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Alternative 3′ UTR polyadenylation of *Bzw1* transcripts display differential translation efficiency and tissue-specific expression ☆

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Abstract

BZW1 is a conserved regulatory factor for transcriptional control of histone H4 gene at the G1/S transition. In this study, three *Bzw1* transcripts were identified in mice with two long forms (~2.9 kb) expressed ubiquitously at low level, and a short transcript of 1.8 kb expressed at high level exclusively in testis. These different transcripts share the same 5′ UTR and coding sequence, but differ in the length of 3′ UTR by utilizing alternative polyadenylation sites. Different translation efficiencies were observed in the cells transfected with chimeric EGFP-Bzw1 genes tailed with different 3′ UTRs. Our results demonstrate that *Bzw1* transcripts are alternatively polyadenylated and expressed in tissue-specific pattern.

Keywords: *Bzw1*; Alternative polyadenylation site; 3′ UTR; Testis; Translational efficiency

Basic leucine zipper and W2 domains 1 (BZW1) was first isolated from HeLa S3 cells by sequence-specific histone H4 gene Site II DNA affinity chromatography [1]. *Bzw1* gene encodes a 45 kDa protein containing an N-terminal basic leucine zipper (bZIP) domain and a C-terminal nucleotide (ATP or GTP) binding domain homologous to that of eukaryotic translation initiation factors, eIF-5 and the Epsilon subunit of eIF-2B [2–5]. At least four closely related genes/pseudogenes could be identified in human and mouse genome by searching genome database. However, the functional relationship between these genes is still unknown.

The presence of a bZIP domain classifies BZW1 as a member of the bZIP superfamily of transcription factors which includes FOS/JUN (AP-1), ATF/CREB, C/EBP, and MAF proteins [6–11]. Leucine zipper was identified as a protein–protein interaction motif, while the basic region was responsible for DNA binding [12,13]. Previously, it was shown that human BZW1 (hBZW1, KIAA0005 or BZAP45) could activate histone H4 gene transcription through Site II which is required for cell cycle. However, this effect is not dependent on direct Site II binding by BZW1, suggesting that hBZW1 serves as an important co-regulator of a subset of transcription factors that control the G1/S phase transition during the cell cycle [1].

Mitra and his colleagues have reported the presence of a 2.9 kb transcript of *hBzw1* in a variety of human tissues and cell lines [1]. In this study, we identified a novel transcript (~1.8 kb) in mouse testis, in addition to two long transcripts (~2.9 kb) that correspond to the previously reported transcript of *hBzw1*. To understand the physiological significance of this short transcript, we first characterized its expression pattern. Furthermore, we examined the translational efficiency of different *Bzw1* mRNA variants. Our results suggest that the alternative polyadenylation

☆ *Abbreviations:* Bzw1, basic leucine zipper and W2 domains 1; RACE, rapid amplification of cDNA ends; UTR, untranslated region.

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in 3′ UTR of *Bzw1* is important for translational efficiency and a possible role of BZW1 in Spermatogonia.

Materials and methods

Constructions. Bzw1 cDNA containing complete CDS and 3′ UTR was cloned from the cDNA of C57BL/6J mouse kidney. The sequence was confirmed by comparison with mouse *Bzw1* cDNA (GenBank No: NM_025824). For construction of EB-SV40 plasmid, *Bzw1* CDS was ligated into the *Bgl*II–*Eco*RI sites of pEGFP-C3 (Clontech). For construction of plasmids EB-UTRa, EB-UTRb, and EB-UTRc, fragments corresponding to different *Bzw1* 3′ UTRs were generated by PCR using *Bzw1* cDNA as a template with respective primer sets UTRa-f (5′-GGAATTCTGTAAA GCAAACAGGAGTTGTAG-3′) and UTRa-r (5′-TCGACGCGTGGAC ATACAGCACACCATTAG-3′) UTRa-f and UTRb-r (5′-TCGACGC GT GTGTGAGGAGTTATCAACC-3′); UTRc-f (5′-GGAATTCTTT GAAACAACATCCTCAGTAAAGC-3′) and UTRc-r (5′-TCG ACGCGT AGAAAACCCAGGGAATAAAG-3′). PCR products were inserted into the *Eco*RI–*Mlu*I sites of plasmid EB-SV40. All the forward primers contain an *Eco*RI site and the reverse primers contain an *Mlu*I site (underlined sequences).

Northern blot analysis. Total RNAs were isolated from adult C57BL/6J mice tissues by TRIzol reagent (Invitrogen). RNA samples were electrophoresed in 1.2% agarose–formaldehyde gel and transferred to Zeta-Probe blotting membrane (Bio-Rad). [α-^{32}P]dCTP-labeled probes named probe-a, Bzw1-a, Bzw1-b, Bzw1-c, Bzw1-d, and Bzw1-e corresponding to nucleotides 1–809, −23–334, 344–748, 872–1270, 1301–1891, and 1744–2370, respectively, were prepared by PCR. Hybridization was performed at 68 °C using ExpressHyb™ Hybridization Solution (Clontech) according to the manufacturer's instructions.

Rapid amplification of 5′- and 3′-cDNA ends (5′- and 3′-RACE). Testis mRNA was purified by Oligotex (Qiagen) from mouse total RNA of testes. Both 5′- and 3′-RACE were performed using SMART™ RACE cDNA Amplification Kit (BD Biosciences, Clontech). Two gene-specific primers, 5′-GCTCATTGCGGAAGGCCTTACATCAC-3′ (GSP1), 5′-GCAAAGGGCAAAAGTGTCTTCCTTGAGC-3′ (GSP2) and two nested gene-specific primers 5′-GCTCCTGCAGCTTGCTCCTCACAC-3′ (NGSP1), 5′-GCTGAGGAGGAATCTGAGTCTGAAGCTG-3′ (NGSP2) were designed for 5′- and 3′-RACE PCRs, respectively. RACE products were cloned and sequenced.

In situ hybridization. Digoxigenin-UTP-labeled antisense and sense RNA probes (probe-a, nt 1–809; probe-b, nt 1744–2370) were generated by in vitro transcription with DIG RNA Labeling Kit (Roche), using linearized plasmid DNA as templates. The testis frozen sections were digested with proteinase K, post fixed, acetylated, dehydrated, and hybridized overnight at 55 °C with the riboprobes, then followed by a stringent wash. Hybridization signal was detected with alkaline phosphatase-conjugated anti-Dig (Roche) and visualized with nitroblue tetrazolium/5-bromocresyl-3-indolylphosphate chromogen (Roche).

Cell culture and transfection. Human embryonic kidney (HEK)-293 cells were transfected with plasmid DNA using Lipofectamine (Invitrogen). Blank pEGFP-C3 vector was transfected simultaneously as a control for transfection efficiency. Cells were harvested 48 h following transfection for RNA or protein extraction. Total RNA was subjected to Northern blot using EGFP CDS as a probe. For protein extraction, cells were lysed in lysis buffer (50 mM Hepes, 1% Triton X-100, 150 mM NaCl, 1 mM EGTA, 1.5 mM $MgCl_2$, 2 mM DTT, and 0.5 mM PMSF, pH 7.6) and lysates were centrifuged at 10,000*g* for 10 min at 4 °C. Protein concentrations were determined with the bicinchoninic acid assay (Pierce).

Western blot analysis. Total proteins (30–50 μg/lane) from transfected cells were separated on 10% SDS–PAGE gel, transferred to PVDF membrane (Amersham Biosciences), blocked for 1 h, and immunoblotted with EGFP antibody (1:2000, the antibody was raised against full-length EGFP in mice) for 2 h at room temperature. Immuno-reactive bands were detected with peroxidase-coupled secondary antibody and visualized with a chemiluminescent substrate for peroxidase (SuperSignal West Pico substrate, Pierce).

Results

Bzw1 is highly conserved during evolution

The human *Bzw1* (KIAA0005) cDNA was initially identified by the Kazusa DNA Research Institute by conceptually assembling two separate cDNA fragments [14] and mouse KIAA0005-homologous cDNA was identified by screening of terminal sequences of cDNA clones randomly sampled from size-fractionated libraries [15]. We cloned mouse *Bzw1* cDNA from kidney and the ORF is deduced to encode a protein of 419 amino acids. Protein sequence alignment across species revealed that BZW1 is phylogenetically conserved and is identical in mouse, rat, and human (Fig. 1).

Bzw1 transcripts are expressed in different forms with tissue specificity

Human EST database showed that 2.9 kb *hBzw1* (BZAP45) mRNA was present in a broad spectrum of cell types and tissues [1]. By performing Northern blot assay, we examined the expression pattern of *Bzw1* in mouse. Our results revealed that *Bzw1* was expressed as RNAs of three different sizes. The larger two transcripts (~2.9 kb) were detected in both somatic tissues and testis, while the short transcript (~1.8 kb) was specifically expressed in testis and was more abundant than the longer transcripts (Fig. 2A). The same expression pattern was also obtained in adult rat tissues by Northern blot (data not shown).

To characterize the 1.8 kb transcript, five probes corresponding to exon 1–4 (Bzw1-a), exon 5–8 (Bzw1-b), exon 9–12 (Bzw1-c), and 3′ UTR (Bzw1-d, Bzw1-e) were used to hybridize total RNAs from mouse testis and ovary. As shown in Fig. 2B, probes Bzw1-d and Bzw1-e locating in 3′ UTR failed to detect the 1.8 kb transcript in testis. The result suggests that the 2.9 kb and 1.8 kb transcripts share the same coding region, but differ in the length of 3′ UTR.

Alternative polyadenylation sites in 3′ UTR result in the size difference of mouse Bzw1 transcripts

Previously, it was reported that alternative polyadenylation is widely employed in both human and mouse to produce alternative gene transcripts [16]. To test whether the short transcript might be an alternative splicing form resulting from alternative polyadenylation in 3′ UTR, we performed 5′- and 3′-RACE. Three bands of PCR products were obtained by 3′-RACE, while only one band by 5′-RACE (Fig. 2C). Sequencing data revealed that there are at least two transcripts of *Bzw1* in testis containing the same coding sequence but distinct 3′ UTRs. The result is consistent with that of Northern blot in testis. Through sequence analysis of 3′ UTR and comparison with genomic DNA sequence (GenBank No. CAAA01210503), we identified three polyadenylation sites within the 3′-terminal exon. The first one is AGTAAA (nt 1280–1285), only 19 bp downstream from the stop codon of coding

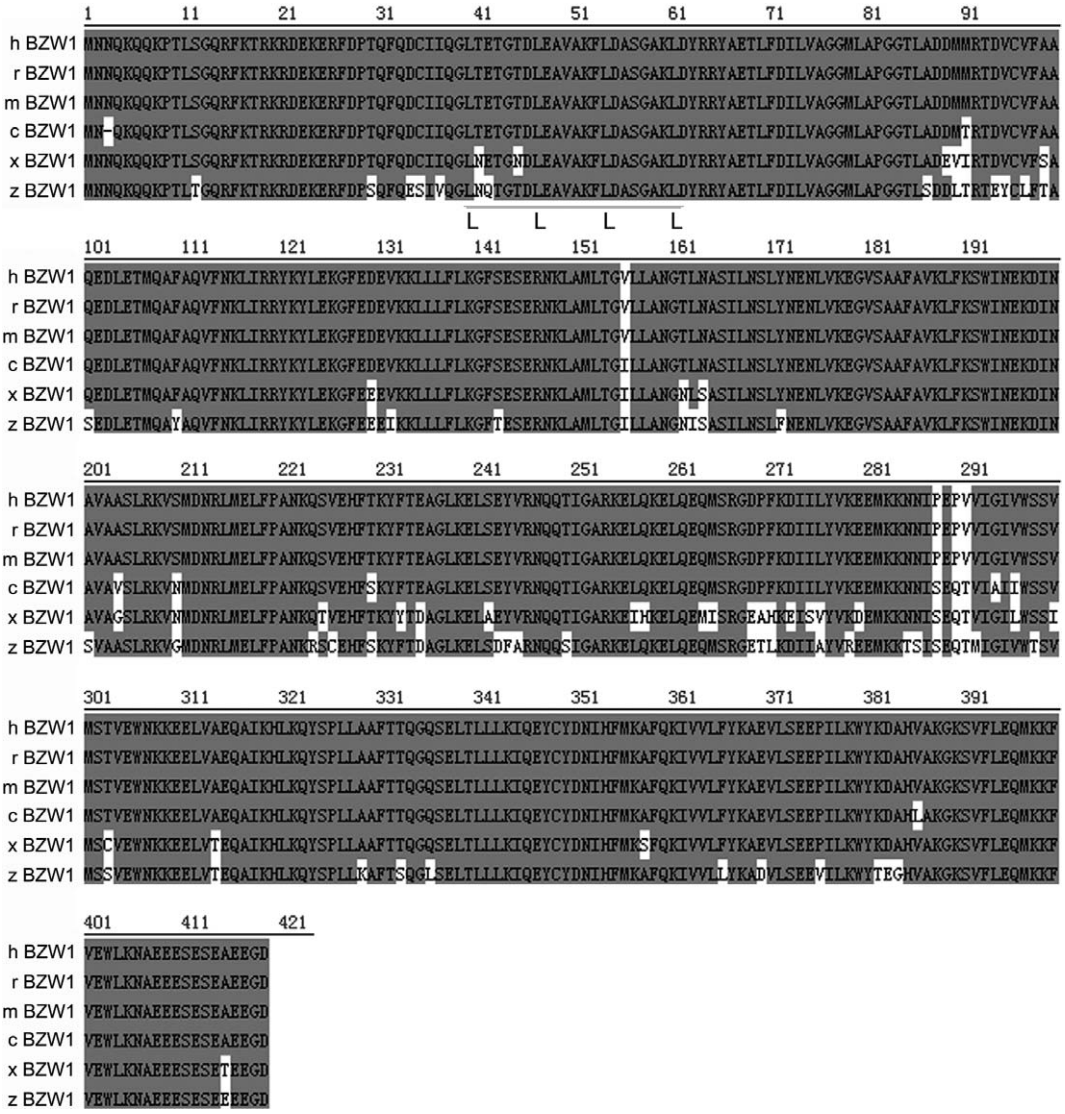

Fig. 1. Phylogenetical conservation of BZW1 revealed by amino acid sequence alignment across species (h: human, AAH26303, r: rat, NP_942084, m: mouse, NP_080100, c: chick, NP_001006516, x: xenopus, AAH41729, z: zebrafish, NP_956002). The leucine-rich repeats of the type L-X(6)-L-X(6)-L-X(6)-L (X = any residue) that define a leucine zipper motif are underlined.

sequence, the second one is a canonical polyadenylation signal AATAAA (nt 2725–2730) at 1445 bp downstream from the first signal (Fig. 2D). They give rise to the 1.8 and 2.9 kb transcripts in testis, respectively. In addition, we found the third polyadenylation signal of ATTAAA (nt 1659–1664) between these two sites. Although we have not detected its corresponding transcript in mouse tissues by northern blot, we actually found a weak band of the expected size in 3′-RACE result (Fig. 2C). Interestingly, all three tandem alternative polyadenylation sites are also conserved in 3′ UTRs of the human and rat *Bzw1*.

Different Bzw1 transcripts resulted from alternative 3′ UTR polyadenylation signals have differential translation efficiencies

As the 3′ UTR often contains regulatory elements that control the spatial and temporal expression of a transcript and determine its translational efficiency or stability [17–21], the choice of alternative polyadenylation sites may affect the expression pattern and level of the gene. To study the effects of different *Bzw1* 3′ UTRs on the translation efficiency, we made multiple constructs containing EGFP-Bzw1 chimeric coding sequence linked with different *Bzw1* 3′ UTRs corresponding to the alternative polyadenylation sites or linked with SV40 poly(A) as a control (Fig. 3A). In the constructs of EB-UTRa and EB-UTRb, the first polyadenylation site was deleted to prevent it from interfering with other polyadenylation sites. These constructs were transiently transfected into HEK-293 cells and a blank pEGFP-C3 vector was transfected simultaneously as internal control for transfection efficiency. Northern blot was performed after transfection. As shown in Fig. 3B, all of the three alternative polyadenylation sites are effective in transcription and produce corresponding mRNA products. However, level of the transcript with

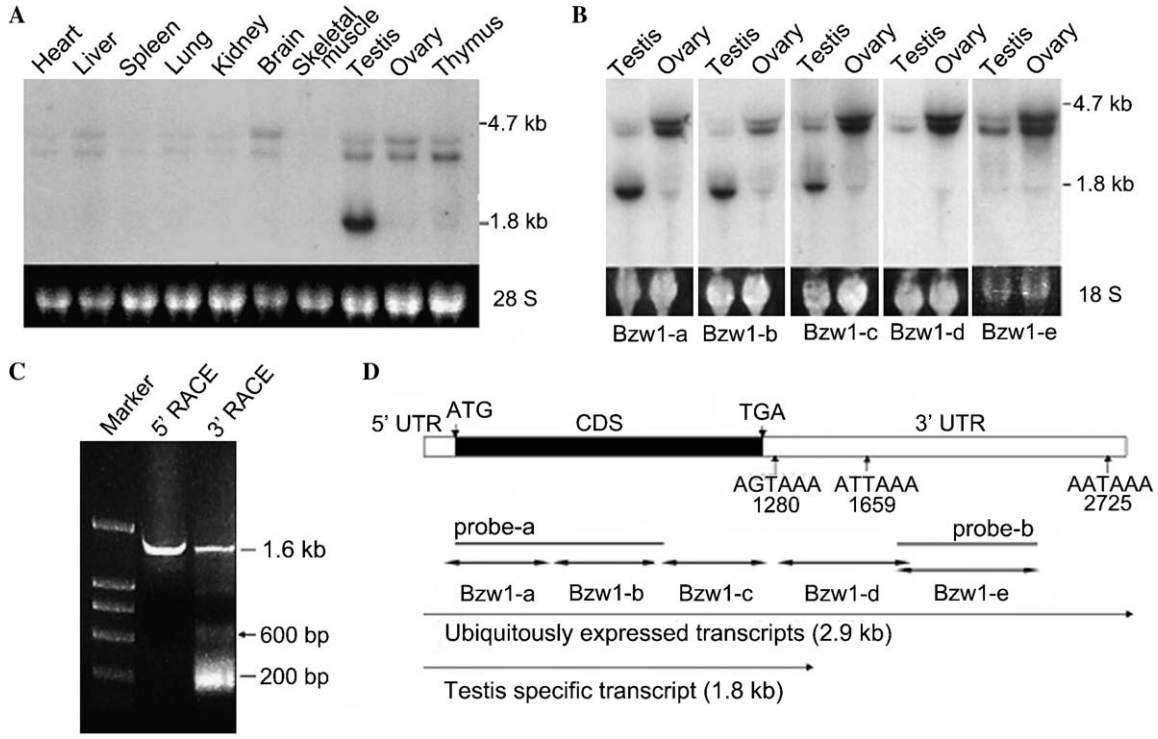

Fig. 2. Characterization of *Bzw1* transcripts. (A) Total RNAs from adult mouse tissues were hybridized with probe-a. A short transcript of 1.8 kb was specifically expressed in testis. (B) Total RNA samples from testis and ovary were hybridized with different probes as indicated. (C) 5′- and 3′-RACE results as indicated. The arrow indicates a weak band which is generated by the second polyadenylation site. (D) mRNA configuration of *Bzw1*. (The ATG is numbered as nucleotides 1–3.)

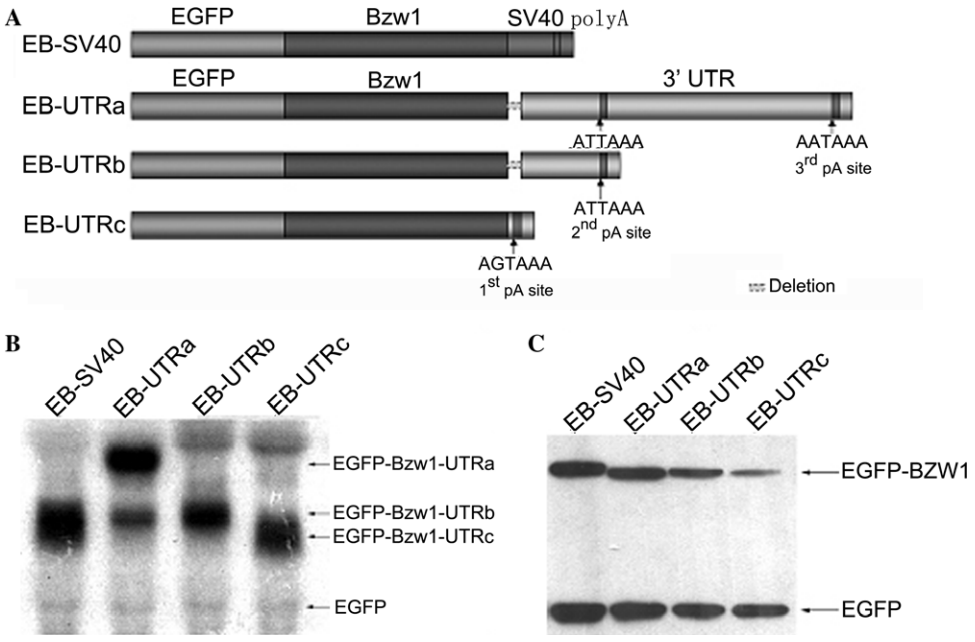

Fig. 3. Effect of different length of 3′ UTRs on transcription and translation. (A) Structures of constructions for transfection. EB-SV40 is linked with SV40 poly(A) as a control. In EB-UTRa and EB-UTRb, the 1st polyadenylation site was deleted. EB-UTRc only contained the 1st polyadenylation site. (B) Northern blot analysis of transfected HEK-293 cells using EGFP cDNA as probe. EGFP mRNA was used as an internal control. (C) Western blot analysis of transfected HEK-293 cells using EGFP antibody. EGFP was used as an internal control.

the second polyadenylation site was much less than that with the third polyadenylation site in EB-UTRa transfected cells (Fig. 3B). This was consistent with the result that the transcript with the second polyadenylation site was not detected in tissues by Northern blot but showed weak signal in 3′-RACE. One possibility is that the second polyad-

enylation site might be less effective or its corresponding transcript is less stable in normal physiological conditions.

The effect of 3′ UTRs on BZW1 translation was examined by Western blot using EGFP antibody. As shown in Fig. 3C, a single band of EGFP-BZW1 fusion protein was detected which is consistent with that all the mRNAs share the same coding sequence. Most interestingly, we observed that EB-UTRc transfection, which produces the shortest transcript by using the first polyadenylation site, has the lowest translation efficiency (Fig. 3C).

Short transcript of Bzw1 is expressed in Spermatogonia

To examine the cell type-specific expression of the short transcript of *Bzw1* in adult testis, we performed in situ hybridization with probes corresponding to *Bzw1* coding sequence (probe-a) and 3′ UTR downstream of first polyadenylation site (probe-b), respectively. The probe-b is used to detect the long transcript (Fig. 2D). As shown in Fig. 4, strong signals were detected with probe-a in the peripheral region of seminiferous tubules where Spermatogonia rest. However, few signals were detected with probe-b. This result is consistent with the observation that the short 1.8 kb *Bzw1* mRNA is highly expressed in testis through Northern blot analysis. To further rule out the possible expression of *Bzw1* in the sertoli cells, we also performed Northern blot with total RNA samples extracted from purified mouse sertoli cells. No signal was detected by probe-a (data not shown).

Discussion

In this study, we examined the expression pattern of *Bzw1* in mouse tissues. Northern blot analysis revealed a testis-specific expression of a 1.8 kb transcript, in addition to long transcripts (~2.9 kb) which are reported to be ubiquitously expressed in a large subset of tissues [1]. Furthermore, results from 5′- and 3′-RACE showed that the transcripts contain identical 5′ UTR and coding region. However, they differ from one another in the length of their 3′ UTRs due to the utilization of alternative polyadenylation sites.

Alternative polyadenylation is widely employed in both human and mouse for gene expression regulation. Tian et al. estimated that ~54% human genes and ~32% mouse genes have multiple alternative polyadenylation sites in 3′ UTR. A large number of human and mouse orthologs have conserved polyadenylation configuration [16]. Moreover, differential polyadenylation is often associated with specific tissue or cell type and development stage, with testis being a hotspot for differential polyadenylation site use [22–24]. In this study, we found three tandem alternative polyadenylation sites in the 3′-terminal exon of mouse *Bzw1* gene. A testis-specific 1.8 kb transcript of *Bzw1* was characterized to be generated by the first polyadenylation site. We believe that the polyadenylation configuration of *Bzw1* is applicable to human and rat since three tandem alternative polyadenylation sites also exist in rat and human *Bzw1* genes. Indeed, we observed the exclusive expression of a short transcript of *Bzw1* in rat testis. It is noticeable that many

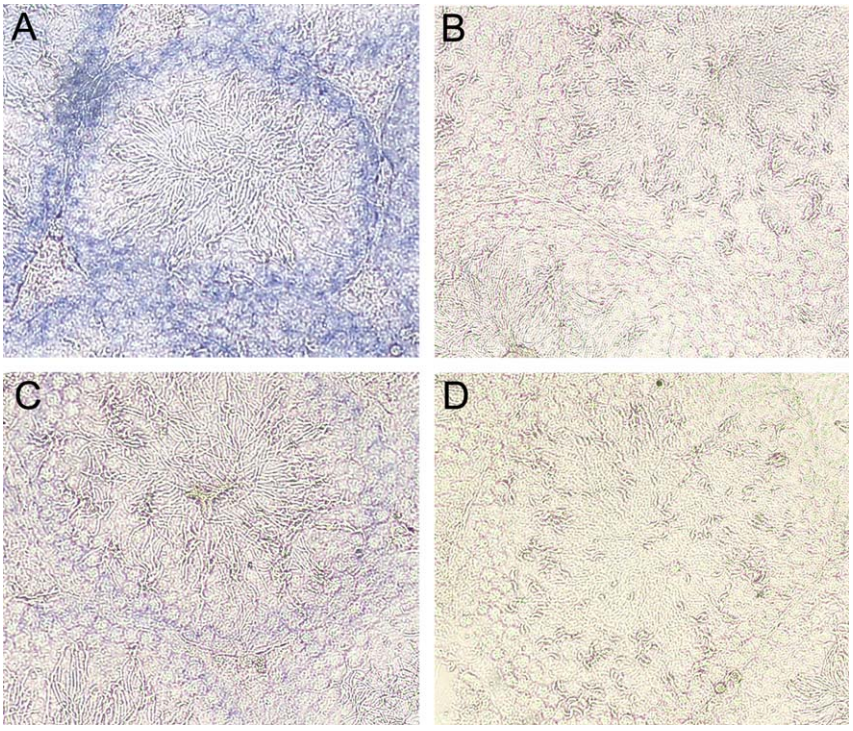

Fig. 4. In situ hybridization of *Bzw1* in seminiferous tubules. Frozen testis sections were hybridized with digoxigenin-UTP-labeled antisense RNA probe-a (A), sense probe-a (B), antisense RNA probe-b (C), and sense probe-b (D).

other genes, including eukaryotic initiation factors for protein synthesis, such as eIF-5, eIF-2α, and eIF-4E which contain a similar GTP/ATP binding fold to BZW1, also generate multiple alternatively spliced mRNAs encoding the same protein but with differential polyadenylated 3′ UTRs. And testis-specific mRNA forms of these genes have been reported [5,25,26].

Interestingly, the transcript with the shortest 3′ UTR showed less translation efficiency than the other transcripts with longer 3′ UTRs in HEK-293 cells. It suggests that both the transcription and post-transcription activities of *Bzw1* are regulated differentially in somatic tissues and testis. Previous study proposed that the length but not the primary sequence of 3′ UTR was important for the effect of 3′ UTR [21]. However, in this study we could not rule out the possibility that the sequence of 3′ UTR of *Bzw1* also contributes to its function. By examining the primary sequence, two 8-mers conserved 3′ UTR motifs (TGTAGATA and TTTGATAA) were found in all mouse, rat, and human *Bzw1* 3′ UTRs. These two motifs are potential binding sites for conserved miRNA [27]. All these evidences raise the possibility that *Bzw1* expression is fine-tuned through 3′ UTR.

Our study localized the 1.8 kb transcript specifically in spermatogonia of testis by in situ hybridization. Spermatogenesis starts with spermatogonia proliferation by mitosis and continues with meiosis of spermatocytes to form round spermatids [28,29]. In mouse, spermatogonia are classified into type A (A0, A1–A4), intermediate, and type B spermatogonia subtypes according to the differentiation status. Although the A0 (As) spermatogonia are generally considered to be the stem cells, regulation of their proliferation and differentiation is poorly understood [30]. As we know, histone genes expression is stringently controlled during cell division to couple with DNA synthesis, mediating the folding of DNA into chromatin through S phase [31–33]. Together with the previous report that BZW1 stimulates cell cycle regulated core histone H4 gene transcription at the G1/S phase transition [1], we postulate that BZW1 might act as a regulatory factor during spermatogonia proliferation.

Acknowledgments

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