Double-Stranded RNA Injection Produces Null Phenotypes in Zebrafish

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Zebrafish is a simple vertebrate that has many attributes that make it ideal for the study of developmental genetics. One feature that has been lacking in this model system is the ability to disable specifically targeted genes. Recently, double-stranded RNA has been used to silence gene expression in the nematode Caenorhabditis elegans. We have found that expression of the green fluorescent protein (GFP) from a microinjected plasmid vector can be suppressed in zebrafish embryos by the coinjection of a double-stranded RNA that is specifically targeted to GFP. To determine that double-stranded RNA can attenuate endogenous gene expression, single-cell zebrafish embryos were injected with double-stranded RNA specifically targeted to Zf-T and Pax6.1. We found that microinjection of double-stranded Zf-T RNA resulted in a high incidence of a phenotype similar to that of ntl. Furthermore, Zf-T gene expression could not be detected by in situ hybridization and the message was decreased by 75% by semiquantitative RT-PCR in 12-h embryos that had been injected with the double-stranded RNA. Expression of the zebrafish genes sonic hedgehog and floating head was altered in the embryos microinjected with the Zf-T double-stranded RNA in a manner that is remarkably similar to the zebrafish no-tail mutant. Microinjection of double-stranded RNA targeted to Pax6.1 was associated with depressed expression of Pax6.1 and resulted in absent or greatly reduced eye and forebrain development, similar to the phenotype seen in mouse mutants. Simultaneous injection of Pax6.1 and Zf-T resulted in embryos lacking notochords, eyes, and brain structures.

INTRODUCTION

Creating mutations in specific genes in the mouse through homologous recombination has significantly enhanced our ability to define the genetic pathways that regulate embryogenesis in vertebrates. Unfortunately, this process is expensive and time consuming and has not been applicable to other vertebrate species. A more efficient method for altering expression of specific genes, which could be used across the spectrum of vertebrate species, would be an extremely useful tool for the study of developmental genetics.

Successful large-scale chemical mutagenesis screens in the zebrafish have led to speculation that this organism will become the vertebrate equivalent of Drosophila for the study of developmental genetics (Nusslein-Volhard, 1994).

In order for this goal to be realized, as the zebrafish genome is further elucidated, it will become imperative to procure techniques for targeted gene knockouts to make maximal use of this vertebrate system. To date, only one technique is available for targeted interference with gene expression in the zebrafish. This technique employs a ribozyme to mediate a gene “knockdown” (Xie et al., 1997).

Recently, Fire et al. (1998) used specifically targeted double-stranded RNA to disable gene function in Caenorhabditis elegans. Double-stranded RNA was substantially more effective at preventing production of endogenous messenger RNA than either strand individually. The phenotypic consequences of disabled gene function were evident both in the injected adult and in their progeny. Very few double-stranded RNA molecules were required to produce an effect in any single cell. It is not known whether such a gene silencing technique is limited to invertebrates. This technique would be very useful in unraveling the genetic pathways that underlie specific developmental processes in the zebrafish where rapid ex utero development of...
MATERIALS AND METHODS

Generation of Double-Stranded RNA and Microinjection of Single-Cell Zebrafish Embryos

A pair of primers for the Zf-T gene was designed based on a unique portion of the coding sequence (Accession Number S57147). The partial cDNA fragment generated by reverse transcriptase-polymerase chain reaction (RT-PCR) from RNA of 8-h zebrafish embryos was 321 bp long (from nucleotide numbers 1764 to 2085). The sequence of upstream primer was 5'-TTGGAACACCTTGAGGGA-3' and the downstream primer was 5'-CCGTCACTTTCCAAGCGTAT-3'.

A 488-bp cDNA clone of the ntl insertion sequence (Accession Number X71596) was also generated by RT-PCR. This served as a negative control for the double-stranded RNA injection. The sequence of the upstream primer was 5'-ACCCATACACCCCCACCTC-3' and the downstream primer was 5'-ATAATAGGCCACCGGTCATG-3'.

Chimeric primers containing T7 promoter-specific sequence and GFP sequence were used to amplify a GFP fragment using PCR. T3 polymerase was used to simultaneously generate sense and antisense strands using this PCR-generated fragment as a template.

Double-stranded RNA was generated from the sequences that had been cloned into pGEM-T vector containing T7 and SP6 promoters. To produce the double-stranded RNA, two sets of PCR products were first generated from the cloned fragment (Fig. 1). Each was generated using antisense primers to the T7 or SP6 promoter sequence. The other primers used were specific to the 3' end of the inserted gene fragment relative to the promoter-specific primer being used. Each PCR product was purified on Centricon-100 columns as described in Publication I-259N (Centricon). Double-stranded RNA was generated by transcribing RNA strands from each template and then annealing the strands. In vitro transcription reactions were incubated at 37°C for 2 h. At completion of each RNA polymerase reaction, RNase-free DNase was added to the reaction to remove the DNA template. The reaction was incubated at 37°C for an additional 15 min and then at 70°C for 10 min to inactivate the DNase. RNA was purified by centrifugation through a QuickSpin column (Boeringer Mannheim). The double-stranded RNA was formed by mixing equal quantities of the single-strand RNAs that had been denatured at 70°C for 5 min in 80 mM KCl and incubating 1-2 h at 37°C. Prior to injection, the efficiency of RNA annealing was determined by RNase A (0.5 \( \mu \)g/ml) digestion for 15 min at 37°C (Fig. 2).

Individual single-cell embryos were microinjected with double-stranded RNA complexes under a dissecting microscope using pulled microcapillary pipettes to deliver the double-stranded RNA solution. The injected embryos were subsequently incubated in embryo medium (Westerfield, 1993) at 28.5°C for 12 h to 5 days.

RT-PCR

RT-PCR was used to quantify the message level after double-stranded RNA treatment. One hundred zebrafish embryos injected with double-stranded RNA targeted to Zf-T, control double-stranded RNA, or un.injected controls were collected at 10 h. RNA was extracted by RNA Easy minicolumn (Qiagen). RNA samples
were treated with 1 unit RNase free-DNase I/1 μg RNA, at 37°C for 15 min. The RNA was extracted with phenol/chloroform and precipitated with 2.5 vol of 95% ethanol and 50 ng RNA was used for PCR to confirm that there was no DNA contamination. RNA (0.1 μg) was combined with 50 ng of oligo(dT)15 primers in 10 μl water at 65°C for 10 min then cooled to room temperature for 5 min. The reverse transcription was carried out at 42°C for 1.5 h with 0.1 μg of total RNA, 0.1 μg of oligo(dT)15, 20 units RNasin, 200 μM dNTPs, 200 U SuperScript II RNase H reverse transcriptase (GIBCO BRL Inc.), 75 mM KCl, 3 mM MgCl2, 20 mM DTT in 50 mM Tris-HCl, pH 8.3, at a final volume of 20 μl. One unit of ribonuclease H was added to digest the RNA at 37°C for 30 min. The reaction was stopped by heating at 94°C for 5 min.

The PCR was performed in 50 μl containing 2 μl cDNA product from the reverse transcription, 200 μM dNTPs, 10 pmol primers, and 1.0 unit of Taq polymerase, 50 mM KCl, 1.5 mM MgCl2, and 0.001% gelatin in 10 mM Tris-HCl, pH 8.3. A GenAmp PCR System 2400 (Perkin Elmer) was used with the following program: 94°C, 1 min; 29 cycles at 94°C for 25 s, 56°C for 20 s, 72°C for 30 s; final extension was at 72°C for 7 min. The PCR product was separated on a 2% agarose gel. The PCR product of the Zf-T gene (Accession Number S57147) is 271 bp. It covers the cDNA region of Zf-T from base 1381 to 1750. The forward primer sequence was 5'-TTGATCTTGGCTTCAGGAG-3' and downstream primer was 5'-TGCAATGGTACCATGGGCTG-3'. Primers for zebrafish β-actin (Accession Number AF 025305) were upstream primer 5'-CCCTTGACTTTGAGCAGGAG-3' (starting from base 665) and downstream primer 5'-ACAGGTCTTACGGATGTCG-3' (ending at base 886). The PCR product size was 221 bp.

In Situ Hybridizations and Histology

The in situ hybridizations were performed as previously described (Wilkinson, 1992). Sense and antisense probes were generated from a region of the Zf-T gene lying outside the region targeted by the double-stranded RNA. Embryos were fixed in 4% paraformaldehyde at 4°C or Bouin’s fixative at room temperature. For histology, embryos were dehydrated in an alcohol series and then cleared in xylene. Embryos were embedded in paraffin and sectioned on a microtome at a thickness of 5 μm and were mounted. The slides were deparaffinized, rehydrated, placed in acid alcohol (1% HCl in 70% ethanol) for 5 min, and then rinsed in distilled water. Giemsa staining was performed as previously described (Vacca, 1985).

RESULTS

Targeted Double-Stranded RNA Specifically Blocks Transient Expression of GFP

Microinjection of the pEGFP-N1 into single-cell zebrafish embryos resulted in the transient expression of GFP in 85% of the embryos (Fig. 3). GFP expression was monitored by fluorescence microscopy throughout early embryogenesis. GFP-targeted double-stranded RNA was generated for the region shown in Fig. 1. When embryos were coinjected with pEGFP-N1 and 2.9 × 105 double-stranded RNA molecules, fewer than 3% of embryos had detectable GFP expression. Eighty-four percent of embryos coinjected with control double-stranded RNA showed abundant GFP expression.

When the embryos were injected at the 16-cell stage, injection of pEGFP-N1 alone or pEGFP-N1 with control double-stranded RNA resulted in embryos with scattered, 

FIG. 2. Demonstration of annealed Zf-T double-stranded RNA. Sense and antisense Zf-T RNA strands (lane 1) were treated with Rnase A before (lane 2) and after annealing (lane 4). Each lane was loaded with 0.5 μg of treated (lanes 2 and 4) or untreated RNA (lanes 1 and 3).
brightly fluorescent cells at 36 h of development (Fig. 3). Coinjection of GFP double-stranded RNA with the pEGFP-N1 completely quenched the GFP signal (Fig. 3). When Zf-T double-stranded RNA was coinjected with the pEGFP-N1 plasmid into single-cell embryos, the embryos had the ntl phenotype described below and showed brilliant GFP expression in scattered cells (Fig. 3). These results show that expression of a transiently transfected plasmid can be specifically attenuated by targeted double-stranded RNA.

**Targeted Double-Stranded Zf-T RNA Blocks Zf-T Gene Expression**

Zebrafish cDNA fragments for Zf-T were amplified using RT-PCR. A fragment of the inserted sequence that disrupts Zf-T in a ntl mutant allele was also cloned to serve as a negative control. The clones were sequenced to ensure their identity. To avoid targeting related genes, each sequence represented a unique portion of the gene; the Zf-T sequence lies outside of the T-box region (Fig. 1).
We found 71% of the zebrafish embryos that had been injected at the single-cell stage with approximately $10^4$ double-stranded RNA molecules generated from the Zf-T cDNA fragment had phenotypes that were grossly similar to those of the ntl mutant, i.e., truncated tails and disorganized somites (Fig. 4a). Injection of Zf-T single-stranded sense or antisense RNA, or double-stranded RNA generated from the ntl inserted sequence, did not lead to a significant incidence of this phenotype (Table 1). Simultaneous injection of sense and antisense RNA that was not annealed did not result in a significant incidence of the ntl phenotype.

After injection of the Zf-T double-stranded RNA, the Zf-T message was undetectable by in situ hybridization in 20% of the embryos (11/56) and weakly expressed in another 50% of embryos injected with Zf-T double-stranded RNA (Fig. 4b). Semiquantitative RT-PCR using $\beta$-actin to control for PCR efficiency and loading, showed an overall 75% reduction in the Zf-T message level from that seen in embryos injected with an unrelated double-stranded RNA (Fig. 5).

To determine whether the Zf-T double-stranded RNA had a global effect on gene expression, we coinjected it with pEGFP-N1 into single-cell embryos (Fig. 3). In these experiments, every embryo that exhibited a ntl phenotype also had significant GFP expression. This supports the view that the phenotypes generated by the injection of double-stranded RNA are not the result of nonspecific effects on gene expression.

**Phenocopy of ntl Generated by Injection of Zf-T Double-Stranded RNA**

To determine whether the mutant zebrafish generated by injection of Zf-T double-stranded RNA into single cell embryos phenocopied the ntl mutant, cross sections of 24-h embryos injected with Zf-T double-stranded RNA or control double-stranded RNA were examined. Embryos injected with the Zf-T double-stranded RNA generally lacked a fully developed notochord as is seen in naturally occurring mutants (Fig. 6), while those injected with the control double-stranded RNA had a notochord similar to that seen in uninjected embryos. Twenty-one percent of the embryos examined that had been injected with Zf-T double-stranded RNA completely lacked notochord (6/28). Somites in the Zf-T attenuated embryos were disrupted in a fashion similar to that seen in ntl zebrafish. The typical chevron appearance of the somites was lacking, but somites were not fused across the midline as is seen in floating head mutants (Figs. 6 and 7).

**Effect of Zf-T Double-Stranded RNA on the Expression of Sonic Hedgehog and Floating Head**

The expression patterns of shh and flh, two genes that are also essential to proper midline development in zebrafish, were examined by in situ hybridization. In 27% of the embryos examined (15/26), the expression pattern of shh throughout the floorplate of the embryos injected with the Zf-T double-stranded RNA was 3-4 cells wide (Fig. 8). This is identical to the expression pattern found for this gene in ntl embryos (Halpern et al., 1997). More than 50% of the
embryos examined had a similar, but less complete, alteration of shh expression. In the embryos injected with the control double-stranded RNA, in situ hybridization showed shh expression limited to a one-cell stripe along the midline as is found in wild-type embryos.

In wild-type zebrafish embryos, flh is expressed in the anterior and posterior nervous system and in a narrow axial strip. In 33% of the embryos examined (6/18), expression of flh in the Zf-T double-stranded RNA injected embryos was unaffected in the anterior and posterior nervous system but was greatly diminished or absent along the axis. Embryos injected with Zf-T double-stranded RNA also show diffuse and broadened flh expression in the tailbud (Fig. 9). This is similar to the expression pattern of flh found in the ntl mutant (Melby et al., 1997). This partial effect on flh expression was observed in more than 80% of the embryos examined.

Dose-Response for Generation of the ntl Phenotype

To determine the number of double-stranded RNA molecules required to generate a phenotype, single-cell embryos were injected with approximately 1 nl of a solution containing double-stranded RNA concentrations ranging from $1.6 \times 10^3$ to $5.0 \times 10^5$ molecules of double-stranded RNA/nl. Phenotype was determined for each embryo at 48-h postinjection. As can be seen in Table 1, embryos that had been injected with $10^4$ or more Zf-T double-stranded RNAs exhibited a very high incidence of the ntl phenotype. A grossly complete phenotype was observed in more than 20% and a partial phenotype was observed in 50% of these embryos (Table 1 and Fig. 10). Embryos injected with $4.0 \times 10^3$ double-stranded RNAs or less did not show a significant incidence of the ntl phenotype. Embryos injected with a control double-stranded RNA were phenotypically normal. Embryos injected simultaneously with sense and antisense single-stranded RNAs also did not display abnormal phenotypes.

### Targeted Double-Stranded Zf-Pax6.1 RNA Blocks Zf-Pax6.1 Gene Expression

Zebrafish Pax6.1 sequence lying outside the paired-box region was cloned for production of double-stranded RNA using procedures similar to those described above. Injection of double-stranded RNA of the zebrafish Pax6.1 resulted in embryos with grossly underdeveloped heads and absent or greatly diminished eyes (Fig. 11). Expression of Pax6.1 message was absent in embryos injected with double-stranded RNA, but undiminished in embryos injected with ntl double-stranded control RNA (Fig. 11). Twenty-five percent of the embryos injected with the Zf-Pax6.1 double-stranded RNA exhibited phenotypes.

### Targeted Silencing of Multiple Genes

Embryos that were injected simultaneously with Zf-T and Zf-Pax6.1 double-stranded RNA exhibited defective development of both the tail and the head, combining the phenotypic defects associated with mutations of either gene alone (Fig. 12). Examination of cross sections through the head confirmed that eye and brain structures were defective in embryos injected with Zf-T double-stranded RNA (Fig. 12). Cross sections through the tail region showed that notochord was lacking or greatly diminished and somites were disorganized (Fig. 12). There was a greater incidence of

<table>
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<th>Injection</th>
<th>Molecules (^a) ((\times 10^3))</th>
<th>Embryo number</th>
<th>Viable embryo</th>
<th>Phenotypic change</th>
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<td>296</td>
<td>239</td>
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<td>768</td>
<td>621</td>
<td>Partial 2 (0.6%) 0</td>
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<tr>
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<td>715</td>
<td>583</td>
<td>Full 3 (0.5%) 0</td>
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<tr>
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<td>708</td>
<td>587</td>
<td>None 2 (0.3%) 0</td>
</tr>
<tr>
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<td>959</td>
<td>815</td>
<td>Partial 5 (0.6%) 0</td>
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<td>468</td>
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<tr>
<td></td>
<td>0.8</td>
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<td>79</td>
<td>Partial 4 (5%) 2 (3%)</td>
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<td></td>
<td>4</td>
<td>328</td>
<td>288</td>
<td>Full 11 (4%) 2 (0.7%)</td>
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<td></td>
<td>20</td>
<td>257</td>
<td>229</td>
<td>Partial 23 (10%) 10 (4%)</td>
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<td>161</td>
<td>129</td>
<td>Partial 38 (29%) 12 (9%)</td>
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<td></td>
<td>100</td>
<td>1975</td>
<td>1618</td>
<td>Full 839 (51%) 322 (20%)</td>
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<tr>
<td></td>
<td>290</td>
<td>769</td>
<td>531</td>
<td>Partial 275 (51%) 163 (31%)</td>
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<tr>
<td></td>
<td>500</td>
<td>1206</td>
<td>822</td>
<td>Full 438 (53%) 287 (35%)</td>
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</table>

\(^a\) The injected RNA numbers for each embryo.

\(^b\) Single-stranded RNA of sense (S) and antisense (A) direction.

\(^c\) S/A indicates the unannealed sense and antisense RNA mixture.

\(^d\) Control (C) double-stranded RNA.
defects in the tail region (greater than 90%) than in the eye or head (25%). This demonstrates that multiple genes can be simultaneously targeted for diminished expression by injection of targeted double-stranded RNAs.

**DISCUSSION**

Double-stranded RNA has been shown to attenuate specific gene expression in C. elegans, Drosophila, Trypanosoma brucei, and planaria (Montgomery et al., 1998; Kennerdell and Carthew, 1998; Ngo et al., 1998; Alvarado and Newmark, 1999). These widely divergent organisms represent three different phyla of eukaryotes. The types of genes attenuated in these organisms include some encoding transcription factors and others that encode growth factor receptors (Montgomery et al., 1998; Kennerdell and Carthew, 1998). There is also evidence that double-stranded RNA may effectively silence gene expression in plants (Wassenegger and Pelissier, 1998; Watergyse et al., 1998). These results suggest that gene silencing through a mechanism that is initiated by gene-specific double-stranded RNA may be a universal phenomenon in eukaryotes.

We have now shown that targeted gene silencing can also be accomplished in a vertebrate embryo by injection of double-stranded RNA into single cell embryos. This method allowed us to disrupt the activity of specific genes encoding the zebrafish homologue of Brachyury, zebrafish Pax6.1, and the reporter gene, GFP. We also have shown that multiple genes can be targeted simultaneously using this method. In addition, embryos that were co-injected with a GFP expression vector and Zf-T double-stranded RNA developed ntl phenotypes while GFP expression was unaffected, showing that the effects of the Zf-T double-stranded RNA are not nonspecific.

Functionally attenuating expression of Zf-T resulted in a reproducible phenotype that mirrored that of the ntl mutant where the same gene was altered by an insertional mutation. Interestingly, we were able to use this inserted sequence as a negative control for these experiments as it produced no phenotype. By a number of criteria, more than 20% of all embryos injected with the Zf-T double-stranded RNA developed in a manner that phenocopied the ntl mutant. Zf-T gene silencing produced by injection of double-stranded RNA was apparent at the message level and by the specific phenotypes that were generated. In the zebrafish embryos, co-injection of unannealed sense and antisense RNA strands did not result in a high percentage of mutants. This is in contrast to results obtained in the nematode where phenotypes could be obtained when the sense and antisense strands were injected separately (Fire et al., 1998).

We would like to emphasize that none of the control treatments, i.e., single-strand sense or antisense, or control dsRNA showed any phenotypes at any concentration that we have tried. The only phenotypes we observed were after injection of Zf-T or Pax6.1 double-stranded RNA, and these phenotypes specifically match the treatment. In this regard double-stranded RNA targeted to Pax6.1 affected head and eye development with no apparent effect on tail development (Fig. 11). On the other hand Zf-T dsRNA affected tail development without any apparent effect on the head or eye (Fig. 4). Thus this treatment does not behave like a nonspecific, toxic effect which would be expected to yield similar phenotypes regardless of the double-stranded RNA injected.

GFP, a reporter gene encoding a protein that fluoresces when exposed to ultraviolet light, allows promoter activity to be monitored in living embryos throughout development.

**FIG. 5.** RT-PCR showing β-actin (lanes 1, 3, 5) and Zf-T (lanes 2, 4, 6) in wild-type (lanes 1 and 2), control double-strand RNA (lanes 3 and 4), and Zf-T double-stranded RNA (lanes 5 and 6) treated embryos. Lane 7 is the negative control for the RT-PCR. The Zf-T RNA expression is reduced by about 75% after Zf-T double-stranded RNA treatment compared to wild-type and control.

**FIG. 6.** Effect of Zf-T double-stranded RNA injection on development of the notochord in 24-h zebrafish embryos. Wild-type zebrafish and embryos injected with the control double-stranded RNA developed an obvious notochord. Ntl mutant embryos lacked a notochord along the entire body axis. Twenty-one percent of the embryos injected with the Zf-T double-stranded RNA also lacked a notochord along the entire body axis. Another 60% lacked notochord in the posterior portion of the body axis. Scale bars = 100 μm.
(Chalfie et al., 1994). Using this reporter gene, it has been possible to identify promoter regions that regulate gene expression in the zebrafish notochord, sympathetic neurons, and hematopoetic lineages (Meng et al., 1997, 1999; Long et al., 1997). We microinjected single-cell embryos with a plasmid vector containing GFP regulated by a promoter that drives ubiquitous expression during early development. We found that coinjection of double-stranded RNA specifically targeted to GFP suppressed expression of this reporter gene in well over 95% of the embryos injected with the plasmid vector.

While it might at first be thought that the GFP plasmid vector would be a good marker for the distribution of double-stranded RNA, the differences in these two nucleotides might predict great differences in their processing by the embryo for two reasons. The GFP plasmid is approxi-
FIG. 10. (A) The range of phenotypes observed in 72-h zebrafish embryos following injection of Zf-T double-stranded RNA. (B) Three embryos with a complete phenotype. Histological sections were used to confirm that the notochord was absent. (C) One embryo with a partial phenotype which was confirmed by histological section to have an anterior notochord.

FIG. 11. Absence of Pax6.1 expression results in severe abnormalities of head development. (A) The 48-h embryos injected with double-stranded control RNA had normal phenotypes. Microinjection of double-stranded Zf-Pax6.1 RNA into single-cell zebrafish embryos resulted in 25% of the embryos having reduced eyes often accompanied by diminished and disorganized forebrains. (B) In situ hybridizations demonstrated that 24-h embryos lack Zf-Pax6.1 expression following double-stranded Zf-Pax6.1 RNA injection into single-cell embryos, although double-stranded control RNA injection had no apparent effect on Zf-pax6.1 expression. Scale bars = 100 μm.
approximately 5000 bp of deoxyribonucleotides while the double-stranded RNA is composed of ribonucleotides and it is a very short piece by comparison (around 200 bp). Data from C. elegans (Montgomery et al., 1998) and our unpublished observations indicate that the double-stranded RNA has much freer access to the cell from the extracellular matrix than the GFP expressing plasmid. Whether this is because of the difference in nucleotide composition or size is not known.

The injection of Zf-T double-stranded RNA into single-cell zebrafish embryos resulted in greatly diminished expression of the Zf-T by in situ hybridization and semiquantitative RT-PCR, and in phenotypes that are very similar to those found in ntl zebrafish embryos (Herrmann et al., 1990; Halpern et al., 1993). Our in situ observations show embryos at 9 h. Most of the published ntl in situ show embryos at 11 h or later and the signal is stronger than that we have shown in Fig. 4. However, it seems clear from this figure that the ntl expression is completely absent after the double-stranded RNA injection and correlates perfectly with the phenotypes of the older embryos. This visual result is substantiated by semiquantitative RT-PCR showing that the Zf-T message is reduced to 75% of control level (Fig. 5). It should be emphasized that semiquantitative RT-PCR was done using pooled zebrafish embryos and the message levels would represent the whole range of phenotypes shown in Fig. 10. Thus a 70% reduction in message level seems quite reasonable.

Not only is the phenotype grossly similar to ntl, it is quite specific. In more than 70% of the embryos injected with the double-stranded Zf-T RNA, the notochord was absent or greatly diminished. Twenty percent of the embryos examined had no notochord. The somites in these embryos also lacked the characteristic chevron appearance observed in wild-type embryos, similar to the ntl mutant. The ntl phenotype can be distinguished from that of another tailless phenotype seen in the floating head mutant by the lack of fusion of the somites across the midline (Halpern et al., 1997). Embryos injected with Zf-T double-stranded RNA did not show fusion of the somites across the midline, providing a differential diagnosis for the ntl versus the similar floating head phenotype.

That these phenotypic characteristics were due to specific attenuation of Zf-T expression was supported by in

FIG. 12. Coinjection of the Zf-T and Zf-Pax6.1 double-stranded RNA generates embryos with defective notochords and eyes. (a) Zebrafish embryos injected with double-stranded Zf-T and Zf-Pax6.1 RNA developed significant defects in the eyes and tail. Zebrafish embryos shown are 5 days old. (b) Cross sections of 48-h embryos show that these defects included diminished eye and brain structures and (c) diminished or lacking notochord along with disorganized somites in the tail. Scale bars = 100 µm.
in a manner similar to that found in the zebrafish midline in these embryos and was also altered in the zebrafish midline in these embryos in a manner similar to that found in the ntl mutant. The expression patterns of these genes appeared to be identical to those found in the ntl mutant in approximately 25% of the microinjected embryos that were examined. The expression patterns were altered in a similar, but less complete, manner in more than 60% of the microinjected embryos.

Twenty-five percent of zebrafish embryos that were injected with Zf-Pax6.1 double-stranded RNA had underdeveloped heads and absent or greatly diminished eyes. In situ hybridizations confirmed that Pax6.1 expression was greatly diminished in more than 90% of the embryos injected with Zf-Pax6.1 double-stranded RNA. Pax6.1 expression was not affected in embryos injected with a control double-stranded RNA. The relatively low occurrence of phenotypes in embryos injected with Pax6.1 double-stranded RNA may be due to functional redundancy of the closely related Pax6.2, which is expressed in overlapping regions of the zebrafish embryo (Nornes et al., 1998). Interestingly, simultaneous injection of double-stranded RNA targeted to Pax6.1 and ntl gives a very clear compound phenotype that is quite distinct from the phenotypes resulting from injection of either double-stranded RNA separately. We believe that these data along with those from simultaneous injection of GFP expression vector with Zf-T double-stranded RNA in which the effect appeared completely independent are a compelling argument to support the idea that treatment with double-stranded RNA causes a relatively specific cellular response.

Although gene silencing has been observed in a number of eukaryotic species and may play a role in cosuppression in transgenic plants, a definitive molecular mechanism that mediates this effect has not been identified (Wassenegger and Pelissier, 1998; Montgomery and Fire, 1998). Recently, Montgomery et al. reported that double-stranded RNA induces specific RNA degradation (Montgomery et al., 1998). This conclusion was based upon the fact that DNA sequences in the targeted regions of the gene were not altered and that 100% of the F2 generation reverted to the wild-type phenotype. In addition, C. elegans has a unique genetic organization. Genes in this animal are organized in operons in which a single promoter controls expression of a number of genes. They showed that the double-stranded RNA affects only expression of the targeted gene.

In contrast, Tahara et al. have observed heritable effects of double-stranded RNA on the expression of a number of genes in C. elegans, suggesting that more than one mechanism may be involved in double-stranded RNA-mediated inhibition of gene activity (Tahara et al., 1998). It has been shown that cosuppression of gene expression in transgenic plants can be mediated through rapid degradation of the mRNA produced by the targeted gene (Smyth, 1997). Others have shown that double-stranded RNA-dependent sequence-specific methylation may mediate the long-term effects of cosuppression in plants (Smyth, 1997; Wassenegger and Pelissier, 1998). Such a methylase may also be dependent on transcription of the targeted sequence since double-stranded RNA targeted to promoter regions in nematode had no apparent effect on transcription. Montgomery et al. did not address the possibility that double-stranded RNA-dependent DNA methylation may play a role in gene silencing in C. elegans (Montgomery et al., 1998).

Certain types of double-stranded RNA, such as mismatched or poly(I)/poly(C) RNA, can be toxic in eukaryotic animals at much higher concentrations than we have employed in these experiments (Kuman and Carmichael, 1998). Although double-stranded RNA can induce interferon-α/β in nonimmune cells, this toxicity is primarily due to an immune system response mediated through interferon production in response to viral infections (Biron, 1998). Immune system or interferon-α/β-mediated toxicity is very unlikely to play any role in generating the phenotypes we have observed. First, the phenotypes that we have generated can be observed in 24-h embryos, long before the zebrafish immune system has been established. The thymus primordium appears in the zebrafish at approximately 54 h, but does not enlarge significantly until 30 h later (Willett et al., 1997b). Rag1 and Rag2 expression cannot be detected until Day 4, indicating a lack of mature T cells in the zebrafish until that time (Willett et al., 1997a). Second, the amount of double-stranded RNA that was used to generate the phenotypes is much less than is necessary to cause this interferon-mediated cell toxicity (Kuman and Carmichael, 1998). We have also found that poly(I)/poly(C) RNA can be toxic both in cultured 3T3 cells and in microinjected embryos. However, none of the 10 double-stranded RNAs that we have so far examined elicit a toxic effect in vitro or in vivo (unpublished results). Third, the phenotypes that have been generated for each gene under study differ substantially from one another and are specifically related to the gene that was targeted (Hermanny et al., 1990; Chalfie et al., 1994). Finally, injection of control double-stranded RNA at the same concentrations does not cause a detectable deviation from the wild-type expression levels or phenotype.

Whatever the particular molecular mechanism, it is clear that double-stranded RNAs can produce long-term disruption of function in specific genes in nematodes and zebrafish and may be an intermediary for cosuppression in transgenic plants. Nematode and zebrafish phenotypes generated using this technique are identical to those identified in randomly occurring natural mutants. Therefore, this technique seems to afford the opportunity to target genes of interest and produce specific mutant phenotypes in a wide variety of organisms. The ability to rapidly generate embryos that have essentially null phenotypes for multiple genes makes this a particularly powerful technology. This...
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method will be especially useful for dissecting genetic pathways that include multiple redundant genes.

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