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Inhibition of Zebrafish *fgf8* Pre-mRNA Splicing With Morpholino Oligos: A Quantifiable Method for Gene Knockdown

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Antisense morpholino oligonucleotides (MO) have been used successfully in zebrafish and *Xenopus* to knock down gene function by gene-specific inhibition of mRNA translation (Ekker, 2000). In addition to their ability to block cytosolic processes, MO can enter the nucleus (Partridge et al., 1996) and have been shown to be effective inhibitors of pre-mRNA splicing in mammalian tissue-culture cell lines (Schmajuk *et al.*, 1999). We show here that MO efficiently block pre-mRNA splicing in zebrafish embryos. Splice-blocking MO have the advantages that the efficacy of gene knockdown can be quantified without the use of antibodies, and that they specifically target zygotic, and not maternal, transcripts.

We targeted the *fgf8* gene (Furthauer *et al.*, 1997; Reifers *et al.*, 1998) with splice-blocking MO. An ENU induced mutation in *fgf8, acerebellar* (*ace*; referred to here as $fgf8^{it282}$), has previously been described (Reifers et al., 1998). $fgf8^{it282}$ is a splice donor mutation that results in the production of an aberrantly spliced mRNA (Reifers et al., 1998). Hence, blocking the same splicing event using MO should result in a phenotype similar to $fgf8^{it282}$.

We determined the *fgf8* exon/intron structure and designed two 25-mer MO complementary to the exon 2 and exon 3 splice donor sites (designated E2I2 and E3I3, respectively; Fig. 1a). Both MO span the exon/intron junction, including the most conserved residues of the splice donor consensus sequence (Fig. 1 legend).

We first asked whether splice site–targeted MO can alter splicing of *fgf8* mRNA. Using reverse transcriptase polymerase chain reaction, we found that injection of the E3I3 MO into early zebrafish embryos results in the production of two aberrantly spliced messages we term variant 1 and 2 (Fig. 1a, b). Sequence analysis of the corresponding cDNAs revealed that variant 1 results from excluding exon 3, whereas variant 2 results from the use of a cryptic splice donor located 32 bases 5′ of the normal exon 3 splice donor (Fig. 1c). Use of the cryptic splice donor creates a premature termination codon at the novel splice junction (Fig. 1c). Additionally, we found that injected splice site–targeted MO only alter the structure of zygotically expressed, but not maternally supplied, mRNA, as expected (Fig. 2d). Thus, E3I3 can

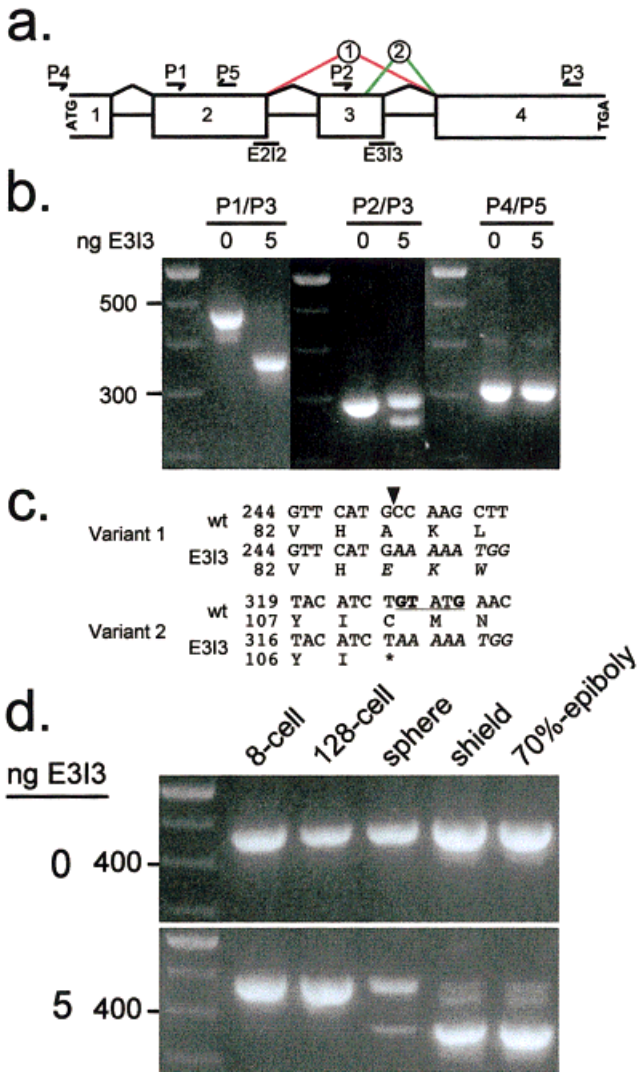

FIG. 1

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alter *fgf8* pre-mRNA splicing, resulting in transcripts that are either known (variant 1; Reifers et al., 1998) or predicted (variant 2) to produce nonfunctional gene products.

We next analyzed the phenotype of embryos injected with E2I2 and E3I3 both singly and in combination at doses ranging from 0.6 ng each to 10 ng each per embryo. At 24 h postfertilization (hpf), *fgf8*^{it282} mutant embryos lack the midbrain-hindbrain boundary (MHB), have reduced ears and subtle somitic defects. Significantly, their tails are of relatively normal length (Reifers et al., 1998). After MO injection, we distinguished three phenotypic classes (Table 1). Class I embryos quite precisely phenocopy *fgf8*^{it282} mutant embryos (Fig. 2b). Class II embryos had significantly shorter tails and disorganized somites (Fig. 2c). Class III embryos were characteristically small with extensive tissue necrosis (Fig. 2d). Hence, injection of *fgf8* splice-blocking MO results in dose-dependent phenocopy of *fgf8*^{it282} mutants as well as produces more severe defects.

We compared the amounts of wild-type *fgf8* transcripts produced by wild-type embryos, *fgf8*^{it282} mutants, and MO-injected embryos by ribonuclease protection. In preliminary experiments, we found that co-injection of E2I2 and E3I3 more efficiently reduced wild-type mRNA levels (data not shown), and thus we present only data from co-injected embryos. We designed the *fgf8* probe to span the exon 2/3 and 3/4 junctions to distinguish wild-type from aberrantly spliced RNA (Fig. 3a). We found that the amount of wild-type *fgf8* mRNA produced by *fgf8*^{it282} mutants was 32% of that produced by wild-type embryos, demonstrating that *fgf8*^{it282} is not a null allele. In contrast, co-injection of 2.5 ng each or 5.0 ng each of E2I2 and E3I3 resulted in levels of wild-type *fgf8* transcripts that were 7% and 2%, respectively, of that produced by uninjected embryos. Thus, reduction of wild-type *fgf8* mRNA levels to 7% of that produced by wild-type embryos, a level fourfold lower than

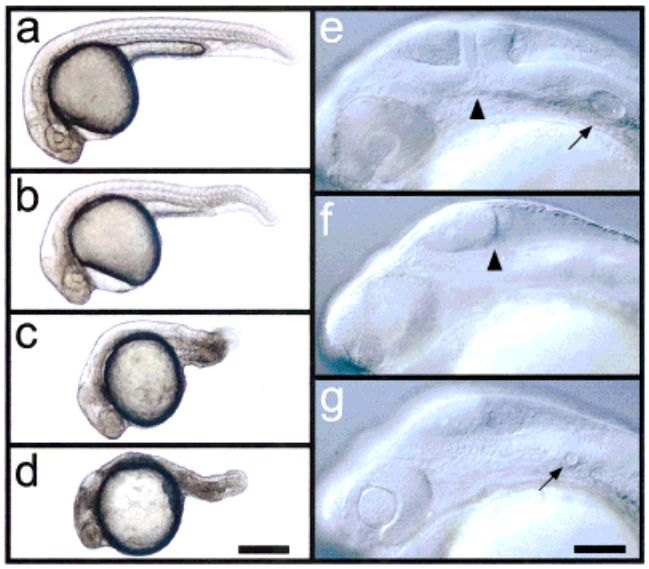

FIG. 2 *fgf8* splice-blocking morpholino oligonucleotides phenocopy *fgf8*^{it282} mutants. Uninjected live 24 hpf wild-type embryo (**a**) and embryos co-injected with E2I2/E3I3 MO (b–d) showing examples of the three phenotypic classes observed: (**b**) class 1, (**c**) class 2, (**d**) class 3 (see Table 1 for phenotypic criteria). High magnification of the head region of an uninjected wild-type embryo (**e**) showing the midbrain–hindbrain boundary (MHB, arrowhead) and the otic vesicle (arrow). Class 1 embryos show clear loss of MHB (**f**, arrowhead) and have smaller otic vesicles (**g**, arrow). Scale bars, 200 μm in a–d; 100 μm in e–g.

Table 1
Phenotypic Responses to fgf8 Morpholino Injections

Morpholino	ng injected	Wild-type	C1	C2	C3	*n*
Uninjected		100%	0%	0%	0%	150
E2I2	2.5	85%	15%	0%	0%	79
	5.0	3%	55%	42%	0%	73
E3I3	5.0	92%	6%	2%	0%	83
	10	42%	43%	15%	0%	83
E2I2/E3I3	0.6 each	100%	0%	0%	0%	76
	1.2 each	75%	25%	0%	0%	76
	2.5 each	4%	88%	3%	5%	69
	5.0 each	0%	0%	56%	44%	77

Injected embryos were scored 24 h postfertilization using a dissecting microscope and the following criteria: Class 1 (C1, Fig. 2a, f, g) embryos were phenotypically identical to *fgf8*^{it282} mutants; Class 2 (C2, Fig. 2c) embryos had significantly shorter tails than C1 embryos; Class 3 (C3, Fig. 2d) embryos were characteristically small and had extensive tissue necrosis.

FIG. 1 Splice-site-targeted morpholino oligonucleotides can alter splicing in zebrafish. (**a**) Genomic structure of zebrafish *fgf8*. Translation initiation and termination codons are indicated. We have identified an additional intron in the previously reported exon 1 (Reifers et al., 1998). Splice sites targeted by E2I2 and E3I3 are shown. Colored lines indicate splice variations observed in E3I3 MO-injected embryos. (**b**) Reverse transcriptase polymerase chain reaction (RT-PCR) analysis of *fgf8* mRNA structure in wild-type and E3I3 MO-injected embryos. PCR primer combinations are shown above lanes and their locations indicated in (a). In addition to wild-type (upper band in P2/P3), two splice variants are distinguished in injected embryos; variant 1 is detected using P1/P3, whereas variant 2 (lower band) is detected using P2/P3. (**c**) cDNA sequence comparison reveals that splice variant 1 lacks exon 3, whereas variant 2 results from aberrant splicing to an upstream cryptic splice donor (underlined). Arrowhead indicates correct exon2/exon3 splice junction. Exon 4 sequences are italicized. (**d**) RT-PCR analysis using the P1/P3 primer pair reveals that E3I3 MO alters *fgf8* mRNA structure beginning at the sphere stage (4 hpf), a point after the initiation of zygotic transcription (Kane and Kimmel, 1993). The faint, larger molecular weight bands detected in MO-injected shield and 70% epiboly lanes correspond to wild-type (upper band) and variant 2 (middle band) splice forms. Methods: Morpholino oligonucleotides were obtained from Gene Tools, LLC. (Corvalis, OR). The sequences of the MO are as follows (intronic sequences underlined): E2I2-TAGGATGCTCTTACCATGAACGTCG; E3I3-CACATACCTTGCCAATCAGTTTCCC. Before injection, MO were diluted in 1X Danieau solution (58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 5 mM HEPES, pH 7.6). Between 2.5 and 5 nl were injected into the yolks of one-to-four-cell stage *AB embryos. Total RNA was isolated from 10 hpf embryos (b), or embryos from stages indicated (d) using RNAwiz (Ambion, Inc., Austin, TX) according to manufacturer's instructions. cDNA for RT-PCR analysis was synthesized using Retroscript (Ambion) according to manufacturer's instructions. Numbers in (c) are according to Reifers et al., (1998).

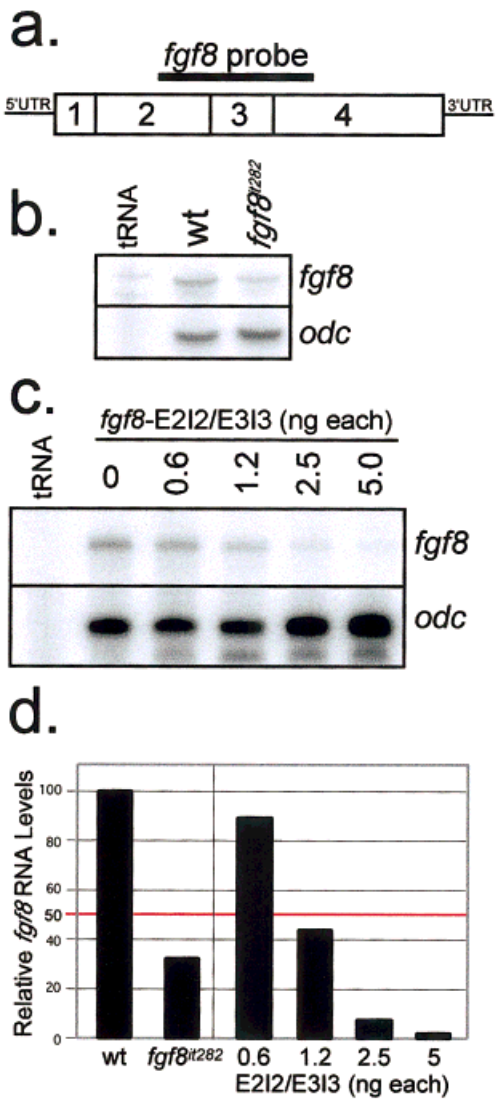

FIG. 3 Quantitation of *fgf8* mRNA levels. (**a**) Position of the *fgf8* RNA probe relative to the wild-type *fgf8* mRNA exon splice junctions. Comparison of *fgf8* mRNA amounts in wild-type and $fgf8^{it282}$ mutant embryos(**b**), or in embryos injected with a combination of E2I2 and E3I3 (**c**). Embryos used in this assay are the same as those scored in Table 1, with the exception that class 3 embryos were not included. (**d**) Amounts of wild-type *fgf8* mRNA levels in (b) and (c). Before comparison, the amount of *fgf8* mRNA in each lane was normalized with respect to the *ornithine decarboxylase (odc)* internal control. Method: Ribonuclease protection assays were performed using the RPA III kit (Ambion), according to manufacturer's instructions. After electrophoresis, the amount of protected fragments were quantified using a Storm 860 storage phosphor system (Johnson et al., 1990) with ImageQuant 4.2 software (Molecular Dynamics, Sunnyvale, CA). Antisense *fgf8* probe was generated by amplifying a fragment of the *fgf8* cDNA using the following primers: TTACACAGCATGTGAGTGAGC, GTACTTCACATTCTGCAGAGC. An EST of zebrafish *odc* was identified by the Washington University Zebrafish Genomic Resource group (clone fc54f04; M. Clark and S. Johnson, WUZGR; http//zfish.wustl.edu). *odc* is expressed at near constant levels during early development (B.W.D., unpublished observations). A template for producing antisense *odc* RNA probe was cloned into pCR2.1 vector (Invitrogen, Carlsbad, CA) after reverse transcriptase polymerase chain reaction using the following primers: GGATGTCCTGAAGAAGCACCT; CCCACTGACTGCACGATCTGG.

that produced by $fgf8^{it282}$ mutants, does not result in phenotypes that are significantly more severe than $fgf8^{it282}$ mutants. In contrast, reduction of *fgf8* mRNA levels to 2% of that produced by wild-type embryos results in more severe phenotypes. Further investigation will be necessary to determine whether the more severe phenotypes are due specifically to reduction of *fgf8* function. These data additionally suggest that the threshold amount of *fgf8* function that is required for normal development of the MHB and ear apparently lies between 66% (the amount estimated to be produced by heterozygotes $fgf8^{it282}$ embryos) and 32% of wild-type levels.

Hence, splice-blocking morpholinos provide a useful tool for studying gene function in zebrafish. Importantly, the efficacy of this method can be readily quantified, and, for the case we studied, MO can reduce functional mRNA levels to below that of the single mutant allele currently available. In addition to what we have demonstrated here, we propose that splice-blocking morpholinos may be effective tools for selectively preventing the production of an alternative splice variant and may prove more effective than translation-blocking MO for reducing the expression of genes that use multiple translational start sites.

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