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Substrate requirements for *let-7* function in the developing zebrafish embryo

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ABSTRACT

MicroRNAs (miRNAs) are involved in the regulation of gene expression at the post-transcriptional level by base pairing to the 3′-UTR (untranslated region) of mRNAs. The *let-7* miRNA was first discovered in *Caenorhabditis elegans* and is evolutionarily conserved. We used zebrafish embryos as a vertebrate *in vivo* system to study substrate requirements for function of *let-7*. Injection of a double-stranded *let-7* miRNA into the zygotes of zebrafish and frogs causes specific phenotypic defects. Only the antisense strand of the *let-7* duplex has biological activity. In addition, co-injected mRNA of *gfp* fused to the 3′-UTR of a zebrafish *lin-41* ortholog (a presumed target of *let-7*) is silenced by *let-7*. Point mutant studies revealed that the two *let-7* target sites in the *lin-41* 3′-UTR are both essential and sufficient for silencing. *let-7* and *mir221* together, but not either of them alone, can silence a construct with one of the *let-7* target sites replaced by a target site for *mir221*, showing that two different miRNAs can provide the required cooperative effect. *let-7* target sites can be moved around: they are also functional when positioned in the coding sequence or even in the 5′-UTR of *gfp*. We took advantage of reporter and phenotypic assays to analyze the activity of all possible point mutant derivatives of *let-7* and found that only the 5′ region is critical for function of *let-7*.

INTRODUCTION

Hundreds of microRNAs (miRNAs) have been discovered in eukaryotes (1–11) and they form an abundant class of post-transcriptional regulators [for reviews see (12,13) and many references therein]. MiRNAs are initially transcribed as longer precursors and subsequently processed into 21–23 nt double-stranded RNAs with 2 nt 3′ overhangs by the RNAse III-like endoribonucleases Drosha (14) and Dicer (15–18), respectively. MiRNAs regulate the gene expression by incorporating into a RISC (RNA-induced silencing complex) complex that binds to miRNA complementary elements in the 3′-UTR (untranslated region) of target genes. Targets have been predicted computationally for many miRNAs, based on the conservation of miRNA targets and known miRNA-target interactions (19–23). Despite the numerous targets predicted for many miRNAs, only few studies have addressed a role for distinct miRNAs in animals: In flies, the *bantam* miRNA was shown to be involved in the control of cell proliferation (24) and *miR-14* suppresses apoptosis and is required for fat metabolism (25). Some miRNAs from mouse are implicated in the modulation of hematopoietic lineage differentiation (26). The *Caenorhabditis elegans lsy-6* and *mir-273* miRNAs regulate chemosensory laterality (27,28). *lin-4* is the founding member of the miRNA class of genes. It acts on *C.elegans* by binding to complementary sites in the 3′-UTR of the heterochronic genes *lin-41* and *lin-28* (29,30). The *let-7* miRNA also regulates developmental timing in *C.elegans* by inhibiting the expression of heterochronic genes, among which *lin-41* (31,32). At least two out of six *let-7* target sites in the *C.elegans lin-41* gene, together with the 27 bp sequence in between, were shown to be necessary for *let-7*-mediated gene silencing (33). Both *let-7* and *lin-41* are conserved in evolution and *let-7* target sites are also present in the *lin-41* orthologs of *Drosophila* and zebrafish (31).

Here, we demonstrate that injection of a synthetic *let-7* miRNA (in double-stranded form) causes specific defects in the vertebrate embryo. Furthermore, we employ the zebrafish embryo to show that two *let-7* target sites from the zebrafish *lin-41* gene mediate silencing. Both target sites are essential for silencing; an mRNA with one *let-7* target site replaced by a *mir221* target site can be silenced by both miRNAs together. Target sites for *let-7* are also functional when placed, in the coding sequence or in the 5′-UTR of a reporter gene.

No study systematically determined the importance of every position of a miRNA. The *let-7* mutant allele (*n2853*) in *C.elegans* harbors a mutation at position 5 from the 5′ end of the miRNA (32). This single point mutation abolishes the function of *let-7* in *C.elegans*. Since the *let-7* miRNA is strongly conserved, we took this miRNA to derive a complete mutational spectrum using zebrafish as an *in vivo* vertebrate system.

MATERIALS AND METHODS

Construction of gfp reporters

A 379 bp fragment containing two putative *let-7* target sites in the zebrafish *lin-41* 3′-UTR (Al794385) was amplified from genomic DNA using primers: lin-41 3′-UTR F,

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GGCATTGAATTCATAAGACTGCTGCAAGCTGAGAG (EcoRI restriction site is underlined) and lin-41 3′-UTR R, GGCATTTCTAGATCAGGGATATAACTTGCGTTC (XbaI restriction site is underlined). This fragment was cloned into pCS2 (Clontech), containing a *gfp* cDNA sequence cloned between restriction sites BamHI and ClaI (p*gfp*) resulting in p*gfp*::*lin-41* 3′-UTR. *pgfp::lin-41* 3′-UTR-1, -2, -1 mut1, -1 mut2, *pgfp::let-7*tar1+2, *pgfp::let-7*tar1-2x, *pgfp::let-7*tar2-2x, *pgfp::let-7*tar1+*mir221*tar and *gfp::let-7*tar cds were made by cloning of double-stranded oligonucleotides *lin-41* 3′-UTR-1 (5′-gaattcataatcttcctgcattacacctacctcatctagctatgtatgaatgtactcgcgtttgtgcagagacctagtcggtgaagttttgttaaaaaaaaaattgtctacctcatctaga-3′), *lin-41* 3′-UTR-2 (5′-gaattcataatcttcctgcattacacctatctcatctagcttatgtatgaatgtactcgcgtttgtgcagagacctagtcggtgaagttttgttaaaaaaaaattgtctatctcatctaga-3′), *lin-41* 3′-UTR-1 mut-1 (5′-gaattcataatcttcctgcattacacctatctcatctagctatgtatgaatgtactcgcgtttgtgcagagacctagtcggtgaagttttgttaaaaaaaaaattgtctacctcatctaga-3′), *lin-41* 3′-UTR-1 mut2 (5′-gaattcataatcttcctgcattacacctatctcatctagcttatgtatgaatgtactcgcgtttgtgcagagacctagtcggtgaagttttgttaaaaaaaaaattgtctatctcatctaga-3′), *let-7*tar1+2 (5′-gaattcataatcttcctgcattacacctacctcatctaggttaaaaaaaaattgtctacctcatctaga-3′), *let-7*tar1-2x (5′-gaattcataatctctcctgcattacacctacctcatctagctgcattacacctacctcatctaga-3′), *let-7*tar2-2x (5′-gaattcataatcttcgttaaaaaaaaaattgtctacctcatctaggttaaaaaaaaattgtctacctcatctaga-3′), *let-7*tar1+*mir221*tar (5′-gaattcataatcttcctgcattacacctacctcatctagcttatgtatgaatgtactcgcgtttgtgcagagacctagtcggtgaagttttaacccagcagtgtatgtagcttctaga-3′) and *let-7* tar cds (5′-gaattctcttcctgcattacacctacctcatctaggttaaaaaaaaattgtctacctcactaagtctaga-3′) in the EcoRI and XbaI sites of p*gfp*. Construct *gfp::let-7*tar 5′-UTR was made by cloning of oligonucleotide *let-7*tar 5′-UTR (ggatcctcttcctgcattacacctacctcatctaggttaaaaaaaaattgtctacctcactaagccatgg) in the NcoI and BamHI sites of p*gfp*. *let-7* and *mir221* target sites are underlined, and the mutations are shown in bold.

Microinjections

Wild-type and mutant *let-7* deprotected and desalted RNA oligonucleotides (Proligo) were dissolved in RNAse free water at a concentration of 100 µM. Pre-*let-7* (UGAGGUAGUAGGUUGUAUAGUUUUAGGGUCACACCCACCACUGGGAGAUAACUAUACAAUCUACUGUCUUUC) was obtained from Biolegio. Oligos were annealed using a 5× buffer containing 30 mM HEPES–KOH, pH 7.4, 100 mM KCl, 2 mM $MgCl_2$ and 50 mM NH_4Ac. 2′-*O*-methyl oligonucleotides (anti-*let-7*, UCUUAACUAUACAACCUACUACCUCAACCUU and control, UCUUCAGCCUAUCCUGGAUUACUUGAAACCUU; Dharmacon) were dissolved in RNAse free water at a concentration of 500 µM. Anti-*let-7* morpholino (AACTATACAACCTACTACCTCA) was dissolved in water at a concentration of 25 ng/nl and injected at a concentration of 10 ng/nl. mRNA derived from Green fluorescent protein (GFP) reporter constructs was obtained by *in vitro* transcription using SP6 (Boehringer) and SacII linearized plasmid as a template. Injection mixtures contained 10 µM of a *let-7* duplex and 100 ng/µl *gfp* mRNA and 50 µM 2′-*O*-methyl oligonucleotide where indicated. This solution was injected into the one-cell stage of wild-type embryos derived from the TL line using 1 nl per embryo. *Xenopus tropicalis* embryos were injected in the two-cell stage using 2 nl per cell.

Northern blot analysis

Total RNA from embryos was isolated using TRIzol Reagent (Invitrogen). GFP mRNA was detected using RNA from 10 embryos (3 µg), separated on 1.5% agarose gels according to the standard procedures. A random primed ^{32}P-dCTP radiolabeled probe covering the complete GFP cDNA sequence was used for hybridization. *let-7* was detected using RNA isolated from 5 embryos (1.5 µg). RNA was separated on a 15% polyacrylamide gel. A radiolabeled probe complementary to *let-7* was used for hybridization.

Western blot analysis

Protein was isolated by boiling 5 embryos (5 mg) for 10 min in 10 µl loading buffer. Prior to loading lysates were centrifuged for 5 min at 14 000 *g*. Western blotting was performed according to the standard procedures. GFP was detected using a rabbit polyclonal antibody.

RESULTS AND DISCUSSION

Effects of injected *let-7* on development

During the first ~48 h of zebrafish development, no endogenous *let-7* miRNA is expressed (31,34). We observed a specific phenotype upon injection of a double-stranded *let-7* miRNA in one-cell stage zebrafish embryos (Figure 1A). At 26 h post-fertilization (hpf), embryos were generally retarded in development. More pronounced characteristics were a lack of proper eye development and reduced tail and yolk sac extension. The embryos died after ~2 days. This phenotype was only induced by a double-stranded version of *let-7*. Injection of either the sense or the antisense strand alone did not affect development, although these species remain stable *in vivo* for at least 48 h (data not shown). A control miRNA bearing five mutations (*mmlet-7*) failed to induce the phenotype, indicating the specificity of the observed phenotype (Figure 1A). Furthermore, an injected pre-*let-7* hairpin is processed *in vivo* and induces a phenotype similar to that of a mature *let-7* duplex (Figure 1B).

Because of its perfect conservation, we also investigated the effects of *let-7* microinjection on *X.tropicalis* embryos. Strikingly, this resulted in similar defects as for zebrafish, i.e. embryos were retarded in development and exhibited a reduced eye size and tail length (Figure 1A). Development was virtually normal in embryos injected with the *mmlet-7* duplex. The phenotypic effects caused by *let-7* misexpression in zebrafish could be specifically inhibited by co-injection with a 2′-*O*-methyl oligonucleotide complementary to the *let-7* miRNA, but not by a control 2′-*O*-methyl oligonucleotide unrelated in sequence to *let-7* (Figure 3B) (35). Injection of the oligonucleotide alone did not cause any developmental abnormalities. Similarly, a morpholino directed against *let-7* inhibited the misexpression phenotype, but did not induce a phenotype when injected alone (data not shown). The results presented here show that the *let-7* miRNA is active in the zebrafish embryo and severely affects normal development upon misexpression. Although the biological significance of the observed effects remains unclear, it seems likely that ectopic expression of *let-7* causes precocious downregulation

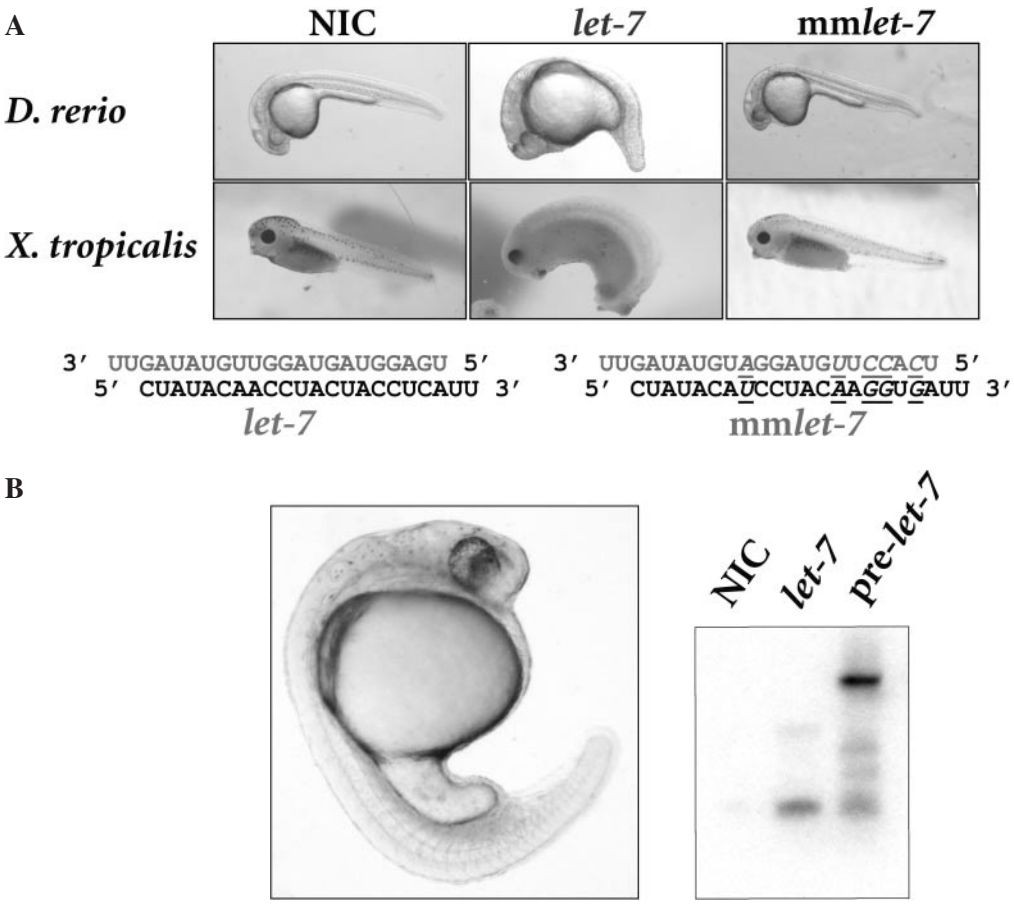

Figure 1. Misexpression of *let-7* in vertebrate embryos. (**A**) Lateral views of *Danio rerio* and *X.tropicalis* embryos injected with *let-7* and a mutant version containing five mismatches (underlined). (**B**) Phenotype induced by injection of a pre-*let-7* hairpin. The northern blot shows processing of pre-*let-7* in zebrafish embryos. Pictures were taken at 28 h post-fertilization (hpf) and 2 days post-fertilization (dpf) for *D.rerio* and *X.tropicalis*, respectively. NIC, not injected control.

of one or more endogenous *let-7* targets. Misexpression studies can be important for defining miRNA function, especially in cases where knockout is difficult because of redundancy.

The antisense strand of the *let-7* duplex is biologically active and an asymmetric duplex exhibits enhanced activity

We next investigated which strand of the *let-7* duplex exerts a biological effect by examining the activity of heteroduplexes (Figure 2). A heteroduplex containing two mutations at the 5′ end of the antisense strand of *let-7* (heteroduplex 1) fails to induce a phenotype, while a heteroduplex with two mutations at the 3′ end of the sense strand (heteroduplex 2) functions extremely well. This indicates that only the antisense strand of the *let-7* duplex exerts a biological effect. The phenotype induced by heteroduplex 2 is even stronger than that induced by the *let-7* homoduplex. In contrast, injection of a heteroduplex containing two mismatches at the 5′ end of the sense strand (heteroduplex 3) resulted in a reduced phenotype. These data support recent work, which shows that both the absolute and the relative stabilities at the 5′ ends of siRNA or miRNA duplexes are critical determinants for incorporation of one or the other strand into RISC (36–38). Our experiments demonstrate that the *in vivo* activity of a miRNA is subject to the same criteria.

Silencing of a *gfp::lin-41* 3′-UTR reporter by *let-7*

The *let-7*/*lin-41* miRNA-target couple was experimentally verified in *C.elegans* (32), but conserved sequences are found in *Drosophila* and zebrafish (31). To show that injected *let-7* acts as a miRNA on the zebrafish *lin-41* 3′-UTR, we fused a 379 bp fragment comprising part of the zebrafish *lin-41* 3′-UTR and containing two *let-7* target sites (Figure 3A) to *gfp*. Co-injection of *let-7* with mRNA derived from this construct resulted in the translational repression of GFP expression (Figure 3B). The *gfp::lin41* 3′-UTR mRNA levels remained unaffected, whereas GFP protein levels were dramatically reduced upon *let-7* overexpression (Figure 3C). Silencing of *gfp* is dependent on the *lin-41* 3′-UTR, since *gfp* mRNA without the *lin-41* 3′-UTR was not silenced by *let-7* (Figure 3B). Furthermore, silencing of *gfp::lin-41* 3′-UTR could be blocked by the 2′-*O*-methyl oligonucleotide complementary to *let-7*, and the *mmlet-7* version did not silence *gfp::lin-41* 3′-UTR. We did not observe downregulation of the *gfp::lin-41* 3′-UTR reporter by endogenous *let-7* as shown by a recent study using mouse embryos transiently expressing *lacZ* miRNA sensor constructs (39). This is likely due to the fact that there are no detectable amounts of *let-7* expressed during these stages of development.

To investigate the interaction between *let-7* and its target sites in more detail, *gfp* was fused to a 100 bp fragment of the

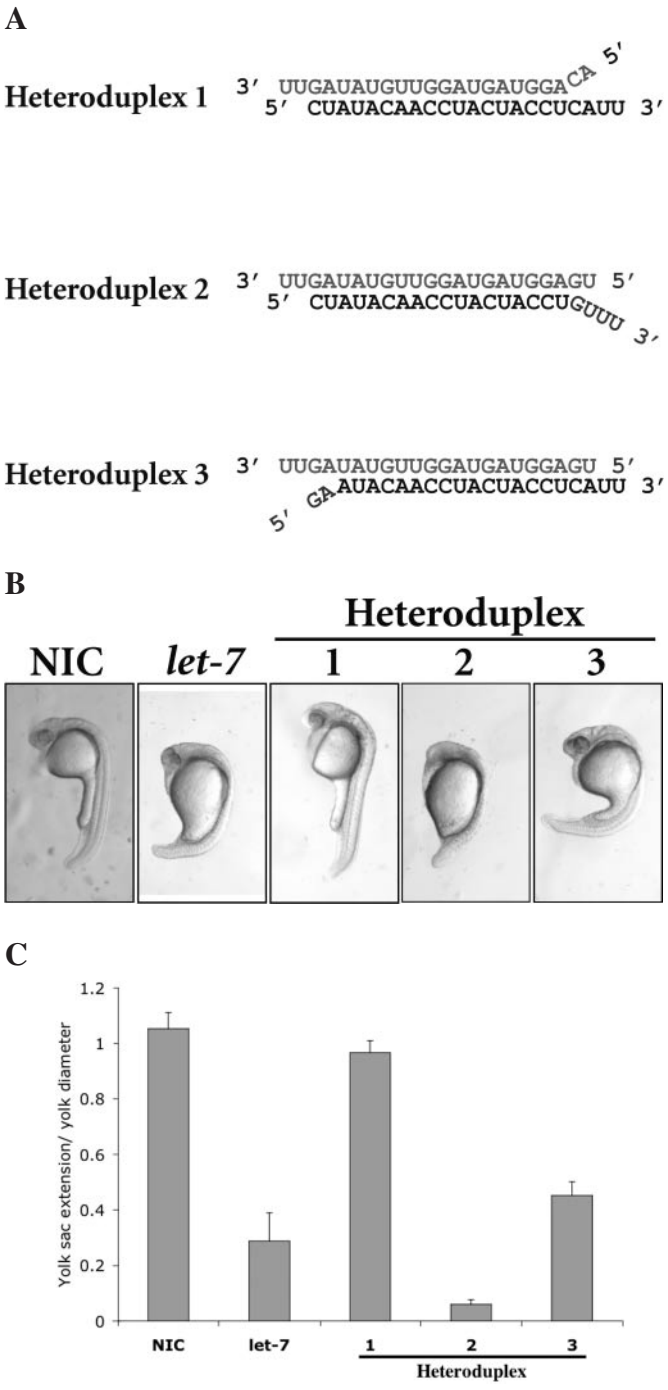

Figure 2. Phenotypic effects of injection of 3 *let-7* heteroduplexes. (**A**) Sequences of heteroduplexes. The upper strand is the antisense strand of the *let-7* duplex. (**B**) Phenotypes of 28 h zebrafish embryos injected with heteroduplexes. (**C**) Bar diagram showing relative length of the yolk sac extension of fish injected with *let-7* heteroduplexes.

lin-41 3′-UTR containing only the two *let-7* target sites including intervening sequence (*pgfp::lin-41* 3′-UTR-1). In addition, a similar construct was made that contains the same fragment, but with a point mutation in both *let-7* target sites (*gfp lin-41* 3′-UTR-2) (Figures 3A and 4A). mRNA derived from *gfp lin-41* 3′-UTR-1 could only be silenced by wild-type *let-7*, while expression of *gfp::lin-41* 3′-UTR-2 was inhibited exclusively by a *let-7* duplex with a compensatory mutation (*let-7mm5*, Figure 3D). In addition, duplex *let-7mm5* does not induce a phenotype in fish embryos. Taken together, our data show that injection of a mature *let-7* miRNA duplex can specifically induce gene silencing via the *lin-41* 3′-UTR in the developing zebrafish embryo.

We next asked whether the sequence intervening the *let-7* target sites is an essential component for silencing. In *C.elegans*, it was shown that silencing of a *lacZ* reporter fused to the *C.elegans lin-41* 3′-UTR is dependent on two specific *let-7* target sites and the 27 bp sequence between the target sites (33). In contrast, we found that a *gfp* reporter containing both *let-7* target sites from the zebrafish *lin-41* gene separated by a 5 bp sequence is still silenced by co-injected *let-7* (Figure 4A, *pgfp::let-7*tar1+2), while in the natural situation both *let-7* target sites are separated by a 57 bp sequence. Similar constructs containing either two copies of target site 1 or two copies of target site 2 fused to *gfp* were also silenced by *let-7* (Figure 4A, *pgfp::let-7*tar1-2x and *pgfp::let-7*tar2-2*x*), showing that both target sites are equally potent in silencing. However, reporter constructs with a point mutation (Figure 3A) in either *let-7* target site 1 or 2 could not be silenced by *let-7*, demonstrating that both target sites act together to mediate a silencing response (Figure 4A, *pgfp::lin-41* 3′-UTR-1 mut1 and *pgfp::lin-41* 3′-UTR-1 mut2). GFP expression from *pgfp::lin-41* 3′-UTR-1 mut1 and *pgfp::lin-41* 3′-UTR-1 mut2 cannot be downregulated by co-injection of a mixture of *let-7* and *let-7mm5*. This is probably due to a competition effect (i.e. *let-7* binding to the mutant target site and *let-7mm5* binding to the wild-type target site), since a mixture of *let-7* and *mir221* could silence a reporter construct with *let-7* target site 2 replaced by an artificially designed *mir221* complementary site (Figure 4A, *pgfp::let-7*tar1+*mir221*tar), whereas *let-7* and *mir221* alone did not affect the GFP expression from this construct. These data are in agreement with previous studies in cultured mammalian cells (40), showing that miRNAs can act cooperatively to repress gene expression, although our data differ from these studies because we used a combination of two copies of one target site instead of two copies of two target sites acting together.

Taken together, we demonstrate that injected *let-7* can act on the zebrafish homolog of the *lin-41* gene, but we have no way of knowing whether endogenous *let-7* silences *lin-41* *in vivo* or whether the phenotype we observe is the result of injected *let-7* acting on *lin-41*.

Silencing of reporters containing *let-7* targets in the coding region or 5′-UTR

To investigate whether silencing of the GFP reporter is restricted to the presence of *let-7* target sites specifically in the 3′-UTR, constructs were made with the *let-7* target unit comprising of both target sites with a 5 bp spacer inserted in the 5′-UTR (*gfp::let-7*tar 5′-UTR) and 12 bp before the stop codon in the *gfp* coding sequence (*gfp::let-7*tar cds) (Figure 4A). Surprisingly, both constructs could be specifically silenced by co-injected *let-7*, but not by *let-7mm5* (Figure 4B). These data indicate that GFP is specifically downregulated due to *let-7* sites in the coding region or the 5′-UTR. In the latter case, it cannot be ruled out that translation initiation is inhibited due

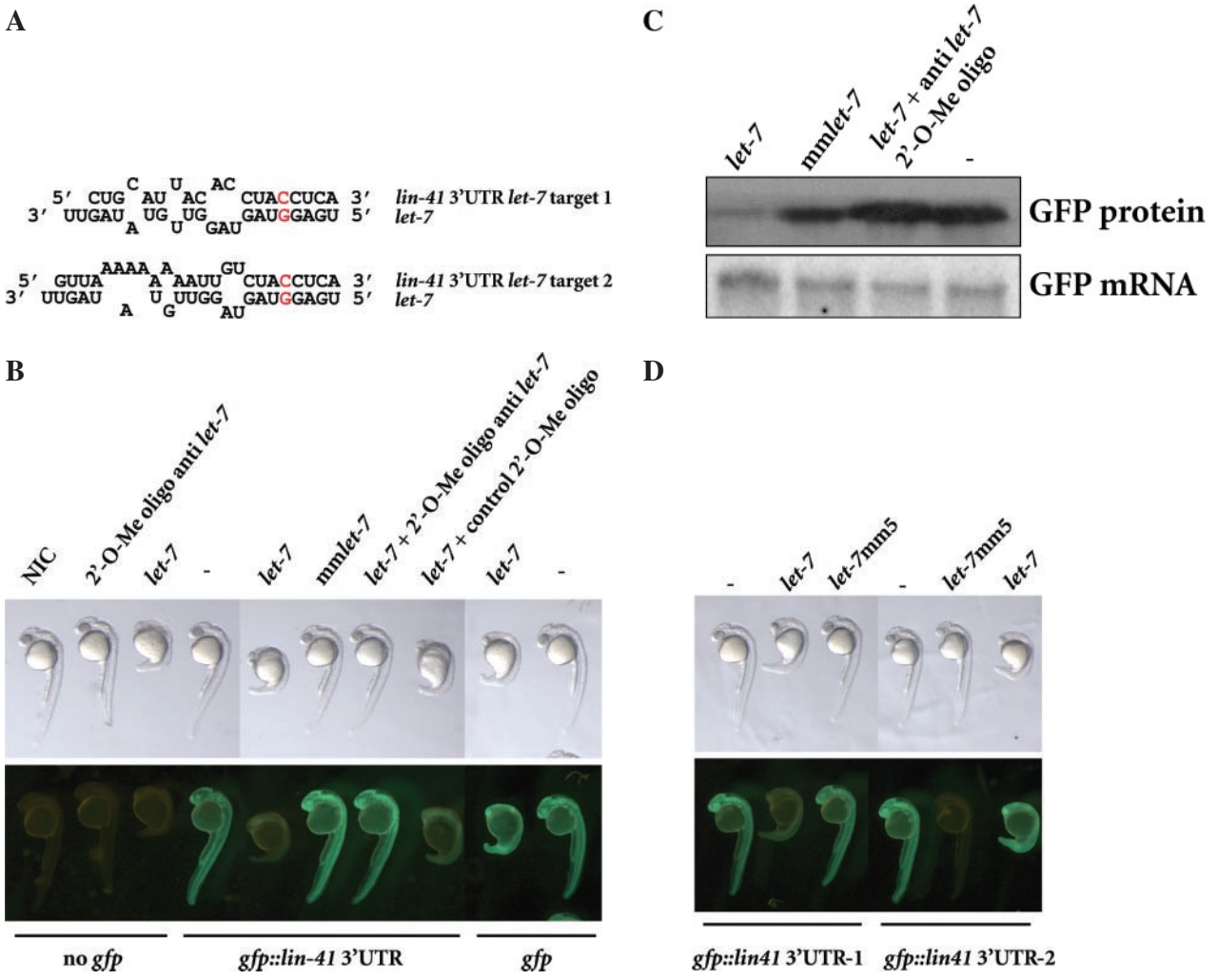

Figure 3. Effects of *let-7* injection on *gfp::lin-41* fusions and zebrafish development. (**A**) Structure of *let-7* bound to its target sites in the zebrafish *lin-41* 3′-UTR. The position of the point mutation in the target and *let-7* is indicated in red. At these positions the C in the target is changed to a T and the G in *let-7* is changed to an A. (**B**) Lateral views of 28 h zebrafish embryos injected with *gfp* with and without the *lin-41* 3′-UTR. The upper panel shows phenotypes and the lower panel GFP fluorescence. (**C**) Western and northern analysis of GFP in 28 h zebrafish embryos co-injected with *gfp:lin-41* 3′-UTR. (**D**) Phenotypes and GFP expression in 28 h embryos injected with mutant (*gfp::lin-41* 3′-UTR-2, *let-7mm5*) and wild-type (*gfp::lin-41* 3′-UTR-1, *let-7*) *let-7* and *gfp::lin-41* 3′-UTR fusions.

to steric hindrance of a *let-7* loaded RISC complex just upstream of the start codon.

In plants, it is known that miRNA targets are also present in the coding sequence of the mRNA, but plant miRNAs often target the mRNA for degradation via single complementary sites (41). No natural examples are known yet of animal miRNAs regulating a target gene via complementary sites in the coding sequence or 5′-UTR, although it was shown that siRNA off-target effects can be mediated through translational repression of mRNAs due to imperfect base pairing of the siRNA with the coding sequence (42).

In our zebrafish system, we find that miRNA-target sites do not necessary need to be located in the 3′-UTR, since *let-7* targets both in the 5′-UTR or in the coding sequence could induce a specific silencing response. This is important information for studies aimed at predicting miRNA targets, which are currently only focusing on 3′-UTR sequences. As also suggested by others (12,13,20), these should include 5′-UTR and coding sequences as well.

The 7 bases at the 5′ end of *let-7* are most important for its activity

To define the most important positions for functionality of the *let-7* miRNA, we performed a mutational scan by introducing point mutations at each of the 22 positions of *let-7*. For all 66 (3 × 22) mutants, we determined phenotypic defects and the influence on *gfp::lin-41* 3′-UTR mRNA translation (Figure 5A and B) as these assays are good indicators for the activity of *let-7*. Similar data were obtained by both the analysis of GFP protein level and the examination of phenotypic defects: mutations induced in the region spanning positions 1–7 from the 5′ end of the antisense strand of the *let-7* duplex eliminate the function of the miRNA (Figure 5). However, small growth defects were observed for *let-7* duplexes with mutations at position 1. Versions of *let-7* with a mutation at position 8 showed a strong phenotype but only a low reduction in the GFP protein level. Remarkably, the change of an A to a G at positions 3 and 7, enabling the formation of a G-U instead of an A-U base pair with the *lin-41*

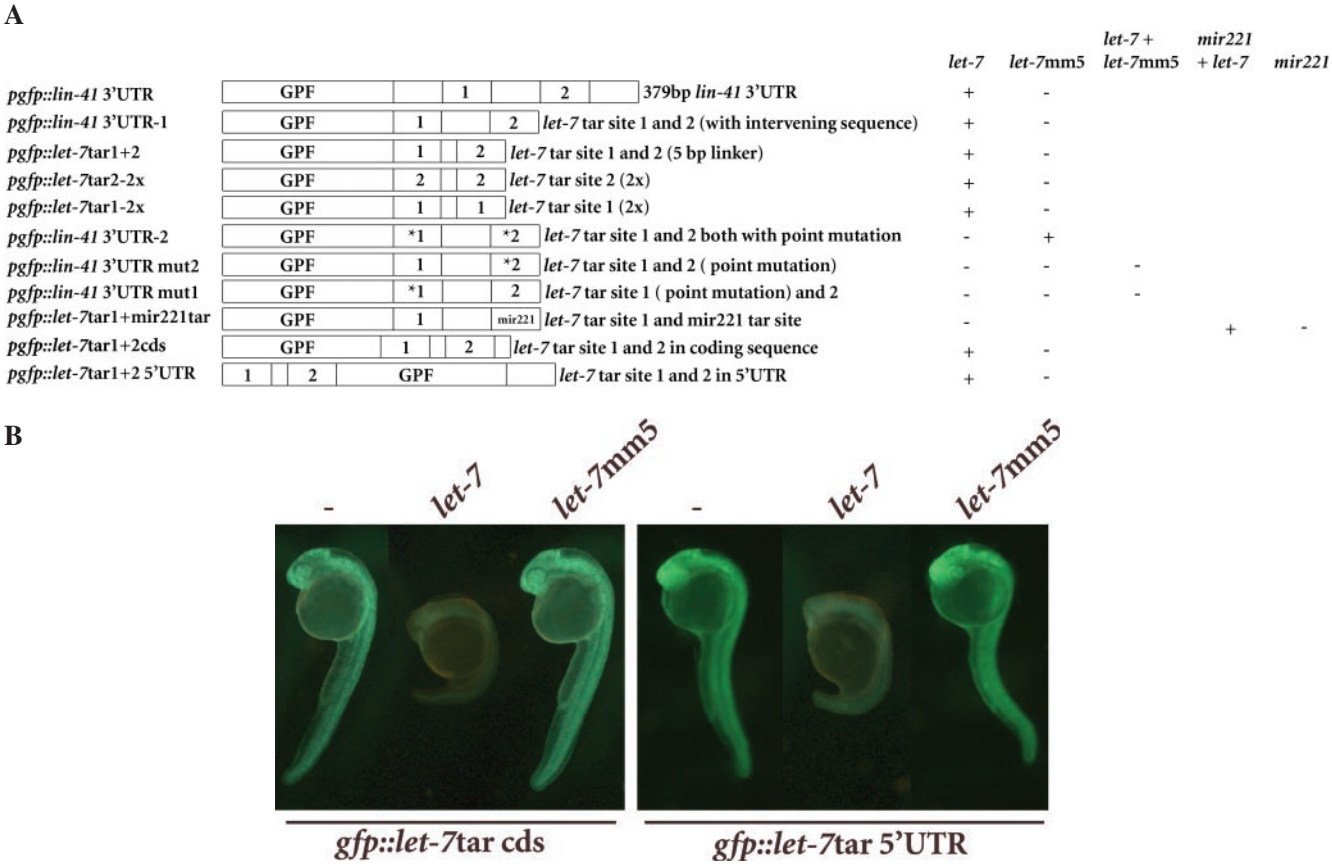

Figure 4. *let-7* target site 1 and 2 are sufficient for silencing. (**A**) Overview of constructs and miRNAs analyzed in this study. + indicates strong silencing, – indicates no silencing (**B**) GFP fluorescence in 24 h zebrafish embryos injected with *let-7* or *let-7mm5* and *gfp::let-7*tar 5′-UTR or *gfp*::*let-7*tar cds.

reporter construct, did not inhibit protein expression or affect normal development.

Most prominently at positions 10 and 13, GFP silencing and phenotypic effects were different for the 3 mutant *let-7* derivatives. These differences probably reflect changes in the *let-7* duplex free energy, which is relatively low for positions 9–14 in natural miRNA duplexes (36,37). For example, at position 10 the change of the original A-U base pair in the *let-7* duplex into an U-A base pair still causes strong silencing. However, the change to a G-C or C-G base pair increases stability of the duplex and decreases its activity. Similar although less pronounced effects were observed for mutations at position 9. Thus, mutations at certain positions in the miRNA duplex can affect its activity, not because of an altered interaction with the target RNA, but because of a change in the stability of the miRNA duplex itself.

Recently, the specificity of miRNA-target interaction was analyzed in cell culture using luciferase reporter assays (40). In this study, the miRNA target instead of the miRNA itself was analyzed for critical positions and, similar to our observations, these reporter studies indicated that base pairing of the first 8 nt in the 5′ region of the miRNA is most important for activity, although the binding of the 3′ end of a miRNA also appeared to contribute to gene silencing. These observations were made with reporter constructs containing multiple miRNA-target sites similar to our *gfp*::*lin-41* reporter. Investigation of rules governing the interaction of a miRNA with a single target site showed that interaction of the proximal (i.e. 5′) part of miRNA with its target is indeed the most susceptible to mutations (43). Only a target:miRNA mismatch in the 5′ part flanked by 4 bp on each side resulted in silencing of the reporter. Similarly, mutational analysis of the plant mir165/166 complementary site of *PHABULOSA* revealed that disrupting miRNA pairing near the 5′ region causes stronger developmental consequences and reduced miRNA-directed cleavage *in vitro* (44).

For our phenotypic assay, it is unclear whether the affected target mRNA(s) that cause(s) the phenotype contain one or multiple *let-7* targets. Our data add to the previous studies that irrespective of the targets, the major sequence determinants for *in vivo* function of *let-7* in zebrafish lie in the 5′ 1–7 residues.

This study represents the first complete mutational analysis of a miRNA in a vertebrate model organism. It is striking and unexplained that the *let-7* miRNA is perfectly conserved throughout evolution, while only mutations in the first 7 residues affect its activity. It could be that the sensitivity of both assays described here is too low, since they make use of a rather artificial over expression setup. Another explanation might be that the mutational spectrum was derived by injection of mature *let-7* miRNA sequences. The processing of the precursor into its mature form might also be affected by mutations in (the 3′ part of) *let-7*, which was not addressed by our screen.

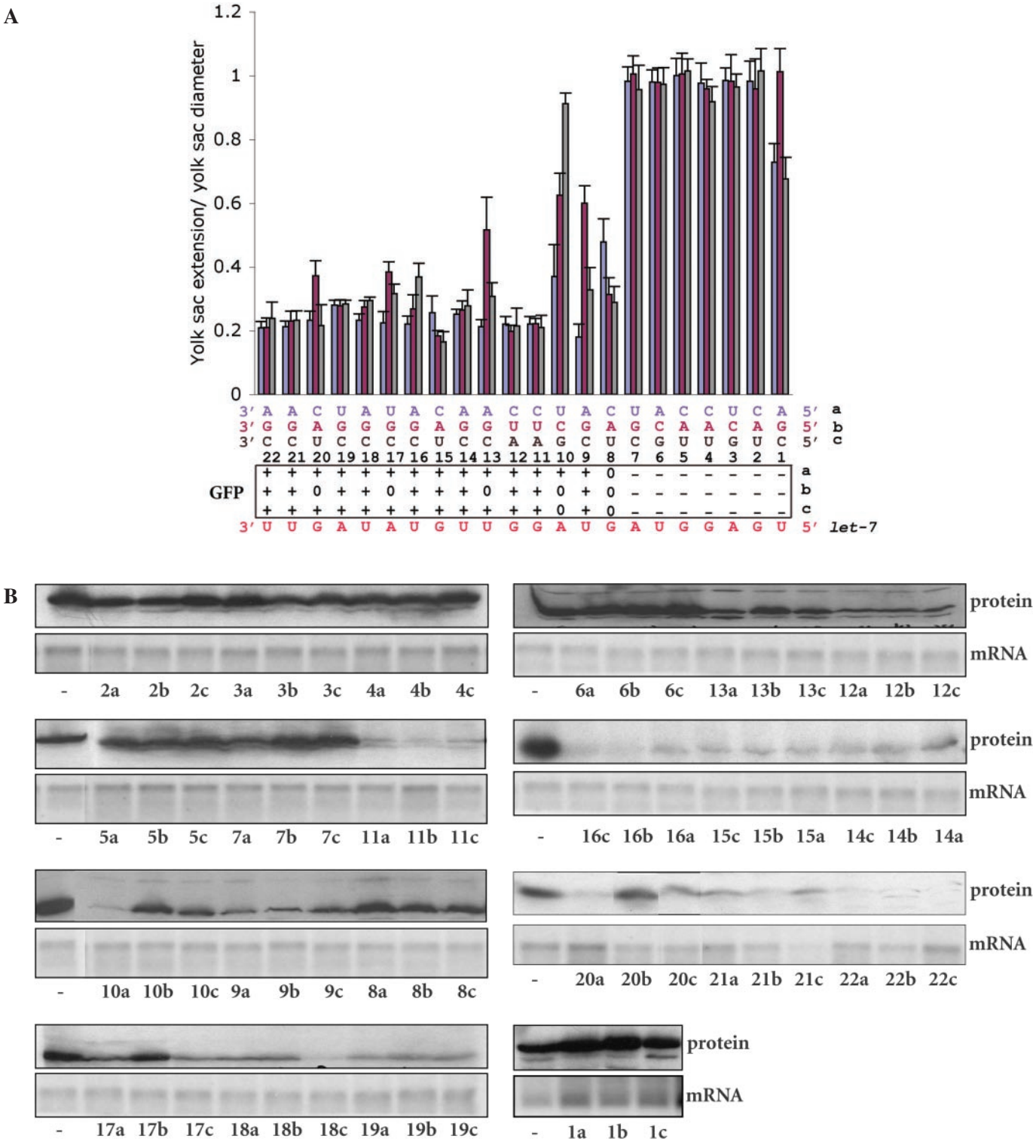

Figure 5. Mutational analysis of the *let-7* miRNA. (**A**) Phenotypes were scored by measuring the length of the yolk sac extension relative to the diameter of the yolk (bar diagram). For each data point, five individuals were measured. Approximate GFP levels, as assessed from western blots (B), are indicated in the table. – indicates that GFP protein level is similar to a control where only the *gfp::lin-41* 3′-UTR mRNA was injected; + indicates a significant decline of GFP protein level compared with the control; 0 indicates a moderate decline of GFP protein level compared with the control. At each position, the three types of possible mutations are shown in the sequences beneath the bars. The first series of mutants (purple bars and sequence a) has an equal number of hydrogen bonds compared with the wild-type *let-7* duplex. Both other series (dark red and gray bars and sequences b and c) have a different number of hydrogen bonds, depending on the nature of the mutation, i.e. U/A to C/G or vice versa. The *let-7* sequence is depicted in red. (**B**) Western blot analysis of GFP protein level for 3 × 22 mutant derivatives of *let-7*. Protein was extracted from 5 embryos. For each sample, 5 mg of protein was loaded. As an injection control, the level of *gfp::lin-41* 3′-UTR mRNA was determined by northern blotting. For each sample, 3 µg of RNA, obtained from 10 individuals, was loaded. Numbering is from the 5′ end of *let-7* and corresponds to that in (A).

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