to the large size of some introns of the *rTf* gene, the Expand Long Template PCR System (Boehringer-Mannheim) was used following the manufacturer's specifications. All elongations were performed at 68°C. For introns 1, 2, 9, 13 and 14, the elongation time was increased from 1 to 3 min. The molecular weight marker used is the 1 kb ladder from Life Technologies.

[2], chicken [1] and medaka [3], the putative exon boundaries in the *rTf* cDNA were hence deduced by comparison with the human cDNA and gene. Seventeen pairs of 18-mer oligonucleotide PCR primers were designed to amplify each exon. These oligonucleotides were named TfN.1 and TfN.2 when they amplified exon N, and their sequences are available and will be sent on request. Each exon was either amplified from genomic DNA and used as a probe with which to map the λ clones, or amplified from λDNA to check the presence of the exon in each λ clone. Three clones, λ23, λ25 and λ6, were selected, characterized and mapped with these exon probes. The entire *rTf* gene was isolated from these three λ clones. The *rTf* gene covers 27.3 kb and is thus smaller than the human gene (33.5 kb) [2], but larger than the murine gene (23 kb) [20], the chicken gene (10.5 kb) [1], and the medaka gene (7.0 kb) [3].

Exon Number	Exon Size						Intron Number	Intron Size					
	Rabbit Tf	Human Tf	Murine Lf	Bovine Lf	Chicken Tf	Medaka Tf		Rabbit Tf	Human Tf	Murine Lf	Bovine Lf	Chicken Tf	Medaka Tf
I	101	93	82	82	119	75	**1**	≈1900	≈2100	1600	≈3100	1313	66
II	173	173	161	164	164	149	**2**	≈2900	≈5000	1000	≈3700	317	364
III	109	106	109	109	109	103	**3**	≈500	≈750	200	293	986	85
IV	177	177	183	183	192	192	**4**	≈600	≈685	370	478	190	287
V	133	133	148	148	136	130	**5**	≈600	≈810	700	942	396	103
VI	56	56	56	56	56	56	**6**	≈400	≈675	800	≈5400	489	693
VII	179	179	179	179	170	164	**7**	≈550	≈765	550	448	124	150
VIII	178	178	175	175	187	154	**8**	≈1350	≈1070	800	527	573	86
IX	155	155	155	155	155	164	**9**	≈3650	≈4900	1120	1967	757	84
X	91	94	91	91	94	97	**10**	≈600	≈900	922	2232	215	97
XI	33	33	48	48	36	21	**11**	≈1200	≈1300	1120	631	633	549
XII	156	156	156	156	156	170	**12**	≈1700	≈1400	1320	1911	749	403
XIII	130	136	142	142	145	146	**13**	≈2300	≈2400	4300	4774	269	638
XIV	65	65	68	69	65	68	**14**	≈4000	≈5300	3000	1936	448	102
XV	185	185	185	184	185	181	**15**	≈1500	≈1600	1360	1145	323	188
XVI	190	190	190	190	187	194	**16**	≈1200	≈2600	1700	1548	418	658
XVII	205	206	132	225	221	318							

Fig. 3. Comparison of the size of exons and introns in transferrin genes from medaka [3], chicken [1], human [2] and rabbit (this study), and in murine [4,5] and bovine [6] lactoferrin genes.

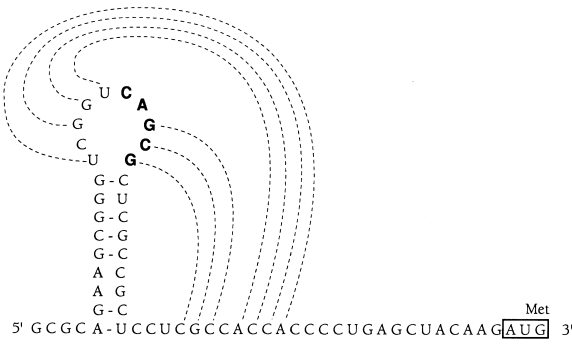

Fig. 4. Possible secondary structure of the 5′-UTR of rTf mRNA following the model of Cox [21]. Dashed lines connecting the loop bases with the 3′ flanking region bases represent Watson-Crick complementarity.

Of note, the *Lf* gene also shows a species-specific size variation: the mouse *Lf* gene covers 23 kb [5], the human *Lf* gene nearly 35 kb [21] and the bovine *Lf* gene 34.5 kb [6].

In order to determine the size of the introns, the same oligonucleotides used to amplify the exons were used to amplify each intron and its two flanking exons. Fig. 2 shows agarose gel electrophoresis of PCR products for introns 1–16 with DNA from the λ clones and with genomic DNA. The length of the amplification products was the same with genomic and λ DNA. Fig. 3 summarizes and compares the sizes of exons and introns of the four *Tf* genes and the two *Lf* genes studied so far. These data demonstrate clearly that the sizes of exons are conserved especially in *Tf* genes of both mammalian species, whereas the sizes of introns vary. In both mammalian *Tf* genes, the relative size of the introns is conserved with the exception of the last one.

The 17 exons and part of their flanking introns were sequenced using an Abi Prism Model 377 automated DNA sequencer (Perkin Elmer) and a Perkin Elmer Terminator kit. The two strands were sequenced using first the 17 couples of oligonucleotides already described, and then newly synthesized oligonucleotides. The sequences of all the exons and of introns 1 and 6 can be found in GenBank under accession Nos. AF031611–AF031625. The 16 introns begin with GT and finish with CAG, following the general rule. In order to determine the transcription start point (tsp), a primer extension experiment was performed. The longer band occurred 58 nucleotides upstream from the ATG codon, and the size of the first exon is thus 101 nucleotides (data not shown). This tsp is entirely homologous to that found for the human gene [2]. It is interesting to note that another strong band was present, suggesting the possible existence of a second initiation site of transcription two nucleotides downstream, a position homologous to the tsp found in the murine gene using a different method of analysis [20].

The 5′-UTR region of the *rTf* gene shows the same interesting feature as the human gene. The 5′-UTR region of the *hTf* gene has been found to bind liver cytoplasmic protein, and this binding was readily inhibited competitively by ferritin IRE RNA [22]. Moreover, purified bovine IRP binds to *hTf* RNA sequences, and mutations therein similarly affect binding by IRP and liver cytoplasmic extract [23]. A model comprising a stem-loop structure and its 3′ flanking region with complementarity to the loop was proposed, and the importance of these two components was demonstrated [23]. Indeed, the possibility to construct such a stem loop (9 bp for the stem, and 10 nucleotides for the loop) with complementarity to the 3′ flanking region is also observed in the rTf sequence (Fig. 4) supporting the proposed model. However, the pentanucleotide sequence CUGUG thought to be an IRE in the *hTf* gene was not found in the rabbit loop. Rather, the pentanucleotide CAGCG was present at this site instead. Moreover, whilst neither the loop sequence nor the 3′ flanking sequence is entirely conserved between rabbit and human genes, the complementarity between these two regions is conserved, allowing the structure to form. It is of note that the complementarity between the loop and the 3′ flanking region does not imply the presence of the same nucleotides in the two species.

The cDNA sequence deduced from the *rTf* gene showed two differences with the published cDNA sequence [15]. The first occurs in the first exon and concerns a GCC trinucleotide encoding the seventh amino acid of the signal peptide which was lacking in the previously published cDNA sequence. In order to verify this point, we sequenced two of our cDNA clones, and confirmed that both harboured this supplementary codon. As this amino acid exists in all other mammalian *Tf* sequences studied so far [24], the most likely explanation is an error during entry of the cDNA sequence in database. The second dif-

ference is a single nucleotide in exon 13 changing amino acid 517 from isoleucine (ATC) to valine (GTC). The two λ clones, λ25 and λ6, showed the same sequence. This GTC codon was also found in our two cDNA sequences. The most likely explanation for this difference is polymorphism. Tf polymorphism at the protein level has been reported in wild rabbits [25,26], but not in New Zealand rabbits where only allele A was found [27]. It is clear that the isoleucine/valine polymorphism could not be detected at the protein level by the methods used (agarose or starch gels). Another possibility which cannot be ruled out, is an error in the previously published sequence, because the 3′ part of the cDNA comprising this nucleotide was amplified by PCR with the accompanying risks of polymerase errors. Moreover, the authors did not mention whether they sequenced the amplification product directly, or whether they cloned it before sequencing [15]. Whilst the cause of this difference remains unknown, we proposed to name our sequence with a valine allele A1, and the published sequence with an isoleucine allele A2. A search for allele A2 is currently in progress in our laboratory. It also would be of great interest to establish the nucleotide sequences of the other known alleles (B, C and D) of the rabbit transferrin, and to look for such a variation in amino acid 517.

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