Differential Translation of Dazap1 Transcripts during Spermatogenesis

Chi-Kai Yang1,2, Pauline Yen1,2,*

1 Graduate Institute of Life Sciences, National Defense Medical Center, Taipei, Taiwan, 2 Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan

Abstract

Deleted in AZoospermia Associated Protein 1 (DAZAP1) is a ubiquitous hnRNP protein that has been implicated in RNA transcription, splicing, and translation. It is highly expressed in testes, predominantly in late stage spermatocytes and post-meiotic spermatids. Dazap1 deficiency in mice results in growth retardation and spermatogenic arrest. The gene produces two major transcripts of 2.4 and 1.8 kb, designated Dazap1-L and Dazap1-S, respectively. Results of our previous RNA in situ hybridization and immunostaining suggested translational regulation of the Dazap1 transcripts during spermatogenesis. The main objectives of the study were to determine the origin of the two Dazap1 transcripts and to investigate whether they were similarly translated. Our Northern and 3' RACE analyses showed that the two transcripts were generated through alternative polyadenylation. In mouse testes, the levels of both transcripts were low at postnatal day 12 (P12), increased significantly at P18, and reached maximum at P27. Sucrose gradient analyses showed that at P12 both transcripts were actively translated. Afterward, an increasing portion of Dazap1-S became associated with the translationally inactive mRNPs, and the translational repression was accompanied by an increase in the length of its poly(A) tail. A much smaller portion of Dazap1-L was also sequestered to mRNPs as testes matured, but there was no changes in its poly(A) tail length. Using RNA pull-down followed by mass spectrometry, we identified DAZL, a germ-cell specific translation regulator, as one of the proteins that bound to the 3' UTR region specific for Dazap1-L. We further showed that DAZL preferentially bound to Dazap1-L in testis lysates and stimulated the translation of a reporter gene carrying Dazap1-L 3' UTR. In summary, our study shows that the translation of the two Dazap1 transcripts is differentially regulated. It also provides a new example of translational repression associated with poly(A) tail elongation during spermatogenesis.

Introduction

Most eukaryotic mRNAs are translated soon after they are processed and exported to the cytoplasm. However, some mRNAs are stored in the cytoplasm as inert messenger ribonucleoprotein particles (mRNPs) for a substantial period of time before they are translated. Such translation repression or mRNA masking is most frequently observed during gametogenesis and early embryogenesis [1]. A classic example of translational regulation during spermatogenesis is the Pm1 gene (encoding protamine 1) which is transcribed in round spermatids and translated several days later in elongated spermatids [2]. Storage of Pm1 mRNA for later translation is a necessary step for male germ cell development because protamine is needed at a stage when the nuclear transcription has been shut down. Premature translation of Pm1 mRNA in round spermatids results in precocious condensation of nuclear DNA and dominant male sterility [3]. Translational repression of Pm1 mRNA is mediated through its 3' UTR, and several proteins that bind to Pm1 3' UTR have been identified although how they regulate the translation remains unclear [4,5,6,7]. Additional patterns of gene repression are also observed in spermatogenic cells [8]. Some mRNAs appear to be partially repressed at all time, while others are almost totally inactivated in free mRNPs and generate little proteins. Recent profiling of testicular mRNAs in polyribosomes (polysomes) and mRNP fractions of prepuberal and adult mice identified over seven hundreds of transcripts that are differentially translated during testis development [9].

Deleted in AZoospermia (DAZ) proteins are a family of RNA-binding proteins that regulate mRNA translation in germ cells [10]. They include DAZ, a spermatogenic factor encoded by the AZFc region of the Y chromosome that is frequently deleted in azoospermic men [11], and the autosomal encoded DAZL and BOULE [12,13,14]. DAZ proteins are expressed predominantly, if not exclusively, in germ cells and their deficiencies impair reproduction [15,16]. A role of DAZ proteins in mRNA translation is supported by their presence on polysomes [17,18] and their effects on translation in cultured cells [18], intact oocytes [19,20] and zebrafish embryos [21]. A recent study using tethered function assay in Xenopus oocytes suggests that DAZL facilitates translation initiation by binding to the 3' UTR of its target mRNAs to recruit poly(A) binding proteins (PABPs) and stimulate the formation of 80 S ribosomes [19]. DAZL recognizes GUU triplet in different sequence context [18,22,23], and several putative natural substrates of DAZL have been identified though some of them need further verification in vivo [20,24,25,26].

DAZ Associated Protein 1 (DAZAP1) is a DAZ interacting protein that is expressed ubiquitously but most abundantly in the...
testis [27,28]. It is a component of the hnRNP particles [29] and has been implicated in several cellular processes including RNA transcription [30,31], splicing [32,33,34], and translation [35]. Very recently, DAZAP1 was suggested as a potential disease gene for amyotrophic lateral sclerosis [36]. Most mice deficient in DAZAP1 die perinatally, and the few survivors manifest growth retardation and spermatogenic arrest [37]. In mouse testes, DAZAP1 first appears abundantly in the nuclei of mid-pachytene spermatocytes, whereas our previous RNA in situ hybridization detected high levels of Dazap1 transcripts in spermatogonia and early spermatocytes [38]. We thus set out to investigate possible delayed translation of Dazap1 transcripts during spermatogenesis. Our results showed that two Dazap1 transcripts were generated through alternative polyadenylation and their differential translation during spermatogenesis involved poly(A) tail length alteration and DAZL binding.

Results

Two Dazap1 transcripts are generated through alternative polyadenylation

Mouse testes express two major Dazap1 transcripts of 2.4 and 1.8 kb, designated Dazap1-L and Dazap1-S, respectively [28]. We failed to detect alternative splicing and promoter usage by RT-PCR and 5’ Rapid Amplification of cDNA Ends (RACE), respectively, suggesting that the two Dazap1 transcripts were generated through alternative polyadenylation (data not shown). The 3’ UTR of Dazap1 contains three canonical AATAAA polyadenylation signals (PASs) (Fig. 1A), but only the second and the third ones are evolutionarily conserved throughout vertebrates (data not shown). To identify the polyadenylation sites of Dazap1 transcripts, we performed 3’ RACE of adult mouse testis RNA and detected two major fragments around 0.8 and 0.3 kb (Fig. 1B). Sequencing of the fragments revealed that polyadenylation of the 0.3 kb fragment initiated from two CA sites downstream of the second PAS, whereas that of the 0.8 kb product started downstream of the third PAS (Fig. 1A). The minor 0.5 kb fragment represented a PCR artifact amplified from the 0.8 kb fragment since it was also produced by PCR amplification of a clone containing the 0.8 kb fragment. Thus the two Dazap1 transcripts were produced through alternative polyadenylation utilizing the second and the third PASs. This was further confirmed by Northern blot (Fig. 1C, D). Probe-A which covers a large portion of the Dazap1 cDNA detected both transcripts, whereas probe-B which contains a segment downstream of the second PAS hybridized only to Dazap1-L.

The Dazap1 transcripts are differentially expressed during spermatogenesis

We next studied the levels of the two Dazap1 transcripts in prepuberal and adult mouse testes. Spermatogenesis is initiated soon after birth, and day 8 mouse testes contain only type A and type B spermatogonia [39]. Spermatocytes start to appear afterward. In postnatal day 12 (P12) testes, germ cells make up 60% of the total cell population and the most advanced germ cell is zygotene spermatocytes. At P18, pachytene spermatocytes dominate the germ cell population, and haploid spermatids start to appear after day 20. P27 testes are enriched in late round spermatids [40], and P34 and adult testes contain spermatids as the major cell types. Northern analyses of mouse testes at different ages showed that the levels of Dazap1 transcripts were low at P12, increased significantly at P18, and reached the highest levels at P27 (Fig. 2A, B), consistent with Dazap1 expression mainly in late pachytene spermatocytes and spermatids. The ratio of the two transcripts also changed as spermatogenesis progresses (Fig. 2B). The two transcripts were present in similar levels at P12 and P18, but Dazap1-S became the dominant form at P27 and after.

The Dazap1 transcripts are differentially translated in the testis

To investigate whether both Dazap1 transcripts are equally translated during spermatogenesis, we fractionated mouse testis lysates on 10–50% sucrose gradients and monitored the distribution of Dazap1 transcripts by 3’RACE as well as Northern blot when Dazap1 transcripts were sufficiently abundant for detection (Fig. 3). Both methods showed that in adult testes Dazap1-L had a bimodal distribution, with a large fraction associated with the polysomes, whereas a vast majority of Dazap1-S was present near the top of the gradient in the mRNPs fraction (Figs. 3A, G). As a control, we also determined the distribution of the Pml transcripts and found it in both mRNP and polysomal fractions as expected [9]. Upon EDTA treatment, the polysomes dissociated and all Dazap1 and Pml transcripts were found near the top of the gradient (Fig. 3B). The polysomal profiles of Dazap1 mRNAs in P27, P24, and P18 testes were similar to that of the adult testes, with a large fraction of Dazap1-S near the top of the gradients and most Dazap1-L associated with polysomes (Figs. 3C, D, E, G). On the other hand, the Pml transcripts in P27 and P24 testes were present only in the mRNP fractions because the most advanced spermatogenic cells in these testes are round spermatids. At P18 and P12, Pml transcripts were undetectable. There was a major change in the distribution of Dazap1 transcripts in P12 testes, with both transcripts mainly associated with polysomes being actively translated (Figs. 3F, G). Since the major difference in germ cell constitution between P12 and P18 testes is the appearance of pachytene spermatocytes in the latter, our results suggest that after the meiotic germ cells reach the pachytene stage, a large fraction of Dazap1-S and a much smaller fraction of Dazap1-L start to be sequestered into mRNPs and become translationally inactive.

Translational repression of Dazap1-S has longer poly(A) tails.

It has been reported that mouse Pml, Pm2, Tnp1, and Tnp2 transcripts being actively translated on polysomes have shorter poly(A) tails than those that are translationally repressed [41]. To investigate whether translational regulation of Dazap1 transcripts also involved changes in poly(A) tail length, we used a recently developed extension Polya(A) Test (ePAT) method [42] to monitor poly(A) tail lengths of Dazap1 transcripts during mouse testis development and in sucrose-gradient fractions. Our initial test of ePAT on Pml transcripts confirmed previous Northern hybridization results, i.e., translationally active polysomal Pml transcripts had much shorter poly(A) tails than the major Pml transcript present in the free mRNP fraction (Fig. 4A and Fig. S1). It is noticed that a small fraction of Pml transcripts in the mRNP contained short poly(A) tails. Based on the size of the TVN-PAT product that had an invariant poly(A) tail of 12 As as well as the sequences of five or more randomly selected ePAT clones (Table S1), the lengths of the poly(A) tails in the short and long Pml transcripts were determined to be approximately 25 and 120 bases, respectively, compared with previous estimations of 30 and 150 bases [41]. We also found that when Pml transcripts were first detected in P27 testes, the vast majority of them had long poly(A) tails (designated as LATS). As the tests matured to contain later stages of germ cells with active Pml translation, the fraction of Pml transcripts with short poly(A) tails (designated as SATs)
increased progressively, consistent with translation activation of Prm1 in elongated spermatids.

We next used a forward primer common to both Dazap1 transcripts in the e-PAT reactions (Fig. 4B and Fig. S1). Dazap1-S also produced two major ePAT products with poly(A) tails approximately 30 and 170 bases long, respectively (Table S1). Before P27 most Dazap1-S had SATs. However, the level of Dazap1-S with LATs increased progressively as the mice grew older, and reached a level comparable to that of Dazap1-S with SATs at P27. Similar to the Prm1 transcripts, Dazap1-S with both SATs and LATs were present in the translationally inactive mRNP fraction whereas only Dazap1-S with SATs was present in the polysomal fraction. In contrast, the poly(A) tail length of Dazap1-L remained unchanged during testis development. The tail was determined to be approximately 30 bases long using a different Dazap1-L specific forward primer that produced shorter ePAT fragments (Fig. 4C and Table S1). Dazap1-L, in both mRNP and polysomal fractions had the same SATs. Additional ePAT reactions showed that the germ-cell specific Sycp3 transcript also had constant SATs of approximately 30 bases (Fig. 4D and Table S1). On the other hand, the house-keeping Gapdh transcript produced a minor band representing LATs in the mRNP fraction, suggesting possible translation repression on a small fraction associated with poly(A) tail elongation (Fig. 4E). Our results therefore showed a correlation between poly(A) tail length and translational status for a subset of transcripts in the testis, with LATs of 120–170 bases associated with translation repression.

Several RNA-binding proteins bind to Dazap1 3’UTR

3’UTRs are known to contain cis-acting elements for the binding of translational regulators. We therefore searched for testicular proteins that bound to Dazap1 3’UTR. We separated Dazap1 3’UTR into three regions (Fig. 5A). The short (S) region (nt1378–1561) consists of the short 3’ UTR present in Dazap1-S, the conserved (C) (nt1568–1793) and the non-conserved (NC) (nt1788–2072) regions include the 5’ and 3’ segments, respectively, of the distal portion of Dazap1 3’UTR that is present only in Dazap1-L. RNA fragments corresponding to these regions were
incubated with testis lysates prepared from mice at various ages, and the RNA-protein complexes were UV cross-linked and analyzed by SDS-PAGE (Fig. 5B). Probe S and probe C, but not probe NC, detected multiple proteins, and some of them showed increased binding during testis development.

We further separated the C region into the CU region (nt1568–1646) that contains a noticeable CU-rich sequence and the AU region (nt1646–1793) that contains several AU-rich elements (AREs), and dissected the CU region again into C_40 (not CU-rich, nt1568–1610) and CU_40 (nt1607–1646) subregions (Figs. 1A, 5A). UV cross-linking assays showed that most proteins represent the averages of three independent experiments. Statistical significance of the differences was determined using one-way ANOVA. * denotes p<0.05, ** denotes p<0.01, *** denotes p<0.001. The relative levels (%) between 1.8 and 2.4 kb transcripts are given inside the bars. doi:10.1371/journal.pone.0060873.g002

DAZL preferentially binds to Dazap1-L

DAZL has been reported to regulate the translation of several mRNAs in germ cells [10], and DAZL consensus binding sequences are present in different regions of Dazap1 3’UTR. We therefore performed RNA gel-shift assays using His-tagged recombinant DAZL to confirm the binding of DAZL to Dazap1 3’UTR (Fig. 6). We first mapped the DAZL binding site to the CU region that is present only in Dazap1-L, and used smaller probes to narrow down the binding site to the CU_40 region which contains three partially overlapping copies of the DAZL consensus sequence (Fig. 6A). The binding between labeled CU_40 and DAZL could be competed out by increasing levels of cold CU_40 but not cold C_40, confirming the specificity of the binding (Fig. 6B).

We next used RNA immunoprecipitation to investigate whether DAZL preferentially binds to Dazap1-L. An anti-DAZL antibody, but not the preimmune serum, specifically precipitated DAZL from adult testis lysates (Fig. 6C, top panel). DAZL-induced translation enhancement of reporter linked to the 3’UTR of Dazap1-L was compared to those of the reporters linked to the 3’UTR of Mvh, Tex19.1, and Gapdh transcripts of which the translation have been previously shown to be regulated by DAZL [20,21,25,26]. Prm1 and Gapdh transcripts which are not known to bind DAZL served as negative controls.

DAZL enhances the translation of a reporter with Dazap1-L 3’UTR

We used luciferase reporter assays to indirectly study the effect of DAZL on the translation of Dazap1 transcripts. We transfected 3T3 cells with a DAZL expression vector or the empty expression vector together with the empty luciferase (Luc) reporter vector or a vector with Luc linked to the 3’UTRs of various genes. Western and RT-PCR analyses showed no significant differences in the levels of DAZL (Fig. S2) and Luc mRNA (Fig. 7) in the cells transfected with the DAZL expression vector and different Luc reporters. However, cells transfected with the reporter linked to the 3’UTR of Dazap1-L exhibited significantly higher luciferase activities than those transfected with the empty vector or the reporter linked to the 3’UTR of Dazap1-S (Fig. 7). Deletion of the entire CU-rich sequence from the Dazap1-L 3’UTR (Dazap1-L-dCU) abolished the translation enhancement. The degree of DAZL-induced translation enhancement of Dazap1-L was comparable to those of the reporters linked to the 3’UTRs of Mvh, Tex19.1, and Tex19.1, which were much lower than what had previously been observed in other systems. DAZL was reported to increase approximately four-folds the translation of Mvh and Tex19.1 luciferase reporters in Xenopus oocytes [25,26] and about 10 folds that of a Tex19.1 reporter in mouse oocytes [20]. Differences in the expression levels of DAZL and other trans-acting factors in the various systems may account for the disparity in the observed translational enhancement.

Discussion

Alternative polyadenylation changes the content of the 3’UTR and offers a means to regulate gene expression at the post-transcriptional stage because most regulatory elements controlling the localization, stability and translation of a transcript are located
in the 3'UTR [43]. Here we show that two Dazap1 transcripts were generated through alternative polyadenylation, and that they were differentially expressed and translated during spermatogenesis. In P12 testes that contain spermatogonia and spermatocytes up to the zygotene stage, the transcripts were present at low levels and were both actively translated. However, with increased Dazap1 expression at P18, there was a major shift in the polysomal profile of Dazap1-S, with an increasing portion of the transcript sequestered to translationally inactive mRNPs, whereas a large portion of Dazap1-L remained on polysomes. In P27 testes that contain mainly postmeiotic spermatids, the level of Dazap1-S increased significantly, consistent with previous EST analyses showing that round spermatids tend to have shorter truncated 3'UTRs [44]. Since most of the increased Dazap1-S was present in mRNPs, no increase in protein synthesis was expected and our previous immunostaining of mouse testicular sections also failed to detect significant increase in DAZAP1 signals in spermatids [38]. The situation is similar to that of the Rnf4 transcripts encoding SNURF/RNF4 transcription modulator [45]. Rnf4 has two transcripts of 3.0 and 1.6 kb that differ in their 3'UTRs. The 1.6 kb transcript is expressed abundantly in round spermatids but most of them are not associated with polysomes. It is unclear why germ cells spend their energy and resources to make mRNAs that are not used for protein production.

The Northern analyses on Dazap1 expression in prepuberal testes, presented here, suggest that the expression of Dazap1 expression in spermatogonia and early spermatocytes is much lower than that in pachytene spermatocytes. This contradicts the
results of our previous RNA in situ hybridization on adult mouse testicular sections which showed highest levels of Dazap1 mRNA in spermatogonia and early preleptotene spermatocytes [38]. We have since repeated the in situ experiments and were able to reproduce the same hybridization pattern with the same 5' probe, but not with a probe from a different region of the Dazap1 cDNA. We also purified different stage germ cells from adult mouse testes and found the highest levels of Dazap1 transcripts in spermatocytes (data not shown). In addition, previous microarray analyses on purified spermatogenic cells also showed almost three times as much Dazap1 transcripts in pachytene spermatocytes as in type A and type B spermatogonia [46]. Therefore our previous RNA in situ hybridization result likely represents an experimental artifact and there is no delayed translation of Dazap1 transcripts as we previously suggested.

We investigated why Dazap1-S was translated on polysomes in early germ cells and sequenced to mRNPs in later germ cells, and found that translational repression of Dazap1-S was associated with an increase in its poly(A) tail length. Translational regulation mediated through poly(A) tail is well documented for some maternal mRNAs during oocyte maturation and early embryogenesis [47]. In the oocytes, masked mRNAs usually have short poly(A) tails fewer than 20 bases, which elongate to about 80 to 150 bases before the mRNAs are recruited to the polysomes for translation. It is thought that longer poly(A) tails facilitate the binding of poly(A) binding protein (PABP) and subsequent recruitment of translational initiation factors. Some translating mRNAs with long poly(A) tails, on the other hand, undergo deadenylation during oocytes maturation, resulting in their translational repression. As first reported by Kleene [41], male germ cells apparently have a reverse pattern of poly(A) tail mediated translational regulation, with the translational activation of several post-meiotically expressed genes accompanied by poly(A) shortening instead of lengthening. The shortened poly(A) tails still contain about 30 As, sufficient for one PABP molecule which requires approximately 27 As for binding [48,49]. Here we confirmed poly(A) shortening of the Pm1 transcript during translational activation using a recently developed ePAT method which offers higher sensitivity and resolution than the previously used Northern blot [41]. The Pm1 short poly(A) tails gave a band that was quite discrete, suggesting that they may not be as heterogeneous as previously suggested. In addition, we found that a small fraction of the mRNP-bound Pm1 transcripts contained SATs. Previous Northern analyses on sucrose gradient mRNAs enriched for transcripts with SATs did detect the presence of trace amount of Pm1, Pm2, Tog1, and Tog2 transcripts with SATs in the mRNP fractions [50]. Our ePAT results are likely biased for transcripts with SATs since it is well known that in PCR reactions short fragments are usually amplified with higher efficiency. It remains unclear whether poly(A) shortening is required for translational activation or it is merely a consequence of translation [49,51]. Our observations that a small fraction of the mRNP-bound Pm1 transcript contained SATs whereas very little polysomal Pm1 transcript contained LATs would argue for the occurrence of poly(A) tail shortening at mRNPs instead of on polysomes as a consequence of translation. The poly(A) tail elongation of Dazap1-S accompanying its translational repression represents the other side of the coin. Similar to the Pm1 transcript, the mRNP-associated Dazap1-S had both LATs and SATs whereas the polysomal Dazap1-S contained only SATs. However for Dazap1-L, which remained largely translationally active during spermatogenesis, there was no obvious changes in its poly(A) tail length. The transcript contained only SATs regardless whether it was sequenced to mRNPs or was actively translating on polysomes. Our results therefore showed that translational repression of Dazap1-S, but not Dazap1-L, was accompanied by poly(A) tail elongation. Whether the poly(A) tail plays an active role in modulating the translational activity of Dazap1-S remains to be investigated. Previous studies on testicular expression of Ldh-x and Rpl32 failed to find an association between poly(A) tail length and translational activity [52,53]. However, the Northern blots in those studies were not of high resolution/quality and the results need to be confirmed with the new ePAT method.

We have identified several proteins, including KSRP, PTBP2, MSY2, MSY1, and DAZL, that bound to Dazap1 3'UTR. The only protein that we found to have any effects on Dazap1 expression when ectopically expressed in cultured cells was DAZL. DAZL is known to stimulate mRNA translation, and requires multiple DAZL binding sites for efficient translation activation [19]. DAZL consensus sequence U2–10[G/C]U2–10 is present in different regions of Dazap1 3'UTR. The S region contains a UUUUUUUU sequence, the CU_40 region contains three partially overlapping UUGUUU sequences, and the AU region contains a UUUGCUUU and a UUUGCUUU sequence. Our RNA gel-shift assays showed that DAZL bound much stronger to the CU_40 region than the S or the AU region. Expression of luciferase reporters in 3T3 cells also indicated that the CU_40

Figure 4. Developmental changes in the lengths of poly(A) tails of Dazap1 and Pm1 transcripts. Total testicular RNAs isolated from mice at the various ages and RNAs extracted from the free mRNP and polysomal fractions of sucrose gradients of adult testis lysates were subjected to ePAT analysis using forward primers specific for the various genes as indicated. In the TVN lanes, the major products contain with an increase in its poly(A) tail length. Translational regulation mediated through poly(A) tail is well documented for some maternal mRNAs during oocyte maturation and early embryogenesis [47]. In the oocytes, masked mRNAs usually have short poly(A) tails fewer than 20 bases, which elongate to about 80 to 150 bases before the mRNAs are recruited to the polysomes for translation. It is thought that longer poly(A) tails facilitate the binding of poly(A) binding protein (PABP) and subsequent recruitment of translational initiation factors. Some translating mRNAs with long poly(A) tails, on the other hand, undergo deadenylation during oocytes maturation, resulting in their translational repression. As first reported by Kleene [41], male germ cells apparently have a reverse pattern of poly(A) tail mediated translational regulation, with the translational activation of several post-meiotically expressed genes accompanied by poly(A) shortening instead of lengthening. The shortened poly(A) tails still contain about 30 As, sufficient for one PABP molecule which requires approximately 27 As for binding [48,49]. Here we confirmed poly(A) shortening of the Pm1 transcript during translational activation using a recently developed ePAT method which offers higher sensitivity and resolution than the previously used Northern blot [41]. The Pm1 short poly(A) tails gave a band that was quite discrete, suggesting that they may not be as heterogeneous as previously suggested. In addition, we found that a small fraction of the mRNP-bound Pm1 transcripts contained SATs. Previous Northern analyses on sucrose gradient mRNAs enriched for transcripts with SATs did detect the presence of trace amount of Pm1, Pm2, Tog1, and Tog2 transcripts with SATs in the mRNP fractions [50]. Our ePAT results are likely biased for transcripts with SATs since it is well known that in PCR reactions short fragments are usually amplified with higher efficiency. It remains unclear whether poly(A) shortening is required for translational activation or it is merely a consequence of translation [49,51]. Our observations that a small fraction of the mRNP-bound Pm1 transcript contained SATs whereas very little polysomal Pm1 transcript contained LATs would argue for the occurrence of poly(A) tail shortening at mRNPs instead of on polysomes as a consequence of translation. The poly(A) tail elongation of Dazap1-S accompanying its translational repression represents the other side of the coin. Similar to the Pm1 transcript, the mRNP-associated Dazap1-S had both LATs and SATs whereas the polysomal Dazap1-S contained only SATs. However for Dazap1-L, which remained largely translationally active during spermatogenesis, there was no obvious changes in its poly(A) tail length. The transcript contained only SATs regardless whether it was sequenced to mRNPs or was actively translating on polysomes. Our results therefore showed that translational repression of Dazap1-S, but not Dazap1-L, was accompanied by poly(A) tail elongation. Whether the poly(A) tail plays an active role in modulating the translational activity of Dazap1-S remains to be investigated. Previous studies on testicular expression of Ldh-x and Rpl32 failed to find an association between poly(A) tail length and translational activity [52,53]. However, the Northern blots in those studies were not of high resolution/quality and the results need to be confirmed with the new ePAT method.

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region was responsible for the DAZL induced translation enhancement of the Luc gene linked to \textit{Dazap1-L} 3' UTR. We also found that an anti-DAZL antibody preferentially precipitated \textit{Dazap1-L} from mouse testis lysates. There is a good correlation between the expression of DAZL and DAZAP1 during spermatogenesis. Previous immunostaining of adult mouse testicular sections detected dramatic increases in the expression of both DAZAP1 and DAZL in pachytene spermatocytes [38,54]. Our current analyses of \textit{Dazap1} expression during spermatogenesis showed that the level of \textit{Dazap1-L} also increased significantly in pachytene spermatocytes. Therefore, increased expression of both \textit{Dazap1-L} and DAZL in pachytene spermatocytes likely accounts for the robust translation of DAZAP1 in the cells.

Materials and Methods

Animals

C57BL/6 mice used in the study were housed in a specific pathogen-free animal facility. Adult mice were 2–4 months old. The experiments were approved by Academia Sinica's Institutional Animal Care & Utilization Committee.

3' Rapid Amplification of cDNA Ends (3' RACE)

3' RACE of \textit{Dazap1} transcripts was carried out according to Schaefer [55]. Testis RNA was reverse transcribed using the universal adapter primer PrRACE-dT: 5'−gctgtcaacgatacgctacgtaacgt24, and PCR amplified using primers PrDAP176: 5'−agtggcttcggacgcgggcagaaccacaac and Pr3' RACE: 5'−gctgtcaacgatacgctacgtaacgt. The PCR products were cloned into pCRII-TOPO (Invitrogen, Carlsbad, CA) and sequenced.

Northern hybridization

Northern hybridization was performed according to the standard protocol [56] using Sure Blot nylon membranes (Intergen, Purchase, NY) and a Stratalinker UV cross-linker 1800 (Stratagene, La Jolla, CA). Blots were hybridized with radioactive probes at 68°C in the ExpressHyb solution (Clontech, Palo Alto, CA) and analyzed with the Typhoon 9410 Imager (GE Healthcare, Piscataway, NJ). For quantification, the hybridization membranes were exposed to X-ray films and radioactive bands were located, excised, and counted.

Sucrose gradient analysis

Post-mitochondrial fractions of mouse testis lysates were fractionated on 10–50% sucrose gradients as previously described [57]. Briefly, mouse testes were homogenized with a motor-driven glass–Teflon homogenizer (Wheaton, Millville, NJ) on ice in 0.5 ml lysis buffer (100 mM NaCl, 3 mM MgCl2, 20 mM Hepes, pH 7.4 and 0.5% Triton X-100). The nuclei and mitochondria were pelleted by centrifugation for 5 min at 12,000 g. The supernatants were loaded onto linear gradients of 10%–50% sucrose (w/w) in the lysis buffer without Triton X-100 and centrifuged for 2 h at 36,000 rpm in a SW-41 rotor (Beckman, Palo Alto, CA). The gradients were pumped through a flow cell (Teledyne Isco, Lincoln, NE) to record the absorbance at 254 nm and fractionated into 0.5-ml fractions. Each fraction was extracted twice with phenolchloroform (1:1) and the RNA was ethanol
precipitated. Polysome profiles of the Dazap1 transcripts were analyzed by 3′RACE-PCR or Northern hybridization. Control gradients, containing 16 mM EDTA in the postmitochondrial supernatants, were centrifuged and analyzed in parallel with the polysome gradients.

UV cross-linking assays

Testis extracts were prepared from mice at indicated age according to Xu and Hecht [58]. 32P-labelled single stranded RNA probes were in vitro transcribed using Riboprobe systems (Promega) and purified on 1.5% agarose gels. UV light (Stratalinker 1800) for 11 min on ice, followed by RNase A (0.5 mg/ml) digestion for 15 min at 37°C. The RNA-protein complexes were resolved by 10% SDS-PAGE. The gels were dried and visualized with the Typhoon 9410 Imager.

RNA affinity pulldown assays

RNA affinity pulldown assay was done according to a published protocol [58] with minor modifications. Biotinylated RNA probes were in vitro transcribed using the Riboprobe system containing [pH 7.4], 2.5 mM MgCl2, 120 mM KCl, 1 mM DTT, 5% [w/v] glycerol, and 1× protease inhibitor cocktail for 30 min at room temperature and treated with 5 mg/ml heparin for 15 min. Streptavidin-agarose beads were then added to the mixture and incubated for 60 min. After five washes with BB, bound proteins were released by boiling in 4× SDS sample buffer, resolved by SDS-PAGE and visualized by SYPRO Ruby staining. The protein bands of interest were excised and analyzed by LC/MS/MS with a Thermo Scientific LTQ XL mass spectrometer (Thermo Scientific, Waltham, MA).

RNA immunoprecipitation assays

RNA immunoprecipitation assay was performed according to Xu and Hecht [58]. Two mg testes extracts were precleaned using 50 μl protein G beads (GE Healthcare) and 100 μg/ml yeast tRNA in 1 ml TKM buffer (20 mM Tris [pH 7.4], 120 mM KCl, 1.5 mM MgCl2, 0.1% NP-40) with 500 U/ml SUPERase-In (Ambion, Austin, TX) for 4 hours at 4°C. After spinning down the beads, the supernatants were incubated overnight at 4°C with 10 μg anti-DAZL-C antibody which was generated against an oligopeptide containing the last 21 amino acid residues of mouse DAZL and affinity purified using the services of Bethyl Laboratories, Inc (Montgomery, TX). The mixtures were then incubated...
with 20 μl protein G beads for 3 hours at 4°C. The beads were
spindown and washed five times by the TKM buffer. Immuno-
precipitated RNAs were extracted by Trizol (Invitrogen) and
analyzed by RT-PCR. Sequences of the PCR primers used in
RNA immunoprecipitation assays are given in Table S2.

RNA gel shift assays

Gel shift assays were carried out according to Venables et al
[22] with some modifications. The 10-μl binding reactions contained 1 μg recombinant DAZL, 20 mM Tris [pH 7.4],
2.5 mM MgCl₂, 60 mM KCl, 1 mM DTT, 5% [w/v] glycerol,
and 10⁴ cpm of 32P-labelled RNA probes. After incubation at
room temperature for 5 min, the reactions were treated with 5 mg/ml heparin and 0.5 mg/ml yeast tRNA for another 15 min.
The RNA-protein complexes were separated on 1.5% agarose gels
in 0.5 x TBE buffer and visualized with the Typhoon9410 Imager.

Plasmid construction

The entire 3'UTRs of Dazap1, Msh, Syp93 and Tex19.1 were
PCR amplified from mouse genomic DNA and cloned into
cPlcII-TOPO. pcDNA-Luc was generated by inserting the Luc
gene into pCDNA3.1 (Invitrogen). The 3'UTRs of these genes in pCRII-TOPO were then cloned into pcDNA-Luc to
generate the reporter vectors. Deletion of the CU-rich sequence in
Dazap1 3'UTR was generated by a PCR-mediated deletion approach
[59]. For in vitro transcription, different regions of
Dazap1 3'UTR were generated by PCR amplification and cloned
into pBluescript (Stratagene). Dazl coding region was RT-PCR amplified from mouse testis RNA and cloned into pCRII-TOPO to
sequence the reporter vector. It was subsequently cloned into p3xFLAG-CMV-14 (Sigma, Saint Louis, MO) to generate pDAZL-Flag, and into pET-28a (Novagen, Madison, WI) to generate pET-DAZL for the production of His-tagged DAZL protein in E. coli [22,60].

Analysis of the translation of luciferase reporters

3T3 cells were maintained in DMEM (HyClone, Fremont, CA)
supplemented with 10% fetal bovine serum and 1% penicillin/
streptomycin (GIBCO, Eggenstein, Germany). Transfection was
performed using the TurboFect reagent (Fermentas, Burlington,
Ontario, Canada). The pDAZL-Flag or the empty p3xFLAG-CMV-14 were co-transfected into 3T3 Cells with different firefly
luciferase reporter constructs and pRL-TK (Promega, San Luis
Obispo, CA) which encodes the Renilla luciferase. The cells were
harvested after 24 hours, and luciferase activities in the cell lysates
were measured using the dual-luciferase reporter assay system
(Promega) and normalized to Renilla luciferase activities. Luciferase
RNA levels were determined by semi-quantitative RT-PCR and
normalized to the levels of Renilla luciferase transcripts. Luciferase
activities and RNA levels in cells transfected with pDAZL-Flag
were normalized to those of cells transfected with p3xFLAG-
CMV-14 to reflect the effects of DAZL, and the results of cells
transfected with different Luc reporter genes were compared using
those of cells transfected with the empty Luc reporter as a reference
(100%). DAZL expression levels were determined by Western blot and
normalized to the levels of tubulin.

Poly(A) tail length determination

A newly developed extension poly(A) test (ePAT) method was
used to measure the poly(A) tail of mRNA [42]. This method
makes use of the ability of the Klenow polymerase to extend the 3' ends
of RNA molecules with dNTPs from an annealed DNA template in standard reverse transcription buffers. The PAT-
anchor primer 5’-cgtcagcaggctagcaggtgctgg contains a unique
universal DNA segment plus 22 Ts at the 3’ end and anneals to the
end of poly(A) tails to serve as the DNA template for the Klone polymerase. After the initial extension, the PAT-anchor primer also serves as the primer for the standard reverse transcription. The incubation was carried out at 55°C to eliminate unwanted priming from internal poly(A) tracts. The protocol started with heating 1 μg RNA together with 1 μl of 100 μM
PAT-anchor primer in a volume of 0 μl at 90°C for 5 min followed by cooling down to 37°C. A master mixture containing 4 μl DEPC-treated water, 4 μl 5X Superscript III buffer
(Invitrogen), 1 μl 100 mM DTT, 1 μl 10 mM dNTPs, 1 μl RNaseOUT (Invitrogen), and 1 μl (5 U) Klenow polymerase
(NewEngland Biolabs, Ipswich, MA) was added and the solution
was incubated at 37°C for 1 hour. The reaction mixture was then
heated at 80°C for 10 min to inactivate the polymerase and cooled
down to 55°C. One μl (200 U) Superscript III (Invitrogen) was
added and incubation was kept at 55°C for 1 hour. The reverse
transcriptase was inactivated at 80°C for 10 min at the end of the
reaction. PCR amplification of the 3’ ends of specific genes was
performed using a universal reverse primer 5’-cgtcagcaggctagc-aggtg 6 C to eliminate unwanted priming from internal poly(A) tracts. The protocol for priming the primer to the polyadenylation site during reverse transcription. The e-PAT products were
analyzed on 2% agarose gels. Fragments of interest were purified from the gels, cloned into pCRII-TOPO and sequenced to
determine the lengths of the poly(A) tails.

Supporting Information

Figure S1 Poly(A) tail length determination of Prmt1 and
Dazap1 transcripts in sucrose gradient fractions. Adult
mouse testis lysate was fractionated on a 10–50% sucrose gradient
and the same proportions of RNAs isolated from alternating
fractions were subjected to ePAT and analyzed on a 2% agarose
gel. The type of ribosome present in each fraction is indicated at
the top. The major bands in the TVN lanes contain an invariant
12-(A) poly(A) tail. Transcripts with long and short poly(A) tails are
indicated with L and S subscript, respectively.

Table S1 Poly(A) tail lengths of ePAT clones.

Table S2 PCR primers for RNAs precipitated from
mouse testis lysate with an anti-DAZL antibody.

Table S3 Gene-specific ePAT forward primers.
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Author Contributions

Conceived and designed the experiments: CKY PY. Performed the experiments: CKY. Analyzed the data: CKY PY. Contributed reagents/materials/analysis tools: CKY PY. Wrote the paper: CKY PY.

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