RESEARCH COMMUNICATION

The RNA-binding protein XSeb4R: a positive regulator of *VegT* mRNA stability and translation that is required for germ layer formation in *Xenopus*

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VegT represents a localized maternal determinant essentially required for endoderm formation in *Xenopus*. Here, we report on the identification of the RNA-binding protein XSeb4R as a positive regulator of *VegT*. XSeb4R interacts directly with the 3'-untranslated region of *VegT* mRNA, stabilizes it, and stimulates translation. Ablation of XSeb4R activity results in impairment of endoderm and mesoderm formation, while ectopic expression of *XSeb4R* in ectodermal cells induces endodermal and mesodermal gene expression. These observations unravel a novel mode of *VegT* regulation at the post-transcriptional level that is essential for germ layer formation in *Xenopus*.

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Germ layer formation in amphibian embryos employs both localized maternal determinants and inductive events (Heasman 2006). In *Xenopus*, the T-box transcription factor *VegT* has been demonstrated to serve key functions in endoderm formation and mesoderm induction. *VegT* is encoded by a maternal mRNA that is localized at the vegetal pole of *Xenopus* oocytes (Lustig et al. 1996; Stennard et al. 1996; Zhang and King 1996). In blastula stages of development it is essential for the formation of the endodermal germ layer (Zhang et al. 1998).

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Two groups of *VegT* target genes can be distinguished in this context. First, *VegT* activates several zygotically expressed, endoderm-specific genes encoding transcription factors, such as *GATAs*, *Sox17*, and *Mixer*, that form a functional network indispensable for the distinction between endodermal and mesodermal territories (Clements et al. 1999; Xanthos et al. 2001; Sinner et al. 2006). Second, *VegT* also induces a group of TGF β -related signaling molecules involved in mesoderm induction and required for proper gastrulation movements (Kofron et al. 1999). During gastrula stages, a second wave of *VegT* expression is detected in the mesoderm; here, *VegT* function is much less understood, but it may be indirectly and/or directly involved in the transcriptional activation of several organizer expressed genes such as *chordin*, *noggin*, and *cerberus* (Xanthos et al. 2002). Interestingly, the switch from maternal to zygotic *VegT* gene transcription is accompanied by formation of an alternatively spliced variant of the *VegT* transcript, which encodes a protein referred to as Antipodean (Apod), carrying a different N terminus (Stennard et al. 1996; Stennard et al. 1999). However, even though structurally diverse, *VegT* and Apod seem to exert the same activities in *Xenopus* gain of function assays (Stennard et al. 1996).

XSeb4R is an RRM-type protein, and therefore assumed to be RNA binding, even though some RRM proteins are also involved in protein-protein interactions (Maris et al. 2005). *XSeb4R* was isolated in *Xenopus* based on its expression at early neurula stages of development, in a pattern indicative for a role in primary neurogenesis. Our previous functional analysis has indeed revealed a proneural function for XSeb4R in the open neural plate and also in the context of retinogenesis (Boy et al. 2004). However, target RNAs that may exist in the context of its proneural activity, as well as its mode of operation remain to be established. *XSeb4R* is also expressed maternally and within the mesoderm at gastrula stages (Boy et al. 2004). Here, we report on the function of XSeb4R in the context of endoderm formation and mesoderm induction. Our results reveal that XSeb4R is a positive regulator of *VegT*, and therefore plays an important role in germ layer formation.

Results and Discussion

XSeb4R activates endodermal and mesodermal marker gene expression in animal cap explants

Xenopus animal cap explants cultivated to the equivalent of neurula stages were utilized to test the inductive activities of a hormone-inducible version of XSeb4R (XSeb4R-GR) (Fig. 1A). In addition to the expected activation of genes characteristic for neuronal differentiation, such as *N-tubulin* and *X-ngnr-1* (Boy et al. 2004), we also observed activation of molecular markers for the endodermal and mesodermal germ layers, such as *Xbra*, *Wnt8*, *GATA4/6*, *Sox17 β* , and *VegT*. Furthermore, microinjection of XSeb4R mRNA into whole embryos also resulted in ectopic expression of *VegT* (Fig. 1B).

The remarkably broad pattern of endo- and mesodermal gene induction as observed in *XSeb4R*-GR-injected animal caps is similar to that described to occur upon ectopic expression of *VegT* in the same explant system (Clements et al. 1999), suggesting that XSeb4R might

Figure 1. *XSeb4R* overexpression activates endo- and mesodermal marker genes via VegT in animal cap explants. (A) Activation of the expression of markers in animal cap explants derived from embryos injected at four- to eight-cell stages with 150 pg of *XSeb4R-GR* mRNA per blastomere and analyzed by RT-PCR. (CE) Control embryos; (CC) control uninjected caps. (B) Whole-mount in situ hybridization using *VegT/Apod* antisense probe demonstrate that *XSeb4R* overexpression causes strong *VegT* activation on the injected side (IS) of stage 15 embryos in the area corresponding to mesoderm formation (100%, $n = 150$). (C) RT-PCR analysis of the expression markers in animal cap explants injected with *XSeb4R* mRNA alone (250 pg), or coinjected with different MOs expected to inhibit translation of their target mRNAs; these were either VegT-MO (40 ng), Apod-MO (40 ng), or a combination of both MOs. The Apod mismatch MO (Apod-MM) (40 ng) was used as a control. (D) Real-time RT-PCR quantification of *VegT* and *XSeb4R* mRNA levels in animal (an), marginal (m), and vegetal (vg) explants from blastula stage embryo. Expression in the ectodermal (animal) explants is arbitrarily set to 1. Values for marginal and vegetal zone explants are thus fold increase in respect to the level of expression detected in the ectodermal explants.

operate via VegT. In order to test this possibility, animal cap explants were coinjected with *XSeb4R* mRNA and antisense morpholino oligonucleotides (MOs) to inhibit translation of the endogenous *VegT/Apod* mRNA, either alone or in combination (Fig. 1C). In the presence or absence of VegT MO, *XSeb4R* induced similar levels of *VegT*, indicating that VegT is not activating its own transcription via a positive feedback loop. However, activation of *GATA4*, *Xbra*, and *Wnt8* gene expression was strongly reduced, providing strong evidence that *XSeb4R* is indeed operating via VegT to obtain endo- and mesodermal gene activity in the animal cap explant system. Apod, on the other hand, seems to be subject to positive autoregulation and not required for a part of the *XSeb4R*-induced endodermal gene expression, correlating with its mesoderm-specific expression in normal embryos (Zhang and King 1996). A combination of MOs directed against both, *VegT* and *Apod*, blocks *XSeb4R* induced endodermal and mesodermal gene transcription.

Before the onset of zygotic gene expression at the MBT, the vast majority of *VegT* mRNA is found in the vegetal portion of the early embryo that will give rise to the endodermal germ layer. Our previous analysis of *XSeb4R* had revealed a maternal mRNA contribution, as well as zygotic expression in the mesoderm at gastrula stages, where *VegT* is also expressed, but the spatial expression before MBT had not been analyzed in detail (Boy et al. 2004). If *XSeb4R* indeed operates via VegT in the

context of endoderm formation, *XSeb4R* mRNA should be present in the vegetal half of blastula stage embryos. Real-time RT-PCR analysis demonstrates that this is indeed the case (Fig. 1D). Taken together, this first set of experimental observations is compatible with the idea that the ability of *XSeb4R* to induce endoderm- and mesoderm-specific gene expression occurs via and/or in cooperation with VegT. Since *VegT* mRNA can be detected in all three germ layers of blastula stage embryos (with very low but significant levels in the ectoderm) (Figure 1D; Clements et al. 1999; Cao et al. 2007), these effects could be either due to *XSeb4R* inducing increased stability of the maternal *VegT* mRNA, or they could be the result of *XSeb4R*-mediated translational activation.

XSeb4R binds to the 3'-untranslated region (UTR) of *VegT* mRNA

XSeb4R is an RRM-type protein and therefore assumed to exert RNA-binding activity. Affinity chromatography on different RNA homopolymers shows that this is indeed the case. Like many other RRM-type proteins, *XSeb4R* binds avidly to poly(G), but not to the other polymers employed, and such RNA-binding activity is lost upon deletion of the RRM motif (data not shown). In order to further unravel the mechanistic correlation of *XSeb4R* and VegT activities, we tested if *XSeb4R* could bind directly to the *VegT* 3'-UTR. UV-cross-linking experiments were performed, making use of bacterially expressed, purified GST or His-tagged *XSeb4R*, as well as of myc/GST-tagged *XSeb4R* produced in microinjected *Xenopus* embryos. As shown in Figure 2A, *XSeb4R* does indeed interact specifically with the 3'-UTR of *VegT* mRNA, but not with other portions of the same mRNA or an unrelated RNA sequence (*lacZ*). A mutant construct lacking the RRM domain (GST-*XSeb4R*ΔRRM) fails to interact with *VegT* mRNA. To localize the RNA sequence responsible for *XSeb4R* binding, the *VegT* 3'-UTR was divided into three fragments (F1–F3). While the C-terminal F1 bound to levels similar to unspecific *lacZ* control, *XSeb4R*, F2, and F3 readily interacted with *XSeb4R*, revealing the presence of multiple, independent binding sites within the 3'-UTR region of *VegT* mRNA (Fig. 2C). This was confirmed by competition of F2 and

Figure 2. *XSeb4R* protein binds directly to the 3'-UTR of *VegT* mRNA. (A) Bacterially purified GST-*XSeb4R* and GST-*XSeb4R*ΔRRM proteins were tested by UV-cross-linking assays for their ability to interact with full-length ³²P-*VegT* mRNA. Unlabeled *VegT* 3'-UTR, ORF, or *LacZ* mRNAs were used as competitors. (B) Schematic representation of the *VegT* 3'-UTR subfragments tested by UV-cross-linking. The corresponding base pairs of the cDNA sequence (Acc. number: U59483) are indicated. (C) UV-cross-linking experiments of bacterially expressed, purified GST-*XSeb4R* or MTGST-*XSeb4R* produced in embryos. Note that *XSeb4R* binds to multiple sites within the 3'-UTR region of *VegT* mRNA.

F3 for binding of XSeb4R to the full-length VegT 3'-UTR (Supplemental Fig. S1) and further deletion mapping of F3 (Fig. 2C). These data suggest that XSeb4R may regulate VegT protein synthesis from maternal mRNA via direct interaction with the *VegT* 3'-UTR.

XSeb4R stimulates translation

As a sequence specific RNA-binding protein, XSeb4R could affect different aspects of RNA metabolism and activity. Several 3'-UTR-binding proteins have been found to be involved in translational regulation and/or control of RNA stability (Maris et al. 2005; Richter 2007). We therefore first tested a possible function for XSeb4R as a translational regulator. For this purpose, XSeb4R was fused to the MS2 RNA-binding protein and the corresponding mRNA coinjected along with a luciferase-reporter mRNA harboring MS2-binding sites in its 3'-UTR (Coller et al. 1998) into *Xenopus* oocytes (Fig. 3A) and embryos (data not shown). Under these conditions, MS2-XSeb4R causes a robust translational stimu-

lation that is even considerably stronger than the one observed with the MS2-PABP fusion protein used as a positive control and injected at a fivefold higher concentration. Moreover, semiquantitative RT-PCR analysis demonstrated that the MS2-XSeb4R did not alter RNA stability of the reporters in microinjected oocytes (Supplemental Fig. S2). This method is sufficient to detect differences in reporter mRNA levels that yield differences of the magnitude of luciferase activity induced by XSeb4R (Supplemental Fig. S3). These results strongly support that the increase in reporter activity is indeed attributed to an influence on translation rather than RNA stability in the *Xenopus* oocyte system (see also below). While the RRM motif of XSeb4R was dispensable for translational stimulation (MS2-XSeb4R Δ RRM), the C-terminal portion was required (MS2-XSeb4R Δ C). This latter finding would suggest that additional protein-protein interactions are involved in the translational activation observed.

The cross-linking experiments described above provide strong evidence for a direct interaction of the *VegT* 3'-UTR and the XSeb4R protein. This would predict that fusion of the *VegT* 3'-UTR to the luciferase ORF should result in a reporter construct that is translationally activated by the XSeb4R wild-type protein. The experiments illustrated in Figure 3B show that this is indeed the case, even though the level of translational activation achieved in oocytes is lower than in the MS2 tethering assay. In this case, as to be expected, translational stimulation was not observed with a mutant version of XSeb4R lacking the RRM; furthermore, translational stimulation with different 3'-UTR fragments (Fig. 3B) correlated well with their ability to interact with XSeb4R described above (Fig. 2D). Semiquantitative RT-PCR analysis demonstrated XSeb4R did not influence the *Luc-VegT* RNA reporter levels (Supplemental Fig. S4). Taken together, these results reveal that ectopically expressed XSeb4R activates translation of microinjected reporter constructs that can bind XSeb4R via their 3'-UTR in *Xenopus* oocytes, strongly suggesting that the endogenous XSeb4R similarly stimulates translation of endogenous *VegT* mRNA in those embryonic cells where the two genes are coexpressed.

XSeb4R binding increases target RNA stability in Xenopus embryos

Interestingly, RNPC1, the human homolog of XSeb4R, has been reported to be a target for p53 and to serve a role in maintaining the stability of the p21 transcript through direct binding to its 3'-UTR (Shu et al. 2006). We thus tested whether XSeb4R, in addition to functioning as a translational activator, might also regulate RNA stability in *Xenopus* embryos, even though such a stabilizing effect was not observed for microinjected reporter mRNAs in oocytes (as reported above). To address this possibility, maternal *VegT* mRNA levels were analyzed by real-time RT-PCR in animal cap explants isolated from blastula stage embryos that were either XSeb4R depleted by MO injection or overexpressing XSeb4R upon mRNA injection (Fig. 4A). Explants were cultivated for different time intervals after dissection. At the different time points analyzed, XSeb4R overexpression resulted in an increase of *VegT* mRNA levels as compared with uninjected control explants. At the time of explant isolation, equal *VegT* mRNA levels were ob-

Figure 3. XSeb4R stimulates translation. (A) A given protein can be targeted to a reporter mRNA harboring MS2-binding sites in its 3'-UTR by fusion to the RNA-binding viral MS2 coat protein. Coinjection of synthetic mRNA encoding either MS2 fused to the full-length XSeb4R (MS2-XSeb4R), or a mutant version lacking the N-terminal RNA recognition motif (MS2-XSeb4R Δ RRM) together with a firefly luciferase reporter mRNA harboring three MS2-binding sites for [Luc-MS2] leads to robust luciferase activity in *Xenopus* oocytes. XSeb4R-MS2 lacking the C terminus, untethered XSeb4R, or MS2 alone fails to induce luciferase activity. Translational activation by MS2-PAB1P serves as positive control. MS2-XSeb4R does not activate a reporter construct lacking the MS2 recognition sites [Luc- Δ MS2]. (B) Delineation of the *VegT* 3'-UTR region required for translational activation through XSeb4R. The *VegT* 3'-UTR and the indicated fragments were tested for conferring translational activation via XSeb4R in *Xenopus* oocytes. All firefly luciferase values were normalized to renilla luciferase; shown is the fold-activation relative to the *Luc-MS2* or the *Luc-VegT* 3'-UTR reporter activity alone.

Figure 4. XSeb4R stabilizes its target mRNAs. (A) Real-time RT-PCR analysis of *VegT* transcripts in animal cap explants from embryos injected with *XSeb4R* mRNA, *XSeb4R*-MO, or control uninjected embryos collected at different time points after dissection. An average of 50 was employed per batch of animal cap explants. Note the significant increase or decrease of the *VegT* mRNA level in caps overexpressing *XSeb4R* or depleted of *XSeb4R*, respectively. Data are expressed in arbitrary units (A.U.). (B) *XSeb4R* overexpression causes persistent staining of a *VegT* 3'-UTR fragment that strongly binds *XSeb4R* (*VegT* F8), but not of a fragment that does not do so (*VegT* F7). The indicated combinations of mRNAs were injected into the animal pole of one blastomere in two-cell stage albino embryos and batches of 20–25 embryos were fixed at various stages. The mRNA fragments of interest were traced by whole-mount in situ hybridization, using *VegT* antisense mRNA as a probe.

served in control and injected caps, which might be due to endogenous XSeb4R that would be present initially at a sufficient level to stabilize maternal *VegT* transcripts. Conversely, MO-mediated XSeb4R knockdown resulted in a complete ablation of *VegT* mRNA in such animal cap explants.

In order to correlate the stability of an RNA molecule more directly with its ability to interact with XSeb4R, two different fragments derived from the *VegT* 3'-UTR, F8 that binds strongly to XSeb4R and F7 that does not (see also above; Fig. 2D), were injected into embryos and their apparent stability traced by whole mount in situ hybridization (Fig. 4B). While both RNA species were readily detected in blastula stage embryos (stage 8), only the XSeb4R-binding fragment was found to persist in late

stage (stage 18, neurula) embryos, even though at reduced levels. Interestingly, overexpression of *XSeb4R* further increased the stability of the XSeb4R interacting fragment, while having no significant effect on the non-interacting RNA injected at a higher concentration. Taken together, the studies evaluating endogenous *VegT* transcript levels when XSeb4R levels are increased or decreased (Fig. 4A) together with the qualitative experiments using fragments derived from the *VegT* 3'-UTR (Fig. 4B) provide evidence that XSeb4R binding confers a stabilizing effect to its target RNAs, such as maternal *VegT* mRNA in *Xenopus* embryos.

XSeb4R is required for endoderm and mesoderm formation in *Xenopus* embryos

Experimental data presented so far argue that XSeb4R functions in endoderm and mesoderm germ layer formation via the positive regulatory activity that it has for *VegT*. If XSeb4R in its ability to stabilize and activate translation of *VegT* mRNA serves an essential function during germ layer formation in *Xenopus* embryos, XSeb4R knockdown should result in a phenotype reminiscent of the one observed upon maternal *VegT* depletion. In order to address this issue, mesodermal *VegT/Apod* mRNA levels were analyzed in XSeb4R MO-injected gastrula stage embryos by means of whole-mount in situ hybridization (Fig. 5A). *VegT/Apod* mRNA concentrations were greatly decreased in XSeb4R morphant embryos; coinjection of *XSeb4R* mRNA along with the MO restores *VegT/Apod* mRNA levels, providing a strong argument for the specificity of the effects observed. Interestingly, and perhaps as a consequence of reduced *VegT* activity in MO-injected embryos, marker gene expression for endodermal (*Sox17β* and *GATA4*) and mesodermal (*Xbra* and *Wnt8*) germ layers was grossly reduced; as above, coinjection of *XSeb4R* mRNA rescued these effects, at least partially (Fig. 5A).

As a second, alternative approach to inhibit binding of XSeb4R to the *VegT* 3'-UTR, a competing XSeb4R-binding RNA fragment derived from the *VegT* 3'-UTR (F8) was microinjected and the resulting embryos tested for mesodermal and endodermal gene expression (Fig. 5B). Interestingly, marker gene expression was again strongly inhibited in the injected portion of such embryos, while microinjection of a nonbinding control RNA (F7) exerts no such inhibitory effects. Coinjection of the 3'-UTR F8 with *XSeb4R* mRNA, however, was not sufficient to rescue the phenotype, suggesting that it may sequester other factors required for germ layer formation (Supplemental Fig. S5). These experiments reveal that XSeb4R, in close similarity to *VegT*, is required for proper endodermal and mesodermal germ layer formation in *Xenopus* embryos.

Concluding remarks

Results reported in this communication reveal increased translation and stability of *VegT* mRNA upon binding of XSeb4R, which thus constitutes a novel element in the regulation of germ layer formation. As indicated by its complex pattern of expression during embryogenesis, which is only partially overlapping with that of *VegT* and *Apod*, XSeb4R is highly likely to operate also on addi-

Figure 5. XSeb4R is required for endoderm formation and mesoderm induction. (A) XSeb4R knockdown decreases *VegT* mRNA and inhibits the expression of endoderm and mesodermal markers (100% inhibited, $n = 41$ for *VegT*, $n = 28$ for *Wnt8*, $n = 31$ for *Xbra*, $n = 36$ for *GATA4*, and $n = 42$ for *Sox17*). XSeb4R-MO was injected into the marginal or the vegetal region of two blastomeres (50 ng each) from four-cell-stage embryos together with *lacZ* mRNA. Embryos were fixed at stage 11 and stained with Xgal (blue). For rescue, 150 pg of *XSeb4R* mRNA were used. Total rescue was obtained in 27 of 42 embryos for *VegT*, whereas only partial rescue was observed for *Wnt8* (13 of 22) *Xbra* (20 of 34) *Sox17* (36 of 42) and *GATA4* (27 of 40). (B) A competing XSeb4R-binding RNA fragment derived from the *VegT* 3'-UTR [F8] inhibits mesodermal and endodermal gene expression. Embryos injected at the marginal or vegetal regions with 3 ng of *VegT* F8 or 4.5 ng of *VegT* F7 mRNA were fixed at gastrula stage 11 and analyzed by in situ hybridization for the indicated probes. The *VegT* probe detects the injected UTR fragments and the endogenous transcripts. *VegT* F8 but not *VegT* F7 UTR injection causes significant suppression of the tested markers (*Wnt8*: *VegT* F8, 36 of 52; *VegT* F7, 19 of 57; *Xbra*: *VegT* F8, 34 of 46; *VegT* F7, 15 of 51; *Sox17*: *VegT* F8, 32 of 40; *VegT* F7, eight of 34).

tional target mRNAs that remain to be identified. As we know from the examples of other RNA-binding proteins (e.g., Wessely and De Robertis 2000; Tran et al. 2007; Chicoine et al. 2007), they can serve different functions that are highly context-dependent. The same may be true for XSeb4R. It will therefore be of prime interest in the future to identify proteins interacting with XSeb4R to form a functional RNP, as well as to decipher the events in the context of translation and RNA stability control that are influenced by XSeb4R and cooperating factors.

Materials and methods

DNA constructs, RNA synthesis, oligonucleotides, and protein production

Expression plasmids were generated by PCR and cloning (EcoRI and XhoI) into the pCS2+ vector and its derivatives as indicated. Templates for the different 3'-UTR fragments were generated as above or using a T7 promoter sequence fused to the forward primer. For in vitro transcription, DNA constructs were linearized with NotI. Capped mRNAs were produced using the mMessage mMachine kit (Ambion). For in situ hybridization or UV-cross-linking, Dig- or 32 P-UTP-labeled probes were produced by making use of the appropriate RNA polymerases. Purification of GST- or His-tagged XSeb4R was achieved as described (GST Gene Fusion System, Amersham Biosciences; QIA expression kit, Qiagen). Apod was depleted by use of antisense Apod-MO (5'-CCTTGTACGTAAGAGACGGCCTAC-3') and controlled by a mismatched Apod-MMO (5'-CCTTCTACCTAACAGACGCCGTAC-3') (GeneTools). XSeb4R-MO and VegT-MO were described previously (Boy et al. 2004; Heasman et al. 2001).

Embryo manipulation, explant dissection, and in situ hybridization

Wild-type and albino *Xenopus* embryos were obtained by hormone-induced egg laying and in vitro fertilization using standard procedures. Embryos were staged as described by Nieuwkoop and Faber (1967). Whole-mount in situ hybridization was done as described (Harland 1991). Animal cap explants were dissected at blastula stage (stage 8) using either a gastromaster (Xenotek Engineering) or an adapted eyelash unit. Induction of GR constructs was performed by treatment with 10 μ M dexamethasone (Sigma) immediately after cap dissection. All injections were performed at least twice, and the effects indicated correspond to one representative experiment.

RT-PCR and real-time quantitative PCR analysis

Total RNAs were extracted using the RNeasy mini kit (Qiagen) or the RNeasy Mini RNA isolation kit (GE Healthcare). All samples were tested for DNA contamination by 30-cycle PCR amplification using histone H4 primers (Niehrs et al. 1994). Complementary DNAs were synthesized with iScript cDNA synthesis kits (Bio-Rad). RT-PCR was done according to Gene Amp RNA PCR kit (Perkin Elmer Biosystem). Real-time RT-PCR was carried out using the Gene Amp TM PCR system 9600 (Perkin Elmer Biosystem) with quantitative PCR core kits for SYBR Green I (Eurogentec). Samples were normalized with *Xenopus GAPDH* (F, TAGTTGGCGTGAACCATGAG; R, GCCAAAGTTGTCGTTGATGA). *VegT* mRNA levels were achieved using specific primers as described by Stennard et al. (1999). All experiments were repeated at least two times with each assay performed in duplicate or triplicate.

UV-cross-linking assays

In vitro UV-cross-linking reactions were performed based on the protocol detailed in Mowry (1996) with modifications as described previously (Claßen et al. 2004). 32 P-labeled probes were incubated with optimized amounts of protein or embryonic extracts in the presence or absence of competitor. Probes were normalized based on their counts per minute.

Luciferase reporter assays

XSeb4R (Fig. 3) was cloned (BamHI) downstream from MS2 in the pET-MS2 vector (Coller et al. 1998). MS2-PAB1P and Luc-MS2 have been described (Gray et al. 2000; Gorgoni et al. 2005). The *VegT* 3'-UTR was cloned (BamHI/NotI) in the pBKMVLuc vector (Stratagene) downstream from firefly luciferase, generating *Luc-MS2* and *Luc-VegT* 3'-UTR, respectively. Renilla luciferase (pHRG-TK, Promega) was cloned (BamHI/EcoRI) into pCS2+. MS2 fusion or XSeb4R mRNA (25 ng) was injected into stage VI *Xenopus* oocytes prepared as described previously (Claßen et al. 2004). After 6 h at 18°C, firefly luciferase (100 pg) and renilla luciferase (3.5 pg) reporter RNAs were injected and the oocytes were cultivated for 14 h. Two samples of 15 oocytes were collected for each injection and homogenized in 150 μ L of 1 $\times$ passive lysis buffer (Promega). Ten microliters of each lysate were assayed using the Dual Luciferase Reporter system (Promega). Each injection was repeated at least three times.

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