Expression of murine novel zinc finger proteins highly homologous to *Drosophila ovo* gene product in testis

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Abstract We have cloned two isoforms of cDNAs encoding novel zinc finger proteins. One form encodes a 274-amino acid protein containing an acidic amino acid and serine-rich domain and a zinc finger domain which shows high sequence homology to that of *Drosophila* Ovo protein. The other form encodes a 179-amino acid protein containing only the zinc finger domain. Expression of both proteins possessing an antigenic epitope in COS cells revealed that they are localized in the nucleus. The 1.3-kbp mRNAs are predominantly expressed in testis, and the expression increases from 3 weeks postnatal, implying that these proteins may play important roles in the development of the testes.
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Key words: cDNA cloning; Zinc finger protein; *ovo*; Homologue; Spermatogenesis

1. Introduction

The development of multicellular organisms is controlled by sequential activation of a hierarchy of regulatory genes, which encode transcription factors having multiple classes of DNA binding motifs. The zinc finger is the most common DNA binding motif among eukaryotic transcription factors, and is classified based on its amino acid sequence involved in the zinc chelation into several subgroups: C_2H_2, C_4, C_2HC and C_6 groups [1–5].

Zinc finger coding sequences have been found in a number of the genes that control *Drosophila* development. The genes *krüppel, hunchback, huckebein* and *buttonhead* are crucial for the proper segmentation of the developing embryo [6–9].

ovo is required for survival and differentiation of female germ line cells and plays an important role in germ line sex determination in *Drosophila* [10–14]. Homologous null mutants produce rudimentary ovaries in which germ cells have completely degenerated, leaving only somatic tissues [10]. Less severe mutations produce viable germ cells that exhibit defective oogenesis [10] or ovarian tumors with male germ line features [10,12]. The three dominant female-sterile alleles either arrest oogenesis at previtellogenic stages or allow the production of defective eggs [15]. Mével-Ninio et al. identified a genomic fragment that rescues *ovo* mutation and isolated cDNA clones of the locus [16]. The molecular structure of the

ovo gene product contains four C_2H_2 zinc finger motifs, suggesting that *ovo* encodes a transcription factor [16–19].

Since molecular mechanisms regulating development and cell differentiation are partly conserved between *Drosophila* and mammals, isolation of mammalian homologues or closely related genes of *Drosophila* genes provides useful clues for elucidating those processes in mammals.

In this study, we report the isolation of two cDNA clones encoding novel proteins that possess a zinc finger domain highly homologous to that of *Drosophila* Ovo protein. We found that these proteins were localized in the nuclei and that the mRNAs were predominantly expressed in testis and developmentally regulated.

2. Materials and methods

2.1. Isolation of cDNA clones and DNA sequencing

A mouse testis cDNA library was constructed in λ Zap II by the method of Huynh et al. [20]. The DNA fragment corresponding to the zinc finger domain of *Drosophila ovo* protein (between amino acids 932 and 1152 [16]) was amplified by the polymerase chain reaction (PCR), and labeled with ^{32}P by random priming. Hybridization was carried out at 42°C with 5×SSPE, 0.1% SDS, 1×Denhardt's solution, 50% dextran sulfate, 0.1 mg/ml denatured salmon sperm DNA and $2×10^6$ cpm/ml probe. The highest stringency wash was 2×SSC-0.1% SDS at 42°C. Out of a screening of $5×10^5$ phages, 11 positive plaques were isolated and excised from the λ Zap II vector according to the supplier's directions (Stratagene). The plasmid clones were grouped into two (two and nine clones) by DNA sequencing. The longest clones of each group were designated M-OVO-A and M-OVO-B and analyzed.

2.2. Transient expression of epitope-tagged M-OVO

The entire coding regions of M-OVO-A and -B were amplified using PCR with sense oligonucleotides containing an *Xba*I site: 5′-GCTCTAGAATGCCCAAAGTCTTTCTGGTA-3′ for M-OVO-A and 5′-GCTCTAGAATGCAGCGCCCGGTTGCCAGG-3′ for M-OVO-B; and antisense oligonucleotides containing a *Hin*dIII site: 5′-CTCTAAGCTTTTTTTCCTCCTCTTCACTC-3′ for both clones. The restriction sites facilitate subcloning the amplified sequences into pCG-HA [21] so that the influenza virus hemagglutinin (HA) epitope (GYPYDVPDYA) would attach to the amino-terminus of M-OVO-A and -B, designated pCG-HA-M-OVO-A and pCG-HA-M-OVO-B, respectively. COS-7 cells were transfected by the calcium-phosphate method [22]. Forty-eight hours later, the cells were fixed in 100% methanol at −20°C for 10 min. The cells were incubated for 2 h at room temperature with a 1:100 dilution of mouse ascites fluid containing 12CA5 monoclonal antibody directed against the influenza HA epitope (Boehringer Mannheim). The cells were washed with PBS and then incubated for 1 h with a 1:50 dilution of rhodamine-conjugated goat anti-mouse IgG (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD). Following several washes with PBS, the cells were viewed with a fluorescence microscope (Olympus GB 200).

2.3. Northern blot analysis

Total RNA and poly(A) RNA of mouse tissues were isolated and analyzed according to standard procedures [23]. The 369-bp *Pst*I frag-

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A

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    1    GGGGGCCGCAGTCGGGAGAGAGGATCCCACCATGCCCAAAGTCTTTCTGGTAGGGAGGAG
                                        M  P  K  V  F  L  V  G  R  R    10

   61    GAGCCCGGGAGTCTCTGTCCGCAGCTGGGATGAGCTCCCGGATGACAAAAGGGCAGACAC
          S  P  G  V  S  V  R  S  W  D  E  L  P  D  D  K  R  A  D  T    30

  121    TTACATCCCAGTGAGCCTGGGCTGTCTGCTCCGCGACCCGCCCGAAGACTGCCGCAGCAG
          Y  I  P  V  S  L  G  C  L  L  R  D  P  P  E  D  C  R  S  S    50

  181    CGGCGGCAGTATCAGCGGCTGCAGCAGCAGCGCCGGGGAGCCCGGAGGGGCCGAAAGCAG
          G  G  S  I  S  G  C  S  S  S  A  G  E  P  G  G  A  E  S  S    70

  241    CTCGTCCCCGCGCGCGCCGGAGCCTGAAACTCCAGAGCTTCACGACGCCCAAGGCACCGA
          S  S  P  R  A  P  E  P  E  T  P  E  L  H  D  A  Q  G  T  D    90

  301    TGGACATCTGGCAGCGATGCAGCGCCCGGTTGCCAGGTCAAAAATCAAGTTTACCACCGG
          G  H  L  A  A  M* Q  R  P  V  A  R  S  K  I  K  F  T  T  G   110

  361    CACATGCGACAACTCTGTGATTCACAACTGTGACCTTTGTGGCAAGAGCTTCCGCCTGCA
          T  C  D  N  S  V  I  H  N  C  D  L  C  G  K  S  F  R  L  Q   130

  421    GCGCATGCTCAACCGTCACCTTAAGTGCCACAACCAGGTAAAGAGGCACCTGTGCACCTT
          R  M  L  N  R  H  L  K  C  H  N  Q  V  K  R  H  L  C  T  F   150

  481    CTGCCGGCAAGGGCTTCAATGACACCTTCGACCTGAAAAGGCACGTACGCACGCACACTGG
          C  G  K  G  F  N  D  T  F  D  L  K  R  H  V  R  T  H  T  G   170

  541    CATCCGCCCCTACAAATGTGAGGTGTGTAACAAGGCCTTCACCCAGCGGTGTTCCTTGGA
          I  R  P  Y  K  C  E  V  C  N  K  A  F  T  Q  R  C  S  L  E   190

  601    GTCCCACCTAAAGAAGATCCACGGGGTACAGCAGCAGTATGCCTACAAGCAGCGTCGCGA
          S  H  L  K  K  I  H  G  V  Q  Q  Q  Y  A  Y  K  Q  R  R  D   210

  661    CAAACTTTACGTGTGTGAGGATTGCGGCTACACGGGCCCCACCCAGGAGGACCTGTATTT
          K  L  Y  V  C  E  D  C  G  Y  T  G  P  T  Q  E  D  L  Y  L   230

  721    GCATGTGAACAGTGACCATTCAGGGAGCACATTTTTCAAAAAGAATTCCAAAAAGTTGGC
          H  V  N  S  D  H  S  G  S  T  F  F  K  K  N  S  K  K  L  A   250

  781    GGCCCTTATGCAGAACAAGCTGACGTCCCCGCTGCAGGAGAATTCCACCCTGAGTGAAGA
          A  L  M  Q  N  K  L  T  S  P  L  Q  E  N  S  T  L  S  E  E   270

  841    GGAGGAAAAAAAGTGAAGAGCGAGCAAGAAAAAGCAGAGGAGAGACGCCGGCGCCACGTT
          E  E  K  K  *                                                274

  901    TACTCCGAATTTTGGTTGTAGGTGTACCCCTTTGCTCCCCTGGATTTTTTTATATTCAGT
  961    CCACCTTTTTTTTTTTTGGGACAGCAACNGTAAGGAGCACCCCAAAGTTGTCAGTCATT
 1021    CTGCCACAACAGGCCCTGTGAATTGTTTCTTGGTGGCTGAGGTCACATACGGGTTTCTGT
 1081    GTGTATGTGTGTCTGTCTGTCTGTCTGTTATGTGTGGGTTTTTTTTTGTTTCCTAGTAGA
 1141    ATTTTCACGTATGAATAGAGAGAGGTGTTCTTAAAGAGACTGTCATCTTAAGGTGCCTTC
 1201    AAATGCTTCCCATGCAAGGAAAATGTATATTCTTCTGAAGAGTTTACAGAAATTATTTTA
 1261    ATAAATGAAATGTTCGTGTG
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B

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    1    CGCTTGGACCCCGGTGCTTCGTGGGTTGGCCTGAGAACCCGTCTCCCGACACCCTAAGTT
   61    AGTTCGAGGGGCGGATGGCTAGGGTTGACTCGTCTACCCTCGGGACGTCGCCCACTTGGC
  121    GAGGTGCCCAGGTCCGGGGCGGGGCGGCGGCACCGCCCGCAGGTCAAACTGCCGTGGGCG
  181    CCCGGGCGTGACGCCGCGTGTGGTTGAGTGTCGGGGTGCGGCCCCCTCCCTCCCCAGTGA
  241    GCCTGGGCTGTCTGCTCCGCGACCCGCCCGAAGACTGCCGCAGCAGCGGCGGCAGTATCA
  301    GCGGCTGCAGCAGCAGCGCCGGGGAGCCCGGAGGGGCCGAAAGCAGCTCGTCCCCGCGCG
  361    CGCCGGAGCCTGAAACTCCAGAGCTTCACGACGCCCAAGGCACCGATGGACATCTGGCAG
  421    CGATGCAGCGCCCGGTTGCCAGGTCAAAAATCAAGTTTACCACCGGCACATGCGACAACT
          M  Q  R  P  V  A  R  S  K  I  K  F  T  T  G  T  C  D  N    19
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Fig. 1. Nucleotide and predicted amino acid sequences of M-OVO-A (A) and 5′ region of M-OVO-B (B). The region of overlap between two clones is indicated by square brackets. The polyadenylation signal is double underlined. In the amino acid sequence, the zinc finger motif is thick underlined, an acidic amino acid/serine-rich domain is underlined. The methionine as an initiation codon of M-OVO-B is indicated by an asterisk. Numbers in the left and right margins refer to nucleotide and amino acid positions, respectively.

ment which includes the zinc finger domain region of M-OVO cDNA was labeled with ^{32}P by random priming. Hybridization was performed at 42°C overnight in 5×SSC buffer with 50% formamide, 1×Denhardt's solution, 1% SDS, 0.1 mg/ml denatured salmon sperm DNA, and 1 ng/ml probe. The filters were washed twice in 2×SSC-0.1% SDS at room temperature for 20 min and once in 0.1×SSC-0.1% SDS at 55°C for 30 min.

3. Results and discussion

Northern hybridization analysis of various mouse tissues with the DNA fragment corresponding to the zinc finger domain of Ovo protein as a probe yielded a faint signal in testis (data not shown). Therefore, we screened a testis cDNA library under low stringency. We isolated two different groups

of clones which were designated M-OVO-A and M-OVO-B. DNA sequencing analysis of the longest clone of each group revealed that the two cDNA clones are different in a small part of the 5′-terminal region, but the other 1154 nucleotides spanning to the poly(A) tail are completely identical (Fig. 1). Genomic Southern blot analysis with a DNA fragment of the common 3′ end part of two clones as a probe revealed that a single band was detected in the genome digested by multiple restriction enzymes, indicating that there is one M-OVO gene in the mouse genome (data not shown). Taken together, M-OVO-A and M-OVO-B are probably generated from a single gene by an alternative splicing event.

The longest open reading frame of M-OVO-A, in which the first ATG (position 1 in Fig. 1A) is assigned as an initiation
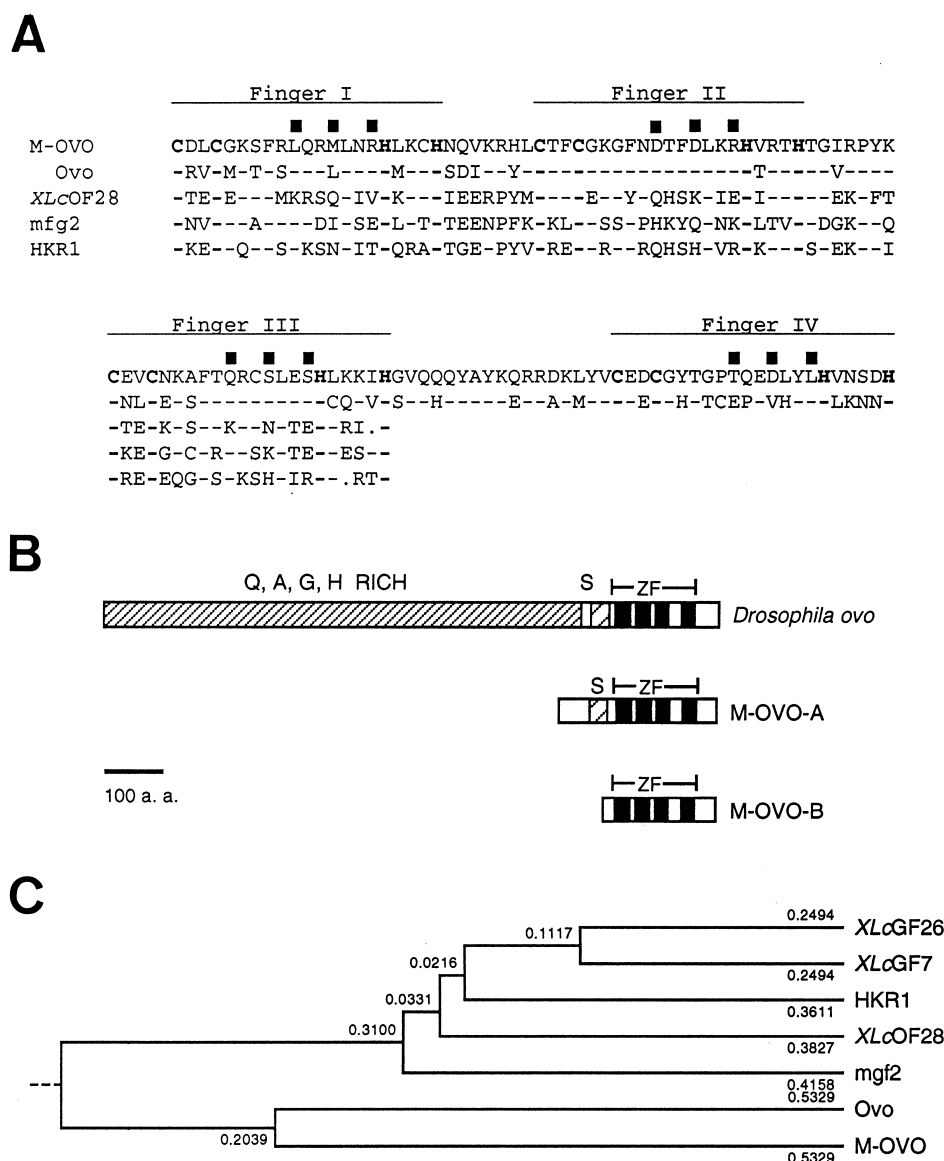

Fig. 2. Comparison of M-OVO with *Drosophila ovo* and other zinc finger proteins. A: Amino acid sequence comparison of zinc finger domain. Ovo, *Drosophila* Ovo protein; *XLc*OF28, *Xenopus* oocyte zinc finger protein *XLc*OF28 [25]; mfg2, mouse zinc finger protein mfg2 [26]; HKR1, human krüppel-related zinc finger protein 1 [27]. Cysteine and histidine residues constituting the zinc finger motifs are presented in bold type. Dashes represent identity to M-OVO. Black squares highlight the DNA contact amino acids as determined by the model of Klevit [28]. B: Schematic comparison of *Drosophila ovo* protein and M-OVO-A and -B. The zinc finger domain and serine-rich region are labeled as ZF and S. C: Phylogenetic tree of M-OVO-A and other zinc finger proteins. On the basis of the distance matrix between aligned amino acid sequences, the tree was inferred by the UPGMA method [33]. The numbers indicate the distance from each branching point. *XLc*GF26 and *XLc*GF7, *Xenopus* gastrula zinc finger proteins [25].

codon, encodes a 31-kDa protein of 274 amino acids. The nucleotide sequence around the first methionine of M-OVO-A fits well the consensus sequence for translational initiation in higher eukaryotes [24]. The predicted protein contains four clusters of C_2H_2 type zinc fingers at the carboxy-terminus (between amino acids 120 and 236), and a domain which is rich in acidic amino acids and serine (acidic/serine-rich domain, between amino acids 11 and 90). On the other hand, due to the difference in nucleotide sequence of the 5′ terminal region, the first methionine (position 1 in Fig. 1B) assigned as an initiation codon of M-OVO-B corresponds exactly to the methionine at position 96 of M-OVO-A. Consequently, M-OVO-B encodes a truncated isoform of M-OVO-A of 21 kDa consisting of 179 amino acids, which possesses the identical zinc finger domain as M-OVO-A, but lacks the acidic/serine-rich domain.

A search of the computer databases for nucleotide and amino acid sequences was conducted. As expected, the highest matches of both sequences were to the *ovo* gene and its product, and to a much lesser degree to other zinc finger proteins such as *Xenopus* zinc finger proteins, *XLc*OF28 [25], *XLc*GF7 [25], *XLc*GF26 [25], mouse mfg2 [26] and human krüppel-related protein, HKR1 [27]. An amino acid alignment of their zinc finger domains is shown in Fig. 2A. There is a high degree of sequence identity between M-OVO and Ovo protein (68%, 80/117), particularly in the region excluding zinc finger IV (74%, 70/95). The amino acids considered to contact the DNA strand are marked by boxes [28]. Considering the fact that erythroid krüppel-like factor, exhibiting 58% amino acid identity with SP I in the zinc finger domain consisting of three C_2H_2 motifs, binds the same CACCC site in the β-globin gene promoter [29], M-OVO and Ovo protein would bind to a similar and perhaps identical DNA sequence. Similar to

Fig. 4. Northern blot analysis of M-OVO. A: Tissue distribution of M-OVO mRNA. Poly(A) RNAs of each tissue (2 μg) are analyzed. Br, brain; He, heart; Sp, spleen; Lu, lung; Li, liver; Mu, skeletal muscle; Ki, kidney; Te, testis; Ov, ovary. B: Analysis of M-OVO expression during testis development. 20 μg of total RNA of testis are analyzed. 2W, 2-week-old mice; 3W, 3-week-old mice; 6W, 6-week-old mice. 28S and 18S rRNAs are indicated on the left side.

Ovo protein, the M-OVO zinc finger domain does not contain a typical TGEKPY(Y/F) consensus sequence in each H/C link, which is well conserved in the krüppel-type zinc finger motif and ubiquitously present in the mammalian genome [30].

A schematic comparison of M-OVO and Ovo protein is shown in Fig. 2B. M-OVO-A is much smaller than Ovo protein, and it lacks characteristic polyamino acid stretches such as polyglutamine [16]. However, a similar serine-rich sequence (between amino acids 49 and 72) is also present at the amino-terminal portion just adjacent to the zinc finger domain. As an acidic amino acid or serine-rich domain is known to act as an activation domain in several transcription factors [31,32], the whole structural feature suggests that M-OVO-A may be a transcription factor which possesses high similarity to Ovo protein. To establish evolutionary relationships among the zinc finger proteins showing high homology with M-OVO-A, a phylogenetic tree was constructed by the UPGMA method [33] (Fig. 2C). M-OVO-A and Ovo protein form a single cluster which is separated from that of other proteins, indicating that they constitute a closed subfamily within the large zinc finger gene family.

To examine the intracellular localization of M-OVO, we carried out transient expression of epitope-tagged M-OVO-A and M-OVO-B followed by indirect immunofluorescence using a monoclonal antibody against the HA tag (Fig. 3). Both transfected cells showed strong immunofluorescence in the nucleus in positive cells.

As shown in Fig. 4A, 1.3-kbp mRNA of M-OVO is predominantly expressed in testis, but apparently not in embryos (data not shown) or ovary where *ovo* mRNA is restrictedly expressed in fruit fly. Although it is often observed that the tissue distribution and expression level of mammalian homologues are different from those of the vertebrate genes, they are commonly expressed in similar crucial tissues where *Drosophila* genes play essential roles. Concerning the apparent absence of M-OVO transcript in ovary, M-OVO may be a structurally homologous protein but not be the homologue of *ovo* involved in ovary development. However, since the mammalian ovary is heterogeneous tissue consisting of different types of cells at various developmental stages, the possibility of restricted or temporary regulated expression of M-OVO in ovary should also not be excluded.
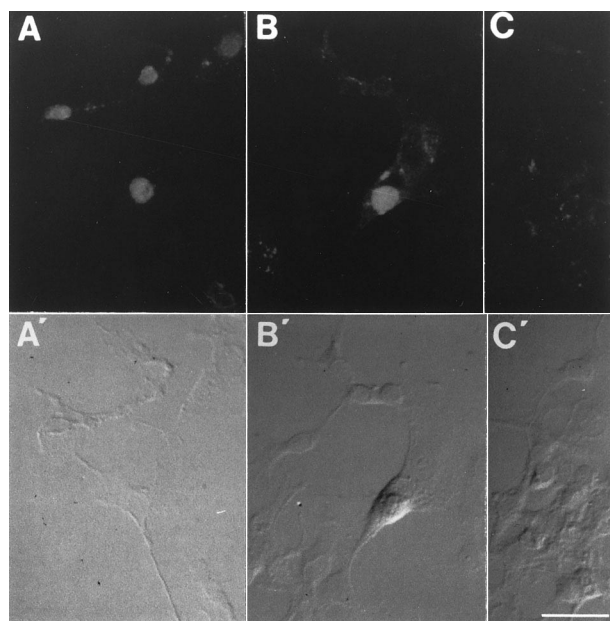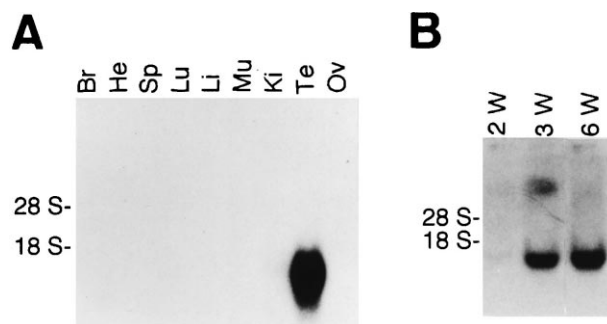

Fig. 3. Nuclear localization of epitope-tagged M-OVO-A and -B. After blocking the fixed cells under permeabilized condition, they were incubated with anti-HA monoclonal antibody 12CA5, followed by detection with rhodamine-conjugated anti-mouse IgG antibody. Immunofluorescence (A-C) and bright-field (A′-C′) photomicrographs of COS-7 cells transfected with pCG-HA-M-OVO-A (A and A′), pCG-HA-M-OVO-B (B and B′), and control pCG-HA (C and C′).

To determine whether the M-OVO mRNA is regulated during development of testis, we prepared total RNA from mouse testis at different stages of sexual maturation, i.e. a 14-day-old prepubertal mouse, a 21-day-old mouse and a sexually mature adult mouse. As shown in Fig. 4B, although no M-OVO mRNA was detected in prepubertal mouse testis, it showed a dramatic increase in 21-day-old mouse testis. Mammalian spermatogenesis is a continuum of cellular differentiation in which three principal phases can be discerned: spermatogonial renewal and proliferation, meiosis, and spermiogenesis, and it is initiated shortly after birth [34]. The first wave of differentiating germ cells have entered early prophase (i.e. leptotene and zygotene) at around day 14, and reached spermatid stage at day 21 [34]. The clear onset of increased M-OVO expression between days 14 and 21 (Fig. 4B) implies that M-OVO may play an important role in the development of testis, possibly in late meiotic or post-meiotic phases of spermatogenesis. Some *ovo* mutants produce small tumors in which germ cells closely resemble early spermatocytes [10,11]. It might be possible that M-OVO gene regulates some unknown process of germ line development which is partly shared between mammals and invertebrates.

The alternative splicing of transcripts or alternative usage of translation initiation sites leads to the generation of multiple isoforms of transcription factors which exhibit different abilities to bind target DNA or to transactivate target genes [35,36]. The interaction of the isoforms is also considered to play important roles in regulating cell differentiation [35,36]. In regulation of terminal liver differentiation, the transcriptional activation of the target gene by the liver enriched activation protein, LAP, possessing both a DNA binding domain and an activation domain, is modulated by its isoform, the liver inhibitory protein, LIP, possessing only a DNA binding domain in dominant negative fashion [36]. Similarly, M-OVO-A and -B are isoforms with or without the putative activation domain, implying that M-OVO-A and -B may be an activation form and an inactivation form, respectively. Therefore, if both forms are expressed in the same cell, they might compete for the binding sequence and regulate the activation of the target gene.

It has recently been reported that chromosome III of the nematode genome contains a sequence which can encode a putative zinc finger protein highly homologous to Ovo protein and M-OVO [37]. Genetic analysis targeted to the gene loci in the nematode might provide insights into the physiological roles of M-OVO. Further investigations, including identification of target genes or gene transfer of M-OVO to *Drosophila ovo* mutants to see whether the abnormalities are rescued, may shed light on the mechanism(s) of controlling testis or germ line development.

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