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Amaury Herpin, Shuhei Nakamura, Toni U. Wagner, Minoru Tanaka, Manfred Scharl. A highly conserved cis-regulatory motif directs differential gonadal synexpression of Dmrt1 transcripts during gonad development. *Nucleic Acids Research*, 2009, 37 (5), 10.1093/nar/gkn1065 . hal-02666631

HAL Id: hal-02666631

<https://hal.inrae.fr/hal-02666631>

Submitted on 31 May 2020

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A highly conserved *cis*-regulatory motif directs differential gonadal synexpression of *Dmrt1* transcripts during gonad development

Amaury Herpin^{1,*}, Shuhei Nakamura², Toni U. Wagner¹, Minoru Tanaka^{2,*}
and Manfred Schartl^{1,3}

¹Physiological Chemistry I, University of Würzburg, Biozentrum, Am Hubland, D-97074 Würzburg, Germany, ²Laboratory of Molecular Genetics for Reproduction, National Institute for Basic Biology 5-1, Higashiyama, Okazaki, Aichi 444-8787, Japan and ³Rudolf-Virchow-Center for Experimental Biomedicine (DFG Research Center), University of Würzburg, D-97078 Würzburg, Germany

Received November 17, 2008; Revised December 16, 2008; Accepted December 18, 2008

ABSTRACT

Differential gene expression largely accounts for the coordinated manifestation of the genetic programme underlying embryonic development and cell differentiation. The 3' untranslated region (3'-UTR) of eukaryotic genes can contain motifs involved in regulation of gene expression at the post-transcriptional level. In the 3'-UTR of *dmrt1*, a key gene that functions in gonad development and differentiation, an 11-bp protein-binding motif was identified that mediates gonad-specific mRNA localization during embryonic and larval development of fish. Mutations that disrupt the 11-bp motif leading to *in vitro* protein-binding loss and selective transcript stabilization failure indicate a role for this motif in RNA stabilization through protein binding. The sequence motif was found to be conserved in most of the *dmrt1* homologous genes from flies to humans suggesting a widespread conservation of this specific mechanism.

INTRODUCTION

Assembly and formation of the gonad primordium is the first step towards gonad differentiation and subsequent sex differentiation (1). Primordial germ cells (PGCs) give rise to the gametes that are responsible for the development of a new organism in the next generation. In many organisms, following their specification the germ cells migrate towards the location of the prospective gonadal primordium (2–5). Similar to other vertebrates, the

structure of fish gonads is composed of germ cells and associated supporting somatic cells (6). The precursors of the somatic cells originate from cells of the lateral plate mesoderm where the gonadal primordium develops, while germ cells are derived from the germline lineage (7,8). To carry out their highly specialized biological functions, together somatic gonadal primordium and germline cells must establish specialized programs of gene expression. However, the early transcriptional and post-transcriptional regulatory events underlying the differentiation of gonad precursor cells through crucial interactions of somatic and germline cells are barely understood.

The *dmrt1* gene is an important regulator of male development in vertebrates (9). It is a highly conserved gene involved in the determination and early differentiation phase of the primordial gonad in vertebrates. In the fish medaka *dmrt1bY*, a functional duplicate of the autosomal *dmrt1a* gene on the Y-chromosome, has been shown to be the master regulator of male gonadal development (10,11), comparable to *Sry* in mammals (12). In males mRNA and protein expression occur before morphological sex differentiation in the somatic cells surrounding PGCs of the gonadal anlage and later on exclusively in Sertoli cells (13). Here it is synexpressed with the autosomal *dmrt1a* (14,15). However, nothing is known about the mechanism(s) that bring about this highly restricted expression pattern.

The expression of most genes is dynamically regulated temporally and spatially. Spatial organization of cells and subcellular compartments arises in part from the sorting and subsequent localization of proteins and RNA. Evidence has been obtained that regulation occurs at multiple steps on the level of gene expression including

*To whom correspondence should be addressed. Email: amaury.herpin@biozentrum.uni-wuerzburg.de
Correspondence may also be addressed to Minoru Tanaka. Tel/Fax: +81 564 59 5851; Email: mtanaka@nibb.ac.jp

transcription, splicing, mRNA transport, mRNA stability, translation, protein stability and post-translational modifications (16,17). Selective advantages could have favored the evolution of regulatory mechanisms at the post-transcriptional level, such as speed of response, reversibility, fine-tuning of protein amounts, coordinated regulation of protein families, potential for spatial control, and efficacy in systems lacking transcriptional control mechanisms. Efficient cell-specific mRNA processing depends on a temporally and spatially orchestrated sequence of protein–protein, protein–RNA and RNA–RNA interactions (16).

Striking examples of localized messengers, transcriptional, post-transcriptional and translational regulations can be found among the maternal mRNAs of fly, fish and frog implicated in the establishment of axial polarity. For example, in the posterior part of the *Drosophila* embryo, Nanos (Nos) protein represses translation of maternal *hb* mRNA (18). Conversely, synthesis of both Bed and Hb proteins in the anterior of the embryo requires that Nos is limited to the posterior (19). The restricted distribution of Nos is generated by selective translation of a subset of *nos* mRNA that is localized to the germ plasm at the posterior of the embryo coupled with translational repression of *nos* mRNA distributed throughout the whole embryonic cytoplasm (20). Both posterior localization and translational repression of *nos* RNA are mediated by the *nos* 3'-untranslated region (3'-UTR) (21). A nucleotide translational control element (TCE) within the *nos* 3'-UTR confers repression through formation of two stem-loop structures, whose functions are temporally distinct (22).

The zebrafish *nanos1* homologue which is required during germline development (23) has also been shown to be remarkably post-transcriptionally regulated. Here, microRNA miR-430 targets the 3'-UTR of *nanos1* during zebrafish embryogenesis in order to confer restriction of mRNA to PGCs (24). This miR-430 target site was shown to reduce poly(A) tail length, mRNA stability and translation, suggesting that differential susceptibility to microRNAs contributes to tissue-specific gene expression (24). Implicit in these mechanisms are the existence of *cis*-acting signals and *trans*-acting factors forming mRNA–protein complexes (mRNPs) that account for specificity and selectivity.

While the importance of complex post-transcriptional regulation—like in the case of *nanos*—has been widely demonstrated for the development of the germline, such mechanisms have not been uncovered so far for the development of the somatic part of the gonad, which determines the development towards testis or ovary. Studying mechanisms regulating localization and translation of gonad-specific genes during early gonad induction, we demonstrate that an 11-nt protein-binding motif located in the 3'-UTR of *dmrt1bY* mediates gonad-specific mRNA stability during embryonic and larval development. Interestingly, the sequence motif was found to be highly conserved in the homologous genes from flies to humans.

MATERIALS AND METHODS

Fish maintenance and breeding

Medaka were taken from closed breeding stocks of the Carbio strain and kept under standard conditions. Medaka embryos were staged according to Iwamatsu (25).

Whole-mount *in situ* hybridization

RNA whole-mount *in situ* hybridization using GFP digoxigenin (DIG)-labeled probe was performed as described for Medaka (26). Briefly, after capped mRNA injection, embryos of the desired stage were fixed with 4% paraformaldehyde and dehydrated with methanol. Anti-sense-DIG-labeled RNA probes were synthesized according to the manufacturers' instructions (Roche, Meylan). Hybridization and detection with alkaline phosphatase (AP)-coupled anti-DIG antibody (Roche, Meylan) were performed according to Thisse *et al.* (27).

Plasmid constructs and RNA injections

To obtain RNA transcripts of *eGFP* the *GFP* open reading frame (ORF) from *pEGFP-N1* (Clontech Laboratories) was inserted (*Bam*HI/*Not*I) into *pCS2+* plasmid (*pCS2:GFP*). To produce *pCS2:GFP:dmrt1bY 3'-UTR*, a PCR product containing the entire 3'-UTR of *dmrt1bY* flanked by *Not*I sites, was amplified from medaka testes and inserted into the *Not*I sites of *pCS2:GFP*. Similarly, *pCS2:GFP:dmrt1a 3'-UTR*, *pCS2:GFP:d. rerio dmrt1bY 3'-UTR*, *pCS2:GFP:h. sapiens 3'-UTR*, *pCS2:GFP:O. curvinotus dmrt1bY 3'-UTR* and *pCS2:GFP:fugu 3'-UTR* and deletion constructs (Supplementary Figure 2) were constructed the same way. Corresponding RFP plasmids were constructed by replacing the *GFP* ORF by *RFP*. *Xenopus β-globin* constructs were produced by inserting the *xenopus β-globin 3'-UTR* (*Not*I/*Kpn*I) from plasmid pRN3 (28) into *pCS2:GFP* (*pCS2:GFP:xlβ-globin 3'-UTR*). For constructing the *pCS2:GFP:BOXxlβ-globin 3'-UTR* plasmid, the *dmrt1bY* box was inserted between the *Not*I sites. The *GFP/RFP 3'-UTR* constructs include the mmGFP5/RFP ORF cloned upstream of the 3'-UTR of the zebrafish *nanos1* gene (23,29). All constructs were checked by restriction digests, diagnostic PCRs and sequencing. Sertoli cell-specific Ds-Red expressing *sox9*prom:DsRed transgenic medaka fish (30) was provided by Prof. Tanaka.

Capped RNAs for injections were transcribed from linearized vectors using the SP6/T3/T7m MESSAGE mMACHINE Kit (Ambion). One nanoliter was injected into the cytoplasm of one-cell stage Medaka embryos as described (31).

Cell culture and transfection

Medaka spermatogonial (Sg3), embryonic stem (MES-1) or fibroblast (OL-17) cells were cultured as described (32–34). Cells were grown to 70–80% confluency in six-well plates and then transfected with 5 μg expression vector using GeneJuice reagent (Novagen) as described by the manufacturer. Luciferase activity was then quantified using the Luciferase Reporter Assay System from

Promega and luciferase activity was normalized against mRNA luciferase copy number. Transcriptional differences between luciferase constructs were evaluated statistically by paired Student's test.

Electromobility shift assay

Nearly confluent cells [Medaka spermatogonial (Sg3), mouse Sertoli (TM4) and Medaka embryonic stem (MES-1)] cell lines grown on plates were washed with ice-cold phosphate-buffered saline (PBS) and removed from the plates with 1 mM EDTA in PBS. The cells were centrifuged at low speed and then resuspended in Passive Lysis Buffer (Promega) supplemented with leupeptin (0.2 µg/ml) and aprotinin (10 µg/ml). Binding assays were carried out using the Gel Shift Assay System (Promega) using radiolabeled RNA oligonucleotides: (Y) UGGUUCACGUCUGCUGCAGGUCUCUGACUCU for the native box target sequence and Mut(2)-box UGGUUCACGUCUGCUGCAGGUCUCUGACUCU; Mut(3)-box UGGUUCACGUCUCUUCACAUGUCUCUGACUCU; Mut(4)-box UGGUUCACGUCUGCUGCAGCAGCUCUGACUCU; Mut(5)-box UGGUUCACGUCUGCCAUGGUCUCUGACUCU as competitors.

RESULTS AND DISCUSSION

Dmrt1a/dmrt1bY 3'-UTRs regulate spatial and temporal expression during early Medaka development

While searching for potentially conserved regulatory sequences in genes involved in gonad induction and formation we analyzed the tightly regulated Medaka *dmrt1a* and *dmrt1bY* duplicated gene pair. Postulating that important *cis*-regulatory motifs required for mRNA regulation in the context of gonad formation might have been retained between the duplicates despite of the processes of co-ortholog gene specialization and subfunctionalization, we noticed that the *dmrt1a* and *dmrt1bY* 3'-UTRs appeared more conserved than expected for independently diverging genes. To test whether this conservation of *dmrt1a/dmrt1bY* UTRs implied common regulatory mechanisms, we examined GFP expression of reporter constructs that contained either the *dmrt1a* or *dmrt1bY* 3'-UTR or the *Xenopus* β-globin 3'-UTR as a control (Figure 1). After injection of the different constructs into one cell stage embryos we initially observed a high and uniformly distributed GFP expression in the whole embryo (Figure 1). After 2 days of development (stages 22–24; 12–16 somites), GFP fluorescence slowly vanished elsewhere except in the primordial gonad area of the fish injected with either *dmrt1a* or *dmrt1bY* UTR (Figures 1A, B, C, D compared to 1E, F, G, H).

GFP fluorescence was clearly detectable in the primordial gonad area until more than 7 days after hatching (stage 40; first fry stage, 2.5 weeks after fertilization) (Supplementary Figure 1). In controls with the β-globin 3'-UTR GFP remained ubiquitously expressed throughout the whole embryonic development. Obviously, the *dmrt1a/dmrt1bY* 3'-UTRs are responsible for specific expression of the GFP protein in the primordial gonad area.

Surprisingly, fusing either human (AJ276801) (Figure 1I, J and K) or takifugu (CAC42778) (Figure 1L, M and N) *dmrt1* 3'-UTRs to GFP mRNA also drove primordial gonad area-specific fluorescence in Medaka (Figure 1I–N compared to 1A–D). In addition, injection of GFP mRNA fused to Medaka *dmrt1bY* 3'-UTR in zebrafish resulted in a similar gonadal persistence of GFP fluorescence (Figure 1O, P and Q) indicating a functional cross-species conserved mechanism mediated by *cis*-regulatory element(s) in these *dmrt1* 3'-UTRs.

A short, highly conserved *cis*-regulatory motif located in *dmrt1a/dmrt1bY* 3'-UTR is responsible for gonadal differential regulation

To delineate the precise RNA sequence and/or secondary structures involved in gonadal-specific fluorescence, GFP expression of a series of reporters (Supplementary Figure 2) that contained deletion mutants of the *dmrt1a/dmrt1bY* 3'-UTRs was investigated (Supplementary Figure 2B, C and D). As a result a core 11-nt box located in the 5' region of the Medaka *dmrt1a/dmrt1bY* UTRs was isolated and shown to be responsible for gonad-specific fluorescence. Consequently, when the box was inserted into the *Xenopus* β-globin 3'-UTR, GFP expression was identical to *GFP::dmrt1a* and *dmrt1bY* 3'-UTR constructs, namely gonad-specific expression (Figure 1R, S, T and U). Conversely, deletion of the box sequence from the *dmrt1bY* 3'-UTR drastically extinguished gonad-specific expression (Supplementary Figure 2D compared to 2B and C).

Considering the functional conservation of gonad-specific expression seen with other fish and human *dmrt1* UTRs we then searched for similar motifs in the 3'-UTR of *dmrt1* genes of other organisms (Table 1). The motif was found to be highly conserved in the fish lineage (*Oryzias latipes*, *Oryzias curvinotus*, *Takifugu rubripes*, *Tetraodon nigroviridis*, *Epinephelus coioides* and *Danio rerio*), but as well in the *dmrt1* 3'-UTR of other vertebrates including man (*Mus musculus*, *Pan troglodytes*, *Macaca mulatta* and *Homo sapiens*) and most surprisingly even in the ecdysozoan clade. For the *doublesex* (*dsx*, the *dmrt1* orthologue) of *Anopheles gambiae* (Table 1), interestingly, the sex-specific differentially spliced *anopheles dsx* transcript results in a male *dsx* form where the box is largely conserved while this is not the case for the female splice form (Table 1). This situation is similar for *dsx* of the olive fruit fly (*Bactocera oleae*) for which a male-specific splicing leads to the preservation of a highly conserved box in the ORF while this fragment is spliced out in the female form (Table 1).

Taken together, a short highly conserved *cis*-regulatory motif located in *dmrt1a* and *dmrt1bY* 3'-UTRs (CUGCU GCAGGU) appears to be mostly responsible for differential expression of the transcripts.

The *dmrt1* box drives specific stability in the somatic mesoderm anlage of the gonadal primordium as well as in a sub-population of PGCs

Dmrt1 is expressed in most species specifically in Sertoli cells and PGCs. To find out the contribution of

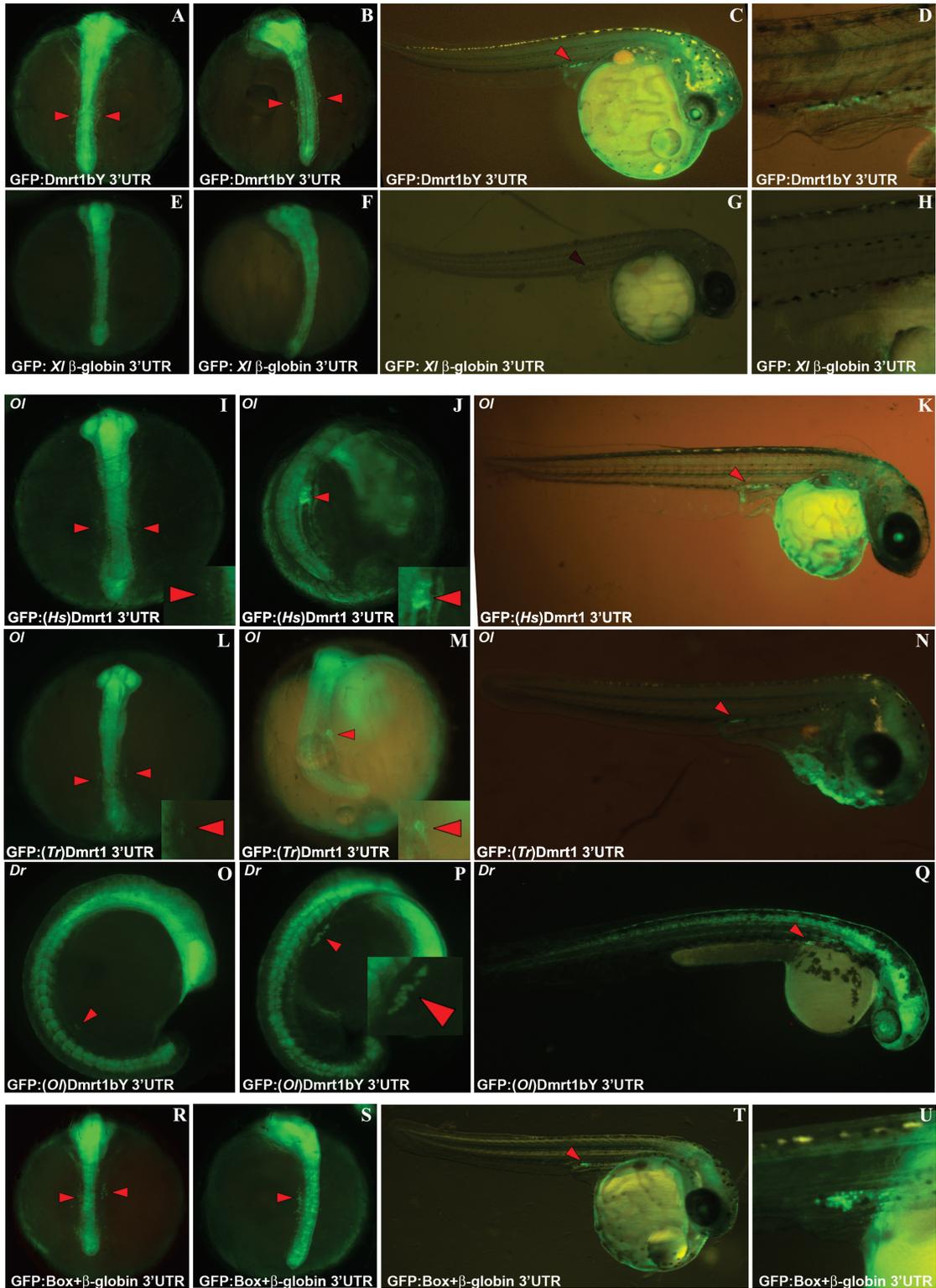


Figure 1. A short highly conserved *cis*-regulatory motif located in *dmrt1bY/dmrt1a* 3'-UTRs regulates spatial and temporal expression during early development. (A–D) GFP expression of a reporter construct that contains Medaka *dmrt1bY* 3'-UTR during somitogenesis (A and B) and at hatching stage (C and D). (E–H) GFP expression of a control reporter construct that contains *Xenopus* β -globin 3'-UTR during somitogenesis (E and F) and at hatching stage (G and H). (I–K) GFP expression of reporter constructs that contain either human *dmrt1* 3'-UTR (I–K) or takifugu *dmrt1* 3'-UTR (L–N) in Medaka embryos during somitogenesis (I, J and L, M) and at hatching stage (K and N). (O–Q) GFP expression of a reporter construct that contains Medaka *dmrt1bY* 3'-UTR in zebrafish embryos during somitogenesis (O and P) and at hatching stage (Q). (R–U) GFP expression in Medaka embryos of a reporter construct that contains *Xenopus* β -globin 3'-UTR in which the Box was inserted. Specific GFP expression in PGCs is indicated (arrow heads).

Table 1. The *dmrt1* 3' UTR *cis*-regulatory motif is well conserved from ecdysozoans to mammals

<i>Oryzias latipes</i>	Dmrt1a3'-UTR(AF319994):	CUGCUGCAGGU
<i>Oryzias latipes</i>	Dmrt1bY 3'-UTR (AB071534):	CUGCUGCAGGU
<i>Oryzias curvnotus</i>	Dmrt1bY 3'-UTR (AB091695):	CUGCUGCAGGU
<i>Oryzias curvnotus</i>	Dmrt1ba 3'-TR (AB091696):	CUGCUGCAGGU
<i>Takifugu rubripes</i>	Dmrt1 3'-UR (CAC42778):	CUGCUGCAGGU
<i>Tetraodon nigrovihdis</i>	Dmrt1 3'-UTR (CAC42783):	CUGCUGCAGGU
<i>Epinephelus coioides</i>	Dmrt1 3'-UTR(EF017802):	TUGCUGCAGGU
<i>Danio rerio</i>	Dmrt1 3'-UTR(AAU11586):	CUGCUGCAGAU
<i>Homo sapiens</i>	Dmrt1 3'-UTR(AJ276801):	CUGCUGCCGAU
<i>Pan troglodytes</i>	Dmrt1 3'-UTR (XM528528):	CUGCUGCCGAU
<i>Macaca mulatta</i>	Dmrt1 3'-UTR (XM001090081):	CUGCUGCCGAU
<i>Mus musculus</i>	Dmrt1 3'-UTR (NM175647):	CUGGUGCUGAU
<i>Anophele gambia</i>	Dsx 3'-UR (Male-spe, AY903307):	CUGCUGUAAGU
<i>Anophele gambia</i>	Dsx 3'-UTR (Female-spe, AY903308):	CUG_UGCAGAA



Bactocera oleae (olive fruit fly) Doublesex ORF (AJ547622):

CUGCUGCAGCU

'Box' present in Male form (dsxm) but absent in Female form (dsxf)

Musca domestica Doublesex ORF (AY461854/AY461853):

CUGCUGCAGGA

'Box' present for both dsxm and dsxf

post-transcriptional regulation by this *cis*-regulatory motif to the restricted expression pattern, Medaka *dmrt1bY* 3'-UTR was fused to the monomeric RFP mCherry and injected either together with *GFP::Nos* 3'-UTR into one-cell stage embryos of wild-type Medaka or of the Olvas transgenic strain. The *GFP::Nos* 3'-UTR construct was previously shown to drive PGC-specific fluorescence in medaka (35–37). Similarly, in Olvas fish the PGCs are marked by GFP expression from stage 25 [18–19 somite stage (25)] onwards (38). RFP expression was compared to germ cell-specific GFP expression due to the *nanos* 3'-UTR or the *vasa* promoter (Figure 2A–F). Starting at stages 14–18 fluorescence was exclusively observed from GFP in PGCs (data not shown; Figure 3A). Only by stages 22–24 (nine somite stage) red fluorescence could also be observed with specific gonadal localization (Figure 2D–F). Noteworthy, although at this stage both fluorescences (green and red) were confined to the same embryonic structures, they are clearly expressed from different cell populations (Figure 2A–C). Additionally, around stage 24–26 (16–22 somite stage) cells expressing both fluorochromes could be observed (Figure 2A–C). Subsequently, at hatching stage, gonadal *dmrt1bY* 3'-UTR-driven GFP expression was then investigated in Sertoli cell-specific RFP expressing *sox9*prom:DsRed transgenic fish (30) either at stage 34 (Figures 2G, I, H and J) or just after hatching (Figure 2K, L and M) in

males and females, respectively. It revealed that although no more supporting cell expression was apparent for these later stages, two different populations of germ cells could be discriminated according to their high or just above background GFP fluorescence expression (arrowheads in Figure 2G, H, K and L). The fact that the RFP reporter gene product was only observed at later stages indicated that mRNAs with the *dmrt1bY* 3'-UTR are translationally repressed in migrating PGCs and in the somatic gonad precursor cells.

Interestingly, such variation and diversity in germ cell-specific Dmrt1 expression is also reported for mouse gonads for which two populations of germ cells could be observed according to Dmrt1 protein presence or absence (39). Consequently, akin to what was observed during medaka gonad formation (Figure 2G–N), it might be possible that mouse Dmrt1⁺ and Dmrt1⁻ expressing germ cells are the result of a similar mechanism since the 'box' motif is also found to be highly conserved in mouse *dmrt1* 3'-UTR (Table 1).

Embryonic expression of *dmrt1* mRNA has been examined in various vertebrates, including mammals (40–42), birds (41), reptiles (41,43) and fish (44). In most cases, *dmrt1* is expressed very early in the genital ridge—the structure from which the gonad derives—and later on in germ and Sertoli cells of the male gonadal primordium [see Zarkower *et al.* (45) for review]. It is then evident that

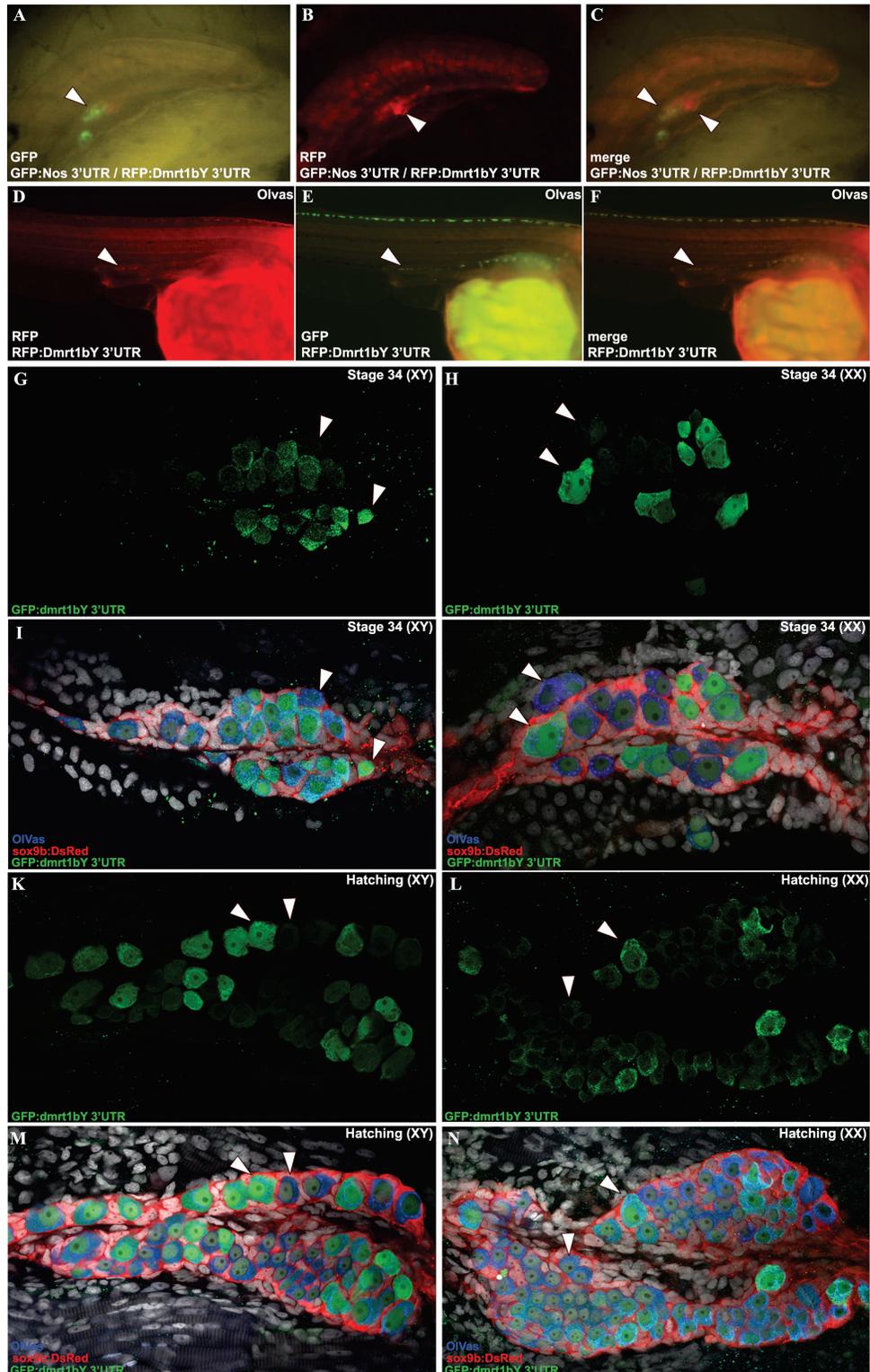


Figure 2. The *dmrt1* box drives specific stability in the somatic mesoderm of the gonadal primordium as well as in a sub-population of PGCs. (A–F) RFP expression from *dmrt1bY* 3'-UTR containing capped RNA compared to germ cell-specific GFP expression due to the *nanos* 3'-UTR at stages 24–26 (A–C). (D–F) RFP expression compared to germ cell specific GFP expression achieved with *vasa* promoter around hatching stage. (G–N) Gonadal *dmrt1bY* 3'-UTR-driven GFP expression investigated in Sertoli cell specific DsRed expressing *sox9prom:DsRed* transgenic fish either at stage 34 (G, I and H, J) or just after hatching (K, M and L, N) in males (XY) and females (XX). Arrow heads indicate either putative somatic gonadal precursor cells (C) or different sub-populations of germ cells (A, B and D–N). Blue: DAPI or Olvas; Red: Sertoli cell specific expression (*sox9prom:DsRed* transgenic fish) and Green: gonadal *dmrt1bY* 3'-UTR-driven GFP expression.

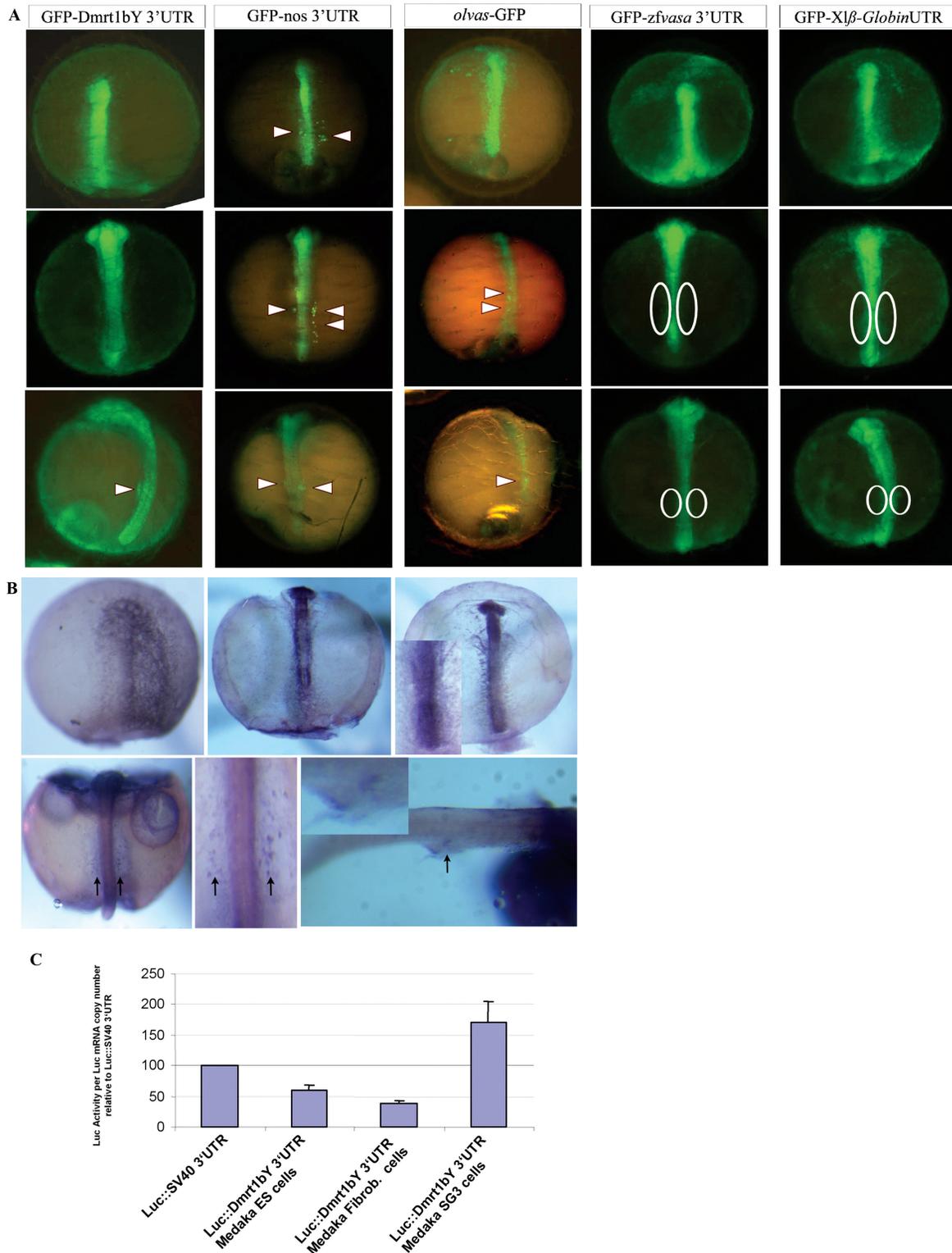


Figure 3. Tissue-specific and temporal-restricted expression by a combination of *dmr1bY* 3'-UTR induced differential mRNA stability and translational regulation. (A) GFP:*dmr1bY* 3'-UTR was injected. As controls, mRNA constructs such as GFP:*nos* 3'-UTR, *olvas*:GFP, GFP:*zfvasa* 3'-UTR and GFP:*xI β -globin* 3'-UTR were also injected to be able to compare the expression with the pattern of known post-transcriptional mechanisms such as micro-RNA mRNA induced decay, specific PGC translational regulation, and ubiquitous stability, respectively. (B) Subsequently *in situ* hybridization using an antisense GFP probe was performed at different stages of development to reveal the spatial distribution of the injected GFP:*dmr1bY* 3'-UTR RNAs. GFP fluorescence and the distribution of RNA were followed at different stages of development. Arrowheads and circles indicate where the PGCs are or should be located respectively. (C) Luciferase expression in different cell lines transfected with a *dmr1bY* 3'-UTR containing construct reveals that translation of the reporter gene was significantly enhanced by the presence of *dmr1bY* 3'-UTR in Medaka spermatogonial cells in contrast to either Medaka embryonic stem or fibroblast cells.

GFP expressing cell are PGCs; likewise are the cells expressing jointly both GFP and RFP. The question remains what the identity of the RFP only expressing cells at early stages (stages 24–26) is. Considering the temporal appearance of these cells and a report identifying and tracing different populations of gonadal precursor cells in medaka (7), it is most likely that these cells are representatives of a subset of gonadal precursors.

Interestingly, like observed for mouse *Dmrt1*, these results point to the existence of at least two populations of PGCs within the primordial gonad of Medaka with possibly different regulations of *dmrt1* in relation to the above described *cis*-acting element located in its 3'-UTR.

Tissue-specific and temporal-restricted expression is caused by a combination of *dmrt1* 3'-UTR-induced differential mRNA stability and translational regulation

Independently of promoter-induced transcription striking examples of specifically localized messengers can be found among the mRNAs of fly, fish and frog, most of them being post-transcriptionally (dynamic relocalization and stabilization) and translationally regulated. To test whether *dmrt1bY* 3'-UTR was more involved in regulating mRNA stability or controlling its translational regulation, embryos were injected with *GFP:dmrt1bY* 3'-UTR and subsequently analyzed for the spatial distribution of the injected RNA by RNA *in situ* hybridization at different stages of development (Figure 3). As controls, *GFP:nos* 3'-UTR, *olvas:GFP*, *GFP:zfvasa* 3'-UTR and *GFP:xlβ-globin* 3'-UTR mRNA constructs were also injected in order to compare the expression with the pattern of known post-transcriptional mechanisms such as microRNA mRNA-induced decay, specific PGC translational regulation and ubiquitous stability, respectively (Figure 3). GFP fluorescence and the distribution of RNA were followed at different stages of development. Remarkably, GFP expressed from the construct containing the *dmrt1bY* 3'-UTR, was shown to be quite stable in the whole body, including the primordial gonad area like with the *GFP:zfvasa* 3'-UTR and *GFP:xlβ-globin* 3'-UTR mRNAs (Figure 3). This is in striking contrast to *GFP:nos* 3'-UTR construct for which mRNA underwent rapid somatic degradation [Figure 3; (22,24,36,46)]. This pattern of decay is typical for a microRNA-mediated process (24). Although an analogous germ cell-specific fluorescence was observed in germ cells of *olvas:GFP* injected embryos, *in situ* hybridizations showed homogenous *olvas* RNA distribution. This pattern implies germ cell-specific translational regulation (35). Accordingly GFP *in situ* hybridization after *GFP:dmrt1bY* 3'-UTR injection revealed that the fused mRNA is first homogeneously distributed in the whole embryonic body until stage 24 and only then becomes progressively restricted to the gonad area to be exclusively present in the gonadal primordium around hatching (stage 39) (Figure 3). Hence, it can be inferred that the apparent specific *GFP:dmrt1*-UTR driven expression in primordial gonad is the result of differential RNA stabilization.

Interestingly these results reflect also the probability of another mechanism affecting mRNAs containing the

dmrt1 UTR. The fact that strong fluorescence is observed in the whole body (including PGCs) at early stages suggests that the *dmrt1bY* box here contributes to enhanced translation. To test whether the *dmrt1bY* 3'-UTR is able to enhance translation, a plasmid construct containing a thymidine kinase promoter driven luciferase fused either to SV40 3'-UTR or *dmrt1bY* 3'-UTR was transfected in different cell lines and translation efficiency measured. Translation of the reporter gene was significantly ($P < 0.01$) enhanced in the presence of *dmrt1bY* 3'-UTR in Medaka spermatogonial cells in contrast to either Medaka embryonic stem cells or fibroblast cells where protein production was even reduced (Figure 3). The suppression of translation in non-gonad cell types is interesting to note in relation to the non-gonad-specific expression of *dmrt1bY* transcripts during early Medaka embryogenesis and in adult male spleen (10,14).

In summary, taking into account the endogenous expression of *dmrt1a* exclusively in the developing gonad and the here observed late gonad-specific translation of injected RFP:*dmrt1bY* 3'-UTR mRNA, it appears that the UTR mediates a translational regulation process specifically in a subset of PGCs and certain somatic cells of the gonad. From RNA localization studies it is apparent that the later gonad-specific expression is then primarily due to the 11-bp *cis*-acting *dmrt1* motif conferring differential RNA stabilization in primordial gonad cells.

The *dmrt1* 3'-UTR box specifically binds a protein possibly involved in gonad-restricted expression

Obviously the 11-mer is involved in a cell-type-specific stability of *dmrt1* RNAs. One explanation could be that it is the target for a protein or small ribonucleoproteins particles. To test this hypothesis, electrophoretic mobility shift assays (EMSAs) were performed using the 11-mer box as a probe and different mutated boxes as competitors (Figure 4). In a first step cell extracts from three different cell lines were tested: (i) a medaka spermatogonia cell line, (ii) a medaka embryonic stem cell line and (iii) a mouse sertoli cell line (TM4) (Figure 4A). While no mobility shift was observed for the medaka embryonic stem cell-like line in all conditions tested, a weak shift was apparent for the TM4 cells and a quite robust one for the Medaka spermatogonial cell line (Sg3) (Figure 4A). As controls, using the medaka spermatogonial cell line, a mutated version of the box used as competitor (Mut(3)-Box) did not interfere significantly with the *Dmrt1bY*-Box binding, indicating the specificity of the interaction (Figure 4B). Furthermore, competition of radioactively with non-radioactively labeled box probes (*Dmrt1bY*-Box) resulted in progressive loss of the apparent shift (Figure 4C). Next, to better characterize the interaction domain(s) of the box, different point mutations were then introduced to the native *dmrt1bY*-box (Figure 4D).

The first mutated RNA oligonucleotide [Mut(2)-Box] regrouped the three main mutations observed for the different *dmrt1* boxes within fish and mammals (see also Table 1). These introduced mutations did not significantly interfere for binding (Figure 4D). Interestingly, this result

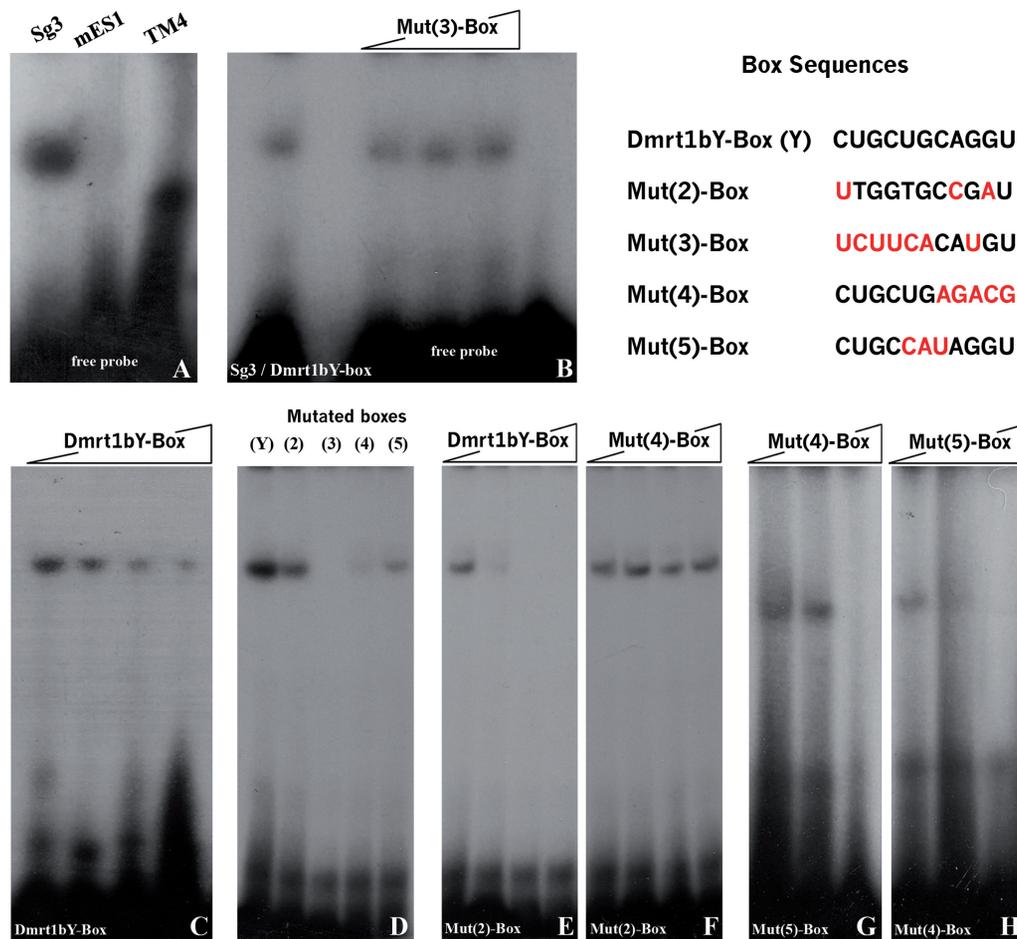


Figure 4. A RNA-binding factor that recognizes the Box could be responsible for primordial gonad restricted stability. (A) Electrophoretic Mobility Shift Assay (EMSA) using cell extracts from three different cell lines (i) a medaka spermatogonial cell line (Sg3), (ii) a medaka embryonic stem cell line (MES-1) and (iii) a mouse sertoli cell line (TM4) and the 11-mer box as a probe shows shift for Sg3 and TM4 cells. (B) A mutated version of the box [Mut(3)-Box] used as competitor (1-1, 1-5 and 1-10 ratios) did not interfere significantly with the binding, indicating the specificity of the binding. (C) As control, using the spermatogonial cell line, competition of radioactively and non-radioactively labeled box probes resulted in progressive loss of the apparent shift (1-1, 1-5 and 1-10 ratios). (D) Different mutated versions of the box [Mut(2-5)-Box] were then tested for binding, and resulted in apparent different binding affinities. (E-H) Relative robustness of the shift was then tested by mean of competition assays among the different mutated versions of the box (E: 1-1, 1-5 and 1-10; F: 1-1, 1-2 and 1-5; G and H: 1-1 and 1-5 ratios).

corroborates *in vivo* observations showing GFP stabilization after injection of either human or zebrafish *dmrt1* 3'-UTR containing constructs in medaka (Figure 4D). Nevertheless, this affinity seems to be significantly weaker when competing against the native Dmrt1bY-Box (Figure 4E). Noticeably, Mut(2)-Box competition against another box mutated in its 3' region [Mut(4)-Box] could not appreciably interfere with the binding (Figure 4F). Similarly, using this box [Mut(4)-Box] and a box mutated in its core region [Mut(5)-Box] alternatively against each other, revealed that although the entire native box is required for efficient binding, its 3' region is important while the 5' and core regions are more likely involved in modulating the affinity (Figures 4G and 4H).

In summary these experiments suggest that the 11-mer box is a preferential target for RNA-binding proteins that may be involved in specifically regulating transcript stability.

CONCLUSIONS

The great majority of sequences that direct the subcellular localization, translation and degradation have been found within the 3'-UTRs [see (17,47) for review]. UTRs are highly diverse in sequence, but often contain regulatory motifs that are common to members of the same metabolic family (48). Nevertheless, the analysis of such *cis*-acting elements has been rather unsatisfying with respect to the identification of common sequences that direct localization of different RNAs (49).

We have identified an 11-nt motif in the Dmrt1 3'-UTR, CUGCUGCAGGU, common to the great majority of Dmrt1 orthologs genes from fly to mammals that is responsible for specific stabilization and translational control of *dmrt1* mRNA in the forming primordial gonad of fish and probably of other species. Interestingly, for the first time such stabilizing motif involving *cis*-regulatory actor(s) has been shown to be not only present in a

single organism [see Kloc *et al.* (49) for review], co-regulating a very specific pool of few synexpressed transcripts (50), but rather to be highly conserved across species for a given transcript family, namely the Doublesex/Mab-3/Dmrt1 genes. The occurrence of such an 11-mer in the 3'-UTR of orthologous genes over a wide range of organisms already indicates that different systems may employ common RNA regulatory mechanisms. Hence, other than a motif involved in specific localization of mRNAs to a well defined subtype of cells, this box would be responsible for mRNA-specific preservation of different subsets of cells all together involved in primordial gonad assembly and formation, namely putative gonadal precursor cells and a specific subclass of PGCs. Consequently, like synexpressed groups reflect the functional compartmentalization of the eukaryote genome (51) and have a striking parallel to the prokaryote operon, our finding might be of particular interest since it might reveal an otherwise hidden logic of cellular regulation where *cis*-regulatory motifs couple spatial and temporal gene expression in different subset of cells during organogenesis.

Finally, our data indicate that transcript stabilization is achieved by interaction of a specific protein with a *cis*-acting stability element located in the *dmrt1* 3'-UTR. Although the identity and the dynamic of action of this stabilizing factor has still to be resolved, our findings point to an obvious level of integrated regulation, namely the presence and the accessibility of this *cis*-regulatory element. The occurrence of multiple *dmrt1*-related spliced variants in corals (52), insects (53–55), lizards (56), fish (57,58), chicken (59), mice (60) and human (61) selecting or splicing out parts of the UTRs also argue for such way of regulation.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

FUNDING

This work was supported by a grant of the Rudolf-Virchow-Zentrum for Experimental Medicine (DFG Forschungszentrum) and DFG-Graduiertenkolleg 1048 (Molecular Basis of Organ Development in Vertebrates) through a PhD fellowship to T.W.

Conflict of interest statement. None declared.

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