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MOLECULAR MECHANISMS OF BIOLOGICAL PROCESSES

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Spliced *oct-1* mRNA Isoforms with Untranslated Exons and a Partly Deleted Region Coding for the POU-Specific Domain

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Abstract—Transcription factor Oct-1 is involved in expression regulation of housekeeping genes, in lymphocyte differentiation, and in the immune response. Tissue-specific *oct-1* mRNA isoforms are known to be expressed in lymphoid cells. Four new mouse isoforms were identified. Of these, two were tissue-specific (*oct-1R*α and *oct-1R*β) and contained exon 1L. The *oct-1R*α was shown to contain an additional fragment, which corresponds to an exon located in the 3'-region of mouse *otf-1*. No homolog was found in human *OTF-1*. The *oct-1R*β isoform proved to lack an exon coding for a fragment of the POU domain. This deletion results in a loss of the first helix of the domain, and the mutant protein is devoid of affinity for octamer ATGCAAAT. Two other mRNA isoforms, *oct-1d* and *oct-1e*, were shown to contain untranslated regions between exons 1U and 2. The regions correspond to exons 1i and 2i located between exons 1U and 1L in the 5'-region of the mouse *oct-1* gene. Human *OTF-1* was not found to contain exon 1i. On evidence of these and published data, it was assumed that a set of *oct-1* isoforms is present in the cell, reflecting the complexity of expression regulation of *oct-l* and the multiplicity of its functions.

Key words: *oct-l*, mRNA isoforms, alternative splicing, POU domain

INTRODUCTION

Oct proteins are classed with those possessing the POU domain, which has first been revealed in transcription factors Pit-1, Oct-1, and Unc-86 [1]. Oct-1 occurs in all types of proliferating eukaryotic cells and plays numerous roles. In particular, Oct-1 participates in regulating the expression of many genes, including various housekeeping genes such as those for snRNA and histone H2B [2, 3]. This implicates Oct-1 in cell proliferation and differentiation. Furthermore, Oct-1 contributes to the tissue-specific expression of various, including immunoglobulin, genes. The factor interacts with promoters and enhancers of the genes for immunoglobulin light and heavy chains; interleukins 2, 4, and 8; and the granulocyte-stimulating factor [4–6]. A knock-out in *oct-1* is lethal [3]. Induction of *oct-1* has been observed in cells exposed to DNA-damaging agents or electromagnetic radiation [7]. Oct-1 participates in regulating *GADD45*, which controls the cell response to DNA damage [8].

The role of Oct-1 in tissue-specific transcription may involve several mechanisms. Thus Oct-1 is capable of complexation with other nuclear proteins [9]. Its interaction with transcription factors and tissue-specific coregulators ensures fine regulation of gene expression. There is evidence that Oct-1 complexates with several transcription factors, including Sp1 [10], Ap-1 [6], and NF-1 [11]; steroid hormone receptor [12]; and homeoprotein Pbx [13]. In lymphocytes, Oct-1 interacts with tissue-specific coactivator OCA-B (OBF, Bob.1) [14]. The interaction is possibly due to the POU domain or other regions of Oct-1. In addition, several *oct-1* mRNA isoforms are expressed only in lymphoid tissue and may play an important role in gene expression in lymphocytes [15].

The multiplicity of Oct-1 functions and the fine regulation of responsive genes may be explained by interaction of Oct-1 with different DNA regions, such as canonical ATGCAAAT, noncanonical oct sites with single-base substitutions, or homeospecific sites. In all cases, Oct-1 affinity for a recognition site strongly depends on its context. On the other hand, the interaction with coregulators depends on the site to which Oct-1 is bound. Thus VP16 interacts only with Oct-1 bound to TAATGARAT (R is a purine) [16], whereas the binding with OCA-B (OBF, Bob.1) is possible only for Oct-1 bound to the canonical oct site (ATGCAAAT) [17].

We have previously revealed *oct-1* mRNA isoforms specific for lymphoid cells and tissues [15]. In this work, we found four new mouse *oct-1* mRNA isoforms, two of which proved to be tissue-specific. A new, untranslated exon was found in mouse and human *oct-1*, and known 5' exons were mapped.

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Finally, we considered the possible role of the alternative *oct-1* mRNA isoforms.

EXPERIMENTAL

Cell lines. We used mouse myeloma cells NS/0, fibroblasts L929, bone marrow stem cells FCDP-2, thymoma cells EL-4. Cells were grown in DMEM or RPMI supplemented with 10% fetal calf serum and 40 μg/ml gentamicin or kanamycin.

RNA was isolated with guanidine thiocyanate from cultured cells and from the spleen, thymus, lymph nodes, and bone marrow of C57Bl/6 mice [18].

Oligonucleotides. We used oligonucleotide primers synthesized by LITEKh (Russia), including

ex2 (5'-GGTTTCTGATGGATTATTCATTC-3') directed to exon 2,

1L (5'-GCCATGCTGGACTGCAGTGAC-3') directed to exon 1L,

1U (5'-GAGCAGCGAGTCAAGATGAG-3') directed to exon 1U,

(5'-1L) (5'-ACTAAGCTTCTCTTCCCACCCTTGTT-3') directed to the 5'-UTR upstream of exon 1L,

1R (5'-ATAGGATCCTTAAGTGCAAACCCATCT-3') directed to the 3' end of *oct-1r,*

T15 (5'-GGGAGGCCCTTTTTTTTTTTTTT-3') directed to the poly(A) tail,

POU (5'-ACCAAAGCGAATTGACACTCC-3') directed to the cDNA region coding for the first helix of the POU domain,

mPOU (5'-ATCACCCTCACGTCCCAGGG-3'),

POU2 (5'-GAGTGGTTGCCACCAATGAGG-3') directed to the POU domain-coding sequence,

and sense and antisense oct primers (5'-TGGTACCTGAG**ATGCAAAT**GAGACTGTCTCTCTAGAG-3' and 5'-TTCTCTAGAGAGACAGTCTC**ATTTGCAT**CTCAGGTACC-3'.

Reverse transcription with M-MuLV reverse transcriptase was carried out as recommended by Fermentas (Lithuania).

Polymerase chain reaction (PCR) was carried out with single-stranded cDNA (10–20% of the reverse transcription product) and *Taq* polymerase (Fermentas). Primer ex2 was labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase as recommended by Fermentas. Amplification included 35 cycles of 40 s at 95°C, 40 s at 57°C, and 2 min at 72°C, with first denaturation for 2 min. The product was resolved by denaturing PAGE in 6% gel. Sequencing products of a known nucleotide sequence were used as markers.

Full-length *oct-1 c*DNA was obtained in two steps. First, PCR with primers 5'-1L and T15 was run for 2 min at 95°C; 5 cycles of 1 min at 95°C, 1 min at 47°C, and 4–6 min at 72°C; and 35 cycles of 40 s at 95°C, 1 min at 57°C, and 4–6 min at 72°C. Then 1 μl of the amplificate was combined with 50 μl of the reaction mixture, and PCR with *Pfu* polymerase (Fermentas) and primers 5'-1L and 1R was run for 35 cycles of 1 min at 95°C, 1 min at 57°C, 4–6 min at 72°C, with first denaturation for 2 min. The 5'-fragments of *oct-1* cDNA isoforms were amplified in one-step PCR with primers 1U and POU2 (2 min at 95°C; 35 cycles of 40 s at 95°C, 40 s at 57°C, 2 min at 72°C).

To estimate the ratio of mRNA isoforms [19], PCR was run with primers 1U–POU2, 1L–POU2, mPOU–POU2, or POU–POU2 for 25–35 cycles of 1 min at 95°C, 1 min at 57°C and 2 min at 72°C, with first denaturation for 2 min. The product was resolved in 1% agarose gel and analyzed by Southern blotting with a probe specific for the POU domain-coding sequence. A portion of isoforms that contained exon 1L was calculated as a ratio between the PCR products obtained with primers 1L–POU2 and 1U–POU2 in 25 or 30 cycles (log phase). A portion of isoforms that had a deletion from the POU domain-coding sequence was calculated as a ratio between the PCR products obtained with primers mPOU–POU2 and POU–POU2 in 35 cycles.

Nucleotide sequences were established with a sequencing kit as recommended by Fermentas.

Expression in *Escherichia coli* and purification of proteins. To obtain the intact POU domain and its deletion derivative, the corresponding cDNA fragments were cloned in pET23b (Novagen) between the T7 promoter and the region coding for six His residues. The resulting constructs were used to transform *E. coli* BL21(DE)3)/pUBS520. Cells were grown and proteins isolated as recommended for the pET system (http://www.novagen.com/SharedImages/TechnicalLiterature/7_tb055.pdf). Cells were transferred in LB (150 ml) supplemented with ampicillin and kanamycin (50 μg/ml each), grown at 37°C until OD_{560} = 0.5, and chilled in ice for about 10 min. Expression was induced with 0.5 mM IPTG (Fermentas). Cells were cultured at 20°C for 4 h; collected by centrifugation; resuspended in 6–7 ml of 50 mM Tris-HCl (pH 8.0), 300 mM NaCl; and disrupted by sonication in a UZDN-A unit for 3–4 pulses of 25 s each at 60% power. The lysate was centrifuged at 12,000 *g* for 10 min at 4°C.

The supernatant was combined with 1 ml of Ni-NTA resin (Novagen) equilibrated with 50 mM Tris-HCl (pH 8.0), 300 mM NaCl. The mixture was incubated for 2 h at 4°C with shaking. The resin was washed thrice with 1 ml of 50 mM Tris-HCl (pH 8.0), 300 mM NaCl, and thrice with the same buffer supplemented with 15 mM imidazole. The protein was eluted with 500 μl of 50 mM Tris-HCl (pH 8.0), 300 mM NaCl, 200 mM imidazole. The eluate was dialyzed against 50 mM Tris-HCl (pH 8.0), 150 mM NaCl,

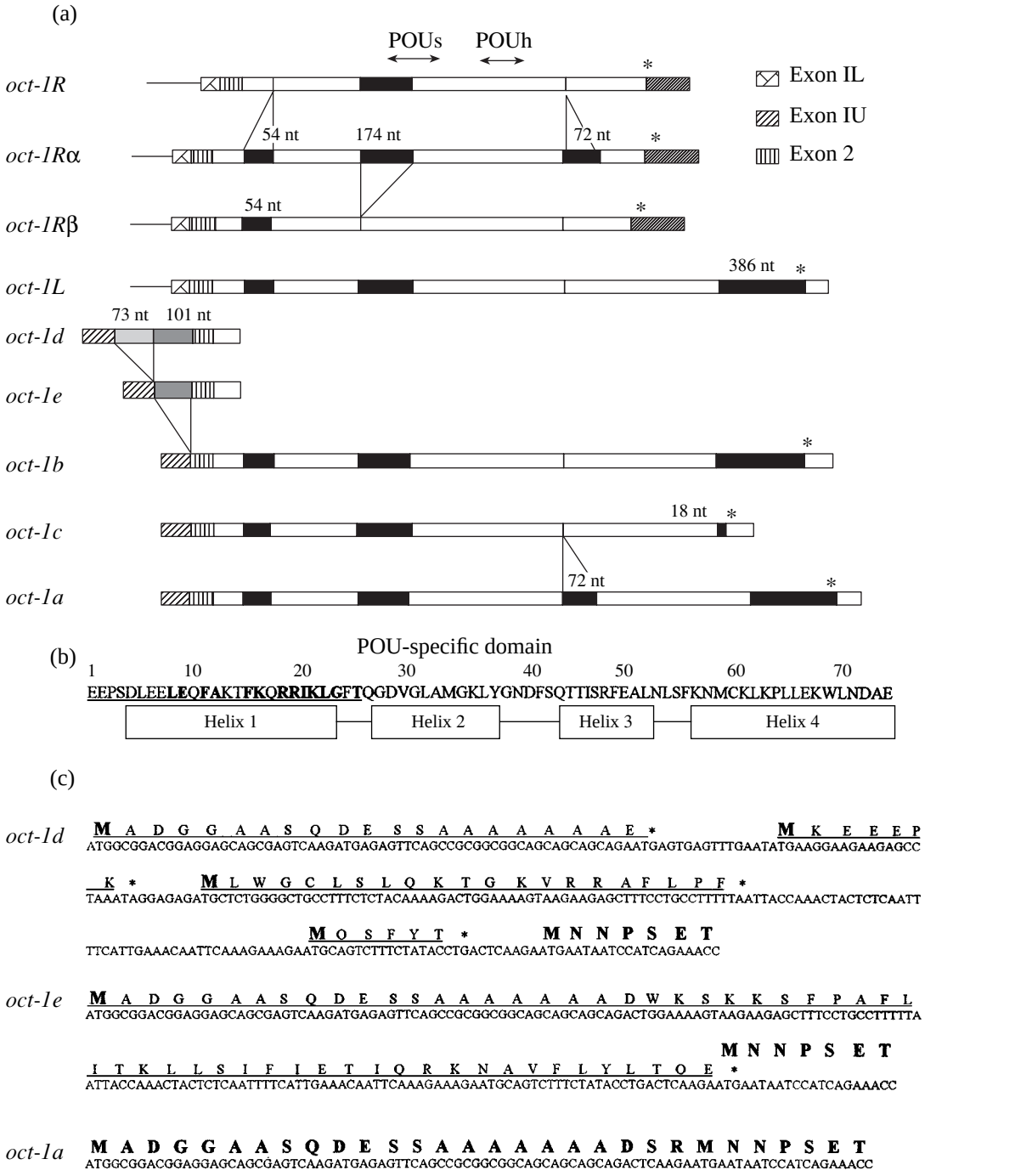

Fig. 1. Mouse *oct-1* mRNA isoforms. The general structure (a) is shown for several *oct-1* mRNA isoforms identified in this and previous works [15, 20, 21]. Stop codons are indicated with asterisks. Filled bars, exons subject to splicing. (b) Structure of the POU-specific domain: a region lost as a result of deletion from *oct-1R*β is underlined, its highly conserved residues are in bold. (c) Structure of the 5'-untranslated region: consecutive short open reading frames are underlined, Met residues and the Oct-1 sequence are in bold.

1 mM EDTA, 1 mM DTT. The protein was analyzed by denaturing PAGE.

Mobility shift assays. Double-stranded DNA probe obtained by annealing of the sense and antisense oct primers was labeled with [^{32}P]dATP and Klenow fragment. Probe binding was performed in 20 μl of the reaction mixture containing 20 mM Tris-HCl (pH 8.0), 75 mM NaCl, 1 mM EDTA, 10% glycerol, 1 mM DTT, 0.1 μg of nonspecific DNA (a 600-nt GC-rich cloned genomic fragment located upstream of exon 1U and lacking any oct sites), and 15–30 ng of

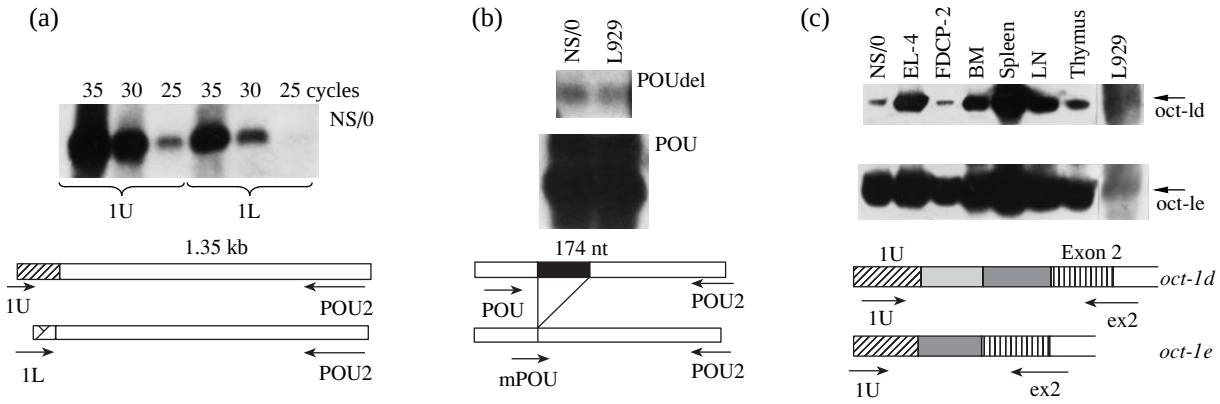

Fig. 2. Expression level of the *oct-1* mRNA isoforms as assessed by PCR. Relative contents were estimated (a) for isoforms with exon 1L or 1U and (b) for isoforms with the native POU domain or its deletion derivative (POUdel). (c) Expression of the *oct-1d* and *oct-1e* isoforms was analyzed in several cell lines and organs, including bone marrow (BM) and lymph nodes (LN). Schemes of amplification and primers employed are shown below.

the protein. The mixture was incubated at 20°C for 30–40 min. The product was analyzed in 4% PAG at 4°C.

RESULTS

New Isoforms of the Transcription Factor Oct-1 mRNA

Two full-length cDNAs (*oct-1R*α and *oct-1R*β), which code for earlier unknown forms of transcription factor Oct-1, were cloned from NS/0 myeloma cells by means of reverse transcription and PCR (Fig. 1a). In 5'-region, both cDNAs were identical to cDNAs *oct-1R* [20] and *oct-1L* [15], which have earlier been cloned from myeloma cells, and differed from other mouse and human *oct-1* cDNAs (*oct-1a*, *b*, *c* [21], *oct-1A*, *B* [22]). The 5'-regions of the *oct-1R*α and *oct-1R*β cDNAs each contained exon 1L and differed from the *oct-1R* cDNA in having an additional 54-nt fragment. Like the *oct-1R*α cDNA, *oct-1*α contained a 72-nt insert at the 3' end. In *oct-1R*β, a 174-nt fragment was deleted from the POU domain-coding region, which results in the absence of the first helix from the POU-specific domain (Fig. 1b).

Two 5'-fragments of the *oct-1* cDNA, *oct-1d* and *oct-1e*, were similarly obtained from mouse L929 cells (Fig. 1a). The *oct-1e* cDNA proved to be similar to the human *oct-1B* cDNA, whereas *oct-1d* contained a new insert at the 5' end. A characteristic feature was that *oct-1e* and *oct-1d* each had a 5'-untranslated region containing open reading frames (Fig. 1c).

Expression Levels of Various *oct-1* mRNA Isoforms

We have previously shown that *oct-1* mRNA isoforms with 5'-terminal exon 1L are expressed only in lymphoid cells and tissues and are thereby tissue-specific [15]. This was also observed for the *oct-1R*α and *oct-1R*β cDNAs. The ratio between the tissue-specific isoforms with 5'-terminal exon 1L and ubiquitous isoforms with exon 1U in NS/0 cells was assayed by PCR (see Experimental). The results showed that the isoforms with 5'-terminal exon 1L amount to 15% of the content of isoforms with exon 1U (Fig. 2a).

Relative content of mRNA isoforms with a deletion from the POU domain-coding region in NS/0 myeloma cells and L929 fibroblasts was analyzed with primer mPOU, which is directed to the sequence flanking the deletion (Fig. 2b). In either cell line, the content of the deletion mRNA isoforms was two orders of magnitude lower than that of isoforms with the intact POU domain-coding region. Thus, isoforms with the deletion from the POU domain-coding region were observed both in lymphoid (NS/0) and nonlymphoid (L929) cells.

The expression patterns of the *oct-1d* and *oct-1e* mRNA isoforms were analyzed with primers 1U and ex2 (the latter was labeled). The PCR product was analyzed by denaturing PAGE in 6% gel. Both isoforms were found in all, lymphoid and nonlymphoid, cells and organs examined (Fig. 2c). Both isoforms accounted for 10–20% of the total *oct-1* mRNA (data not shown).

Analysis of Mouse and Human *OTF-1*

All known exons of the *oct-1* gene were localized using the BLAST program (www.ncbi.nlm.nih.gov/BLAST). The human (NT_004668) and mouse (AC093371) *OTF-1* loci were respectively 200 and 140 kb in size. In either species, exon 1L is in the center of the locus, being about 100 (human) or 70 (mouse) kb away from exon 1U (Fig. 3a).

In the *oct-1d* mRNA, the 5'-untranslated region results from insertion of two intermediate (i) exons, 1i and 2i, between exons 1U and 2. In the *oct-1e*

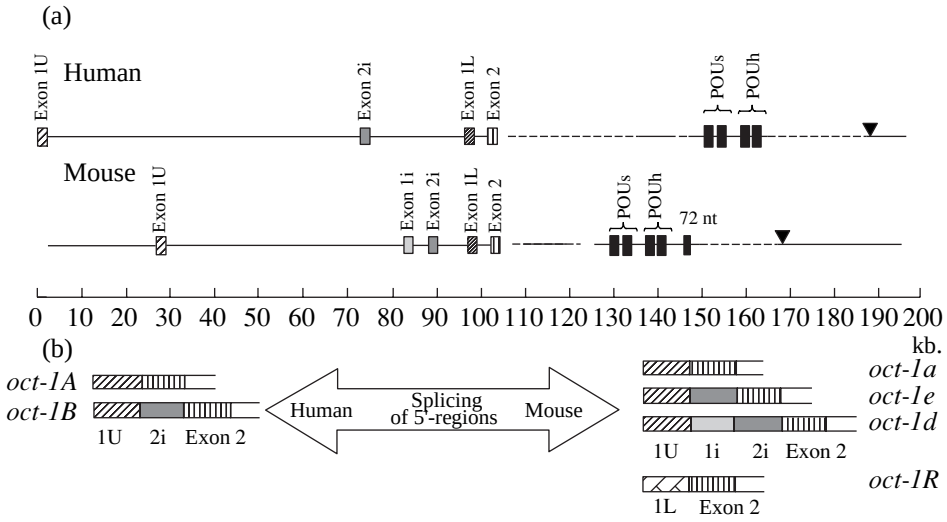

Fig. 3. Comparative structure (a) and alternative splicing (b) of mouse and human *OTF-1*. Exons involved in alternative splicing of the 5'-region and position of the last exon (▼) are indicated.

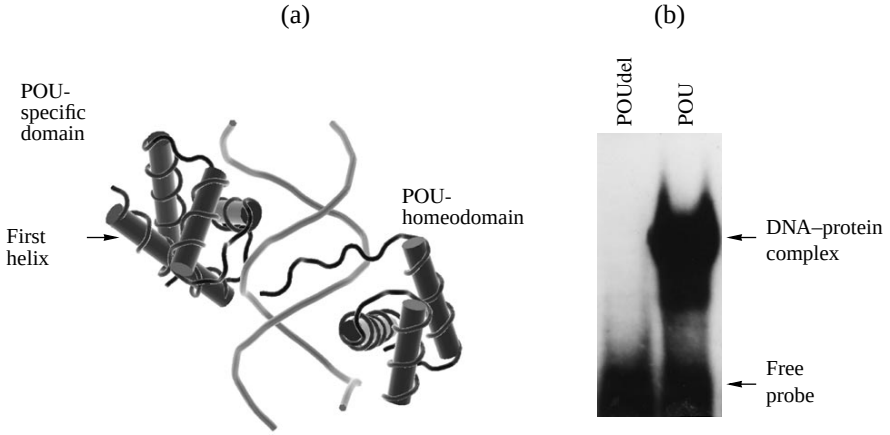

Fig. 4. Comparative DNA-binding properties of the POU and POUdel domains. (a) Complex of the POU domain with the oct DNA site as inferred from the X-ray data [25]. (b) Mobility shift assay of the POUdel and POU domains with the oct probe.

mRNA, only exon 2i is between exons 1U and 2 (Fig. 1a). As a comparison showed, human *OTF-1* lacks a homolog of mouse exon 1i and has a homolog of mouse exon 2i (Figs. 3a, 3b).

On evidence of the structure of mouse genomic *otf-1*, a full-length 72-nt exon is integrated into the 3' end of the *oct-1R*α mRNA (Fig. 3a). Human *OTF-1* lacks a homologous exon. The *oct-1R*β mRNA lacks the first exon (174 nt), which codes for the first helix of the POU-specific domain (Fig. 3a).

Analysis of the genomic nucleotide sequence showed that the splicing sites of exons 1i and 2i agree with the consensus (table).

DNA-Binding Properties of the Product of *oct-1R*β

The *oct-1R*β mRNA has a deletion from a region coding for the DNA-binding domain, which may alter the DNA-binding ability of the protein product. The native POU domain and its deletion derivative (POUdel) were overexpressed in *E. coli* BL2(DE3)/pUBS520 and purified on Ni-NTA. Binding was analyzed with a labeled probe containing the oct site ATGCAAAT (Fig. 4). The native POU domain, but not its deletion derivative, bound with the oct probe.

Splicing sites of exons 1i and 2i of the mouse *oct-1* gene

Exon	5'-Donor sequence	3'-Acceptor sequence
1i	AAG**gt**aaat	TTGC**ag**/
2i	TGG**gt**aaat	TCTT**ag**/
Consensus	(A/C)AG**gt**aagt	$(Py)_n$NPy**ag**/

DISCUSSION

In eukaryotic cells, mRNA isoforms result from alternative splicing of the primary transcript. This is common for many genes, as evident from the high prevalence of transcripts over the corresponding genes located on one or another chromosome. Thus the average number of mRNA forms per gene is 2.6 for chromosome 22, and 3.2 for chromosome 19 in humans [23]. The number is expected to increase with accumulation of experimental data.

In this work, we cloned and characterized four new mRNA isoforms of mouse *oct-1*. Of these, two (*oct-1R*α and *oct-1R*β) proved to be tissue-specific.

The *oct-1R*β isoform is most interesting, as it lacks a fragment of the POU domain-coding sequence and its product, the first helix of the domain. The deletion involves several conserved amino acid residues (Fig. 1b). Upon POU domain binding with the oct site, Arg20 interacts with the DNA sugar-phosphate backbone and with Glu51 to form hydrogen bonds, which are involved in a hydrogen bonding network within the POU-specific domain [24]. On X-ray evidence, Leu9, Phe12, Phe16, and Leu23 form multiple hydrophobic contacts within the domain, which is important for its correct folding. We did not observe the binding of the *oct-1R*β product with the oct probe, which testifies that the deletion impairs the function of the POU domain. This finding agrees with the data of deletion analysis of the POU domain of Oct-1 [25].

The *oct-1d* and *oct-1e* isoforms, which contain the 5'-untranslated exons, are also of interest. As shown with many genes, translation is suppressed when 5'-untranslated exons contain short open reading frames upstream of the major reading frame or assume a secondary structure that hinders translation initiation [26]. Possibly, exons 1i and 2i present at the 5' end of the *oct-1* mRNA affect its translation. The human locus lacks a homolog of mouse exon 1i found in the *oct-1d* isoform, suggesting a difference in post-transcriptional regulation of *oct-1* expression for mouse and human.

The function of the *oct-1R*α isoform is obscure. We can only note that the mouse *otf-1* locus contains the 72-nt exon, which is absent from the human locus.

Thus, expression of *oct-1* yields several mRNA isoforms, including ubiquitous *oct-1a*, *b*, *c*, *d*, and *e* and tissue-specific *oct-1R*, *L*, *R*α, and *R*β. Of these, *oct-1R*β codes for a protein with impaired DNA-binding properties, while *oct-1d* and *oct-1e* contain 5'-untranslated exons. Probably, this reflects the complexity of *oct-1* expression regulation and the multiplicity of Oct-1 functions.

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