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A CONSERVED 8 BP MOTIF (GCYATCAY) IN THE 3'UTR OF TRANSITION PROTEIN 2 AS A PUTATIVE TARGET FOR A TRANSCRIPT STABILIZING PROTEIN FACTOR

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Summary: The 3'UTR of the transition protein 2 gene of several mammalian species was sequenced and the transcript level of this gene was determined by Northern blots. In all species tested so far, a Northern blot detectable transcript level was associated with the presence of a conserved 8 bp motif (GCYATCAY) 50 bp downstream of the stop codon. RNA-bandshift experiments indicate that this region is capable of binding a cytosolic protein factor from rat testis. These findings support our hypothesis that the low transcript level of the transition protein 2 gene in human is due to insufficient storage of the mRNA as ribonucleic/protein particle.

Transition proteins (TNPs), like protamines are small,extremely basic proteins which are involved in the reconstitution of the chromatin structure during late spermatogenesis (1). These genes are exclusively expressed in round spermatids and mRNA is stored in a translationally repressed state until translation starts 4-6 days later (1,2). Several reports indicate that the 3'UTR (3' untranslated region) is involved in the translational control of protamine 1 and 2 mRNA in mouse testis (3,4). This regulation is assumed to be achieved by the formation of RNA/protein complexes. The human TNP2 gene, in contrast to its counterparts in other mammalian species, is expressed at a very low level and transcripts can only be detected by RT-PCR or by RNase protection assay (5). Since there is an intriguing deletion in the 3'UTR of the human gene, we reasoned that the deleted sequence might influence the TNP2 mRNA level. This sequence harbours a conserved 8bp motif GCCATCAC, present in mouse (6), rat (7), bull (8) and boar (9) which all express the TNP2 gene at a high level. We have now tested further species for consistency of this association. In all species tested so far, a Northern blot detectable expression was observed when the motif was present in the 3'UTR. Therefore, we reason that this motif might

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represent a binding site for a transcript stabilizing factor. Storage of TNP2 mRNA in human testis would then fail due to the absence of the appropriate protein binding site. This hypothesis is supported by the results of RNA-Bandshift experiments. Wildtype rat TNP2 3'UTR is able to bind a cytosolic protein factor from rat testis while a mutated 3'UTR, lacking the 8 bp motif, is not bound by the same protein extract.

MATERIALS AND METHODS

Polymerase chain reaction (PCR) and sequencing: Degenerative oligonucleotides ATNP2-1 (5'GTA CAA GCT TTA C/ATC/T C/AG/AT GTT GC 3') and ATNP2-2 (5' AAG AG/CG CA/GG/T AG/CG/C TCA GGT ACC 3') were synthzised on an ABI Oligonucleotide Syntheziser and allowed amplification of the TNP2 intron, exon 2 and 3'UTR of *Maccaca mulatta* (Rhesus monkey), *Callithrix jacchus* and *Tupaia belangeri* (Tree shrew). Genomic DNA was prepared from liver or testis following standard protocols (10). PCR-mix contained 50 pmoles of each primer ATNP2-1 and ATNP2-2, 0,2 mM dNTPs, 0.3-0.6 µg of genomic DNA, Taq-DNA-polymerase buffer and 2.5 U Taq-DNA-polymerase (Amersham). Cycle profiles were 1 min 93°C, 1 min 56°C and 1 min 72°C. TNP2 containing fragments were identified by hybridizing with the TNP2 cDNA of bull (8) and human (5), agarose gel purified and reamplified. All fragments were then sequenced directly as described earlier (5).

Northern blots: Isolation of RNA from testis and Northern Blots were performed according to standard methods (10). Blots with RNA from primates were hybridized with the human TNP2 cDNA (5), rodent RNA was hybridized with the rat TNP2 cDNA (7), and bull and boar RNA was hybridized with bull TNP2 cDNA (8). All blots were stripped and rehybridized with bull TNP1 cDNA (11) to demonstrate integrity of the RNA. Hybridization temperature for TNP2 probes was 60°C and final washes were carried out at 60°C in 1x SSC. TNP1 probes were hybridized at 65°C and washed at 65°C in 0.2x SSC.

Protein extraction: Testes of adult SIV 50 rats were prepared and immersed for 15 min at 4°C in PBS. The tunica was stripped off and tubuli were scissored thoroughly. Cytoplasmic proteins were prepared according to Dignam et al. (12). Usually we extracted 8 - 10 mg cytoplasmic protein out of 2.5 - 3.0 g testis material.

RNA-bandshifts: By PCR a rat TNP2 3'UTR fragment was generated which contained 9 nt of exon 2, the stop codon and 125 nt 3'flanking region (position 376 to 512 in (7)). Primer sequences were 5'CGGTCTAGATACAAGTGACACAC 3' (5'oligo) and 5'CCAGGGTACAAGCTTTAATCCATG 3' (3'oligo). The resulting fragment was cloned into pSPT19, employing the natural occuring HindIII site beneath the poly A signal and a XbaI site, introduced by the 5'oligo. The 8 bp motif GCCATCAC in this clone was deleted by in vitro-mutagenesis based on PCR. The mutated fragment was cloned into pSPT18 with XbaI/HindIII. Both constructs were verified by dideoxysequencing. For in vitro transcription templates were linearized by Hind III. SP6- or T7-RNA-polymerases were used to prepare sense transcripts of the wild type (wt) or the mutated (del) template, respectively, with limiting amounts of [α^{32}P] UTP. One nanogram transcript was heat denatured and after cooling was incubated with 80 µg cytoplasmic protein extracts of whole rat testis and up to 100 µg yeast tRNA as unspecific competitor. Binding reactions were allowed for 20 min at 30°C. Stringency of binding was further achieved by incubation with heparin (5 mg/ml) at 30°C for 10 min and reaction was stopped with 0.25 vol loading buffer (40 % sucrose, 0.01 % bromphenolblue, 10 mg/ml tRNA). Samples were immediatly loaded and electrophoresed on 9% polyacrylamide/TBE gels containing 0.6 % LMP agarose.

RESULTS AND DISCUSSION

In an earlier paper (5) we have shown that the human TNP2 gene is expressed at a much lower level than those of bull, boar, rat and mouse. Our working hypothesis to explain this

finding rested on the observation that the 3'UTR of the human gene differs markedly from that of others species. A deletion of 18 bp in the human gene includes the highly conserved motif GCCATCAC. If this motif acts as a regulatory sequence, one has to expect a similar association of transcript level and 3'UTR sequence in further species. We have now sequenced the TNP2 3'UTR of *Macacca mulatta, Callithrix jacchus* and *Tupaia belangeri* and aligned these sequences with the TNP2 3'UTR of mouse (6), rat (7), bull (8), boar (9) and human (5) (Fig. 1.) This alignment was produced by the DNA Star program MULALIGN and was reconstructed by hand. There is about an 18 bp deletion in the human 3'UTR when compared with the sequence of all other specis. This stretch harbours a conserved motif (GCYATCAY) in *Macacca mulatta, Callithrix jacchus, Bovis bovis, Sus sus, Mus musculus* and *Rattus norvegicus*. The position of this motif, about 50 bp downstream of the stop codon, is remarkably constant among these species. In the 3'UTR of *Tupaia belangeri* this motif is altered in positions 5 and 6 from TC to GG thus changing two pyrimidines into purines. We then tested the TNP2 transcript level of these species in Northern blots and found a strong TNP2 expression in all species except human and tupaia which both lack the motif in question (Fig.2). It seems that only those species which possess the conserved 8 bp motif (GCYATAY) in their 3'UTR show levels of TNP2 mRNA detectable by Northern blot analysis. Regardless of which kind of regulation applies here (formation of nucleic acid secondary structures or binding of protein factors), pyrimidine to purine transversions in two positions (as occured in *Tupaia*) would most likely impede this function while C/T transitions are tolerated in many cases (13,14).

Since the absolute amount of any mRNA is determined by the rate of synthesis ("source") and the rate of decay ("sink"), a positive regulatory sequence can act either as enhancer of transcription or as a mRNA stabilizing factor. The latter principle of regulation (mRNA binding by protein factors) is assumed to be important for the translational control of TNPs as well as protamines. mRNA is synthezised and detectable 3-4 days before they appear on polysomes (1,2). For this period of time, transcripts are thought to be stored (and protected

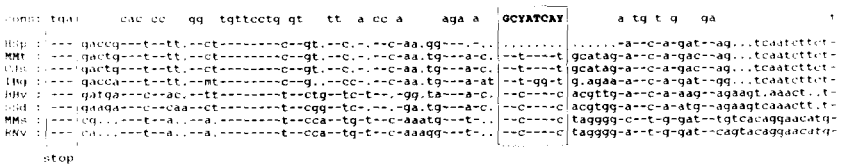

Fig 1.
Comparison of TNP2 3'UTR sequences from the stop codon (stop) to approximately 40 bp upstream of the polyadenylation signal. The comparison was produced by the DNA STAR program MULALIGN and was reconstructed by hand. Nucleotides matching the consensus sequence (cons) are given by dashes (-), gaps are marked by points (.). HSp is *Homo Sapiens*, MMt *Macacca Mulatta*, CJs *Callithrix Jacchus*, TBg *Tupaia Belangeri*, BBv *Bovis Bovis*, SSd *Sus Sus domesticus*, MMs *Mus Musculus*, RNv *Rattus Norvegicus*.

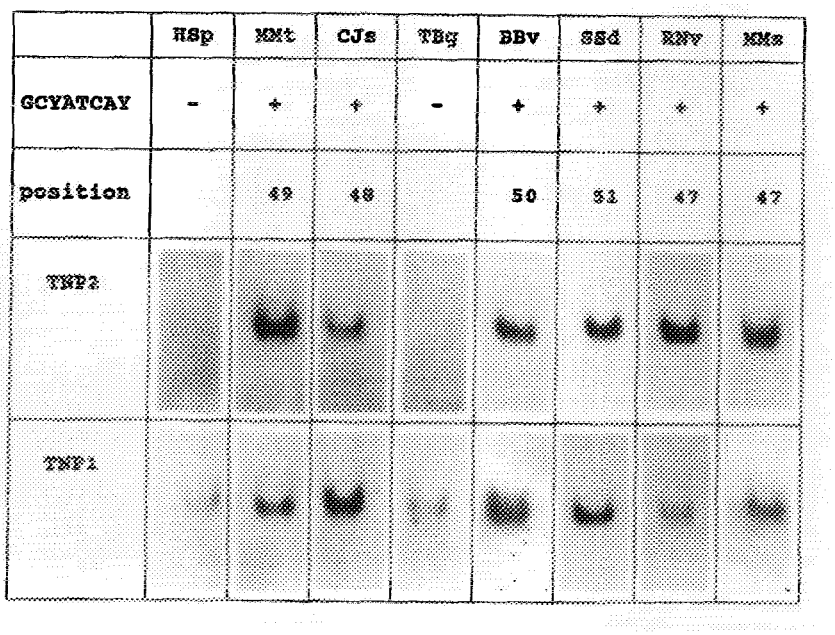

Fig. 2.
Expression of TNP2 and TNP1 in different mammalian species. Species designations as in fig. 1. TNP2: transition protein 2, TNP1: transition protein 1, position: distance from stop codon to the conserved motif GCYATCAY in bp.

from digestion) as RNA/protein-complex (15). In case of mouse protamine 1, Kwon and Hecht (3) identified protein factor(s) which bind specificly to two different motifs in the 3'UTR of this gene. It is now tempting to speculate that a similar mechanism is responsible for the storage of TNP2 transcripts with the motif GCYAUCAY acting as a binding site for the putative protein factor. Human TNP2 transcripts lacking the appropriate binding site, would become rapidly degraded. To test this hypothesis, we cloned the rat TNP2 3'UTR in a transcription vector and analyzed the in vitro synthezised RNA for its ability to bind cytosolic protein factors from rat testis. Incubation of transcripts from the wildtype rat TNP2 3'UTR with cytosolic proteins of rat testis resulted in a lowered electrophoretic mobility of these RNAs (see Fig. 3). Formed complexes were stable against a 100,000 fold excess of unspecific competitor. No complexes were observed with protein extracts of various other tissues (data not shown). If the 8 bp motif was removed by in vitro mutagenesis, specific complexes were not formed any longer (Fig. 3). Our data strongly support the view that the above described motif indeed can act as binding site for a transcript-complexing protein factor which is a major candidate for the translational regulator of the protamine/transition protein gene family. Since all protamine and transition protein genes are regulated in a similar manner, it is conceivable that a common protein binds to all the different transcripts. In this case one would expect a common RNA recognition sequence. Kwon and Hecht (3) described two possible binding motifs of which one shows some similarities to the 8 bp motif described here. If we allow C/T exchanges,

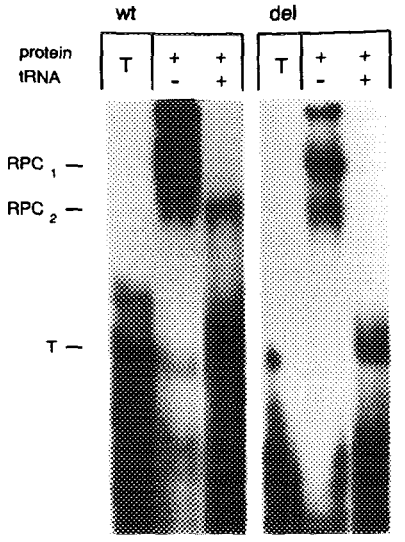

Fig. 3.
Comparison of the RNA/protein complexes (RPCs) formed by wild type (wt) and mutant (del) transcripts with cytoplasmic proteins of rat testis. T lanes show transcripts alone, the presence of protein and tRNA is indicated by (+) or (-). Unspecific complexes are suppressed by tRNA; a competitor resistant complex (RPC_2) is only found with wt trancripts.

the "Z" motif (AAAGCCACCTGCC) has 6 bases in common with GCCATCAC (underlined in both sequences). It remains to be determined whether both factors are identical or whether there is a group of several different factors, each responsible for an individual transcript.

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