**Construction of the fluorescently tagged Protamines A and B of *D. melanogaster***

To make transgenic flies whose sperm heads express a fluorescent signal, we tagged *Mst35Ba* and *Mst35Bb* (also known as *CG4479* and *CG4478*) that encode similar sperm-specific chromosomal proteins called ProtamineA and ProtamineB, respectively (Raja and Renkawitz-Pohl, 2005). Fusion constructs were made in which either the Enhanced Green Fluorescent Protein (EGFP) or DsRed-Monomer (DsRed-M) coding sequence was joined in-frame with the C-terminus of the ProtamineA or ProtamineB coding region, and trangenic flies created, using P-element or piggyback mediated germline transformation methods.

A BAC clone (RPCI-98 33.C.24) of the genomic region containing the *Mst35Ba* (*ProtamineA*) and *Mst35Bb* (*ProtamineB*) genes was obtained from Children's Hospital Oakland Research Institute. The published sequence of this clone (accession number AC092246) predicted a 4.2 kb *Kpn*I-*Pst*I fragment containing the *ProtamineB* gene region, with 1.3 kb of sequence upstream of the transcription start site and 1.2 kb of sequence downstream of the polyadenylation site. BAC DNA was therefore treated with these enzymes and the 4.2 kb fragment gel-purified and ligated into pBluescriptKS+ (Stratagene) to obtain pBS/ProtB4.2KP. Site-directed mutagenesis, using primers NdeMut-1 = 5' CGCCACAAGCGCCGACGCATATGCAAGTAATACTG 3', and NdeMut-2 = 5' CAGTATTACTTGCATATGCGTCGGCGCTTGTGGCG 3', was carried out to generate an *Nde*I restriction site at the 3' end of the *ProtamineB* coding region to yield plasmid pBS/ProtB4.2NdeKP. A DsRed-Monomer *Nde*I cassette was made by PCR amplifying the coding region of DsRed-Monomer from pDsRed-Monomer (Clontech) using the primers Nde-DsRedM5 = 5' CCATATGCATGCATGGACAACACCGAGGAGGTCATC 3' and Nde-DsRedM3 = 5' TCATATGTCTACTGGGAGCCGGAGTGGCGGGC 3'. The 692 bp product was cloned into the TOPO cloning plasmid pCR2.1 TOPO using the TOPO TA cloning kit (Invitrogen) to give pTOPO/DsRedM-Nde-1. The DsRed-Monomer sequence was cut out with *Nde*I and ligated into the *Nde*I site of pBS/ProtB4.2NdeKP. Orientation of the inserted DsRed-Monomer sequence was checked by digestion with *Stu*I and *Eco*RI. The 4.2 kb insert of one correct clone, pBS/ProtB4.2KP-Nde-DsRed13, was cut out with *Kpn*I and *Bam*HI and ligated into the transformation vector pW8 to give pW8/ProtB-DsRedM (Figure 1). This DNA was used for P-element transformation and multiple independent transgenic lines were established.

To create GFP-tagged ProtamineB, an EGFP *Nde*I cassette was made by PCR amplifying the coding region of EGFP from pEGFP (Clontech) using the following primers: NdeGFP5B = 5' TGCATATGCAAGGTGAGCAAGGGCGAGGAGCTGTTCACC 3' and 3'GFP-Ndejb = 5' TCCATATGCAGAGGTTTTCACCGTCATCACCGAAACG 3'. The 875 bp product was cloned into the TOPO cloning plasmid pCR2.1 TOPO using the TOPO TA cloning kit (Invitrogen) to give pTOPO/GFPNde3, which was sequenced to confirm that no mutations had occurred. The *Nde*I EGFP cassette was cut out and cloned into the *Nde*I site of pBS/ProtB4.2NdeKP to give pBS/ProtB-GFP4. The orientation of the inserted EGFP cassette was checked by digestion with *Eco*RI, and the 5.1 kb *Kpn*I/*Bam*HI fragment was cut out and ligated into pW8 to give pW8/ProtB-GFP1 (Figure 1), which was used for P-element germine transformation.

In order to create EGFP- and DsRed-tagged ProtamineA constructs, BAC clone RPCI-98 33.C.24 was digested with *Nru*I and *Asp*700 (an *Xmn*I isoschizomer) and electrophoresed on a 0.7% agarose gel. A 6.0 kb fragment containing the ProtamineA coding region with 4.8 kb of sequence upstream of the transcription start and 0.5 kb downstream of the polyadelylation site, was gel purified using the QIAQuick gel extraction kit (Qiagen) and made blunt-ended using the Stratagene Polishing Kit (Stratagene). This DNA was then ligated into the *Hin*CII site of pBluescript KS+ to give pBS/ProtA6.0NA13. Site-directed mutagenesis, using primers ProtAMut-1 = 5' CGCCACAAGCGCCGACGCATATGCCAGCAATACTGAG 3', and ProtAMut-2 = 5' CTCAGTATTGCTGGCATATGCGTCGGCGCTTGTGGCG 3', was carried out to generate an *Nde*I restriction site at the 3' end of the *ProtamineA* coding region to yield plasmid pBS/ProtA6.0NA-Nde2. The *Nde*I DsRed-Monomer cassette from pTOPO/DsRedM-Nde-1 was inserted into the *Nde*I site of pBS/ProtA6.0NA-Nde2 to generate pBS/ProtA-NA/DsRedM20. The orientation of the inserted cassette was checked by cutting with *Stu*I and *Eco*RV. The 6.7 kb *Kpn*I-*Bam*HI fragment of this clone was subcloned into pW8 to give pW8/ProtA-DsRedM3. To create an EGFP tagged ProtamineA construct, the *Nde*I EGFP cassette of pTOPO/GFPNde3 was ligated into the *Nde*I site of pBS/ProtA6.0NA-Nde2 to give pBS/ProtA-EGFP6, and the orientation checked by cutting with *Eco*RI. The 6.9 kb *Kpn*I/*Bam*HI insert was cut out and subcloned into pW8 to give pW8/ProtA-GFP8 (Figure 1), which was used for P-element germline transformation.



Figure 1. Fluorescently-tagged Protamine A and Protamine B gene constructs. The gray boxes are the coding regions. The green and red rectangles represent the EGFP and DsRed-Monomer coding regions, respectively, that are inserted into the *Nde*I site.