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# Double–Stranded RNA Induces Specific Developmental Defects in Zebrafish Embryos

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**Treatment with double-stranded RNA (dsRNA) has been shown to interfere with the function of specific genes in various invertebrate species. However, it has not yet been reported that this technique can be applied to vertebrates as well. We have investigated whether dsRNA treatment will inhibit gene function in zebrafish embryos. By microinjecting dsRNA corresponding to three genetically characterised genes we produced embryonic defects that were similar to the known mutant phenotypes of these loci. The efficiency of inducing specific defects (20–30%) was about 10-fold higher than in experiments with antisense RNA. We also observed that the level of the endogenous mRNA in zebrafish embryos was substantially reduced throughout the embryo following dsRNA injection. However, the interference of gene function showed a strong dependence on the amount of dsRNA. These findings suggest that dsRNA-mediated interference will become an important tool for analysing the functional roles of genes in zebrafish and other vertebrates.** 

For various animal models different approaches have been used to directly manipulate gene expression *in vivo.* The most common strategy has been to microinject excess amounts of RNA (or other nucleic acids) that may cause gain- or loss-of-function of a particular gene. Whereas overexpression by mRNA injection can provide useful information about the gene's functional potential, attempts to generate loss-of-function phenotypes by such direct experimental manipulation have in many cases proved to be difficult or impossible. Recently it was discovered that double-stranded RNA (dsRNA) can be a potent and specific inhibitor of gene activity in the nematode *Caenorhabditis elegans* (1), and similar results have now also been reported for other invertebrates (2–4) and the protozoan *Trypanosoma brucei* (5). This type of RNA-mediated gene interference (RNAi) seems to involve a catalytic mechanism in which dsRNA function to target homologous mRNAs for degradation (5, 6). Furthermore, experimental evidence in plants suggests that dsRNA may have a more general role in gene regulation and antiviral processes, while in vertebrate systems dsRNA has been shown to induce a global antiviral response leading to general translational arrest and RNA degradation (7, 8). However, gene-specific dsRNA-mediated effects have not yet been reported for vertebrate species.

Among the vertebrates zebrafish (*Danio rerio*) has many advantages as a genetic model and these have been exploited in large-scale screens for developmental mutants (9, 10). In addition, a large number of zebrafish genes with putative developmental control functions have been cloned and characterised. Only a few of these genes have so far been shown to correspond to any of the known mutants. For most of the other cloned genes it has not yet been possible to determine the functional roles due to the lack of efficient experimental methods to generate loss-of-function phenotypes in zebrafish. In an effort to solve this problem we have investigated whether microinjection of dsRNA into fertilised zebrafish eggs can cause genetic interference during embryonic development. These experiments revealed that dsRNA treatment induces gene-specific defects at a relatively high frequency and the mechanism involves a reduction in the amount of endogenous mRNA. Hence dsRNA can be used to disrupt gene activity in zebrafish embryos and possibly in other vertebrates as well.

## MATERIALS AND METHODS

*RNA synthesis.* Linearized plasmids were used as templates for production of sense and antisense *pax2.1* RNA corresponding to the complete *pax2.1* cDNA. The templates used for dsRNA synthesis of ds-*ntl*, ds-*flh*, ds-*pax2.1*, and ds-*lacZ* were PCR products synthesised from primers amplifying limited regions of the cDNA sequence for each gene. All primers contained a T7 promoter sequence at the 5′-end (5′-TAATACGACTCACTATAGGGCGA-3′) which allows direct synthesis of dsRNA. The PCRs amplifying templates for ds-*ntl*

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and ds-*flh* were performed using a 6-9 hpf zebrafish cDNA library, while templates for ds-*pax2.1* and ds-*lacZ* were amplified from the plasmids pG3*pax2.1* (11) and CMV-$\beta$ (Promega), respectively. All DNA templates were purified using phenol/chloroform extraction and ethanol precipitation. RNA synthesis was performed using T7 or SP6 (antisense *pax2.1*) RNA polymerase (RiboMAX Large Scale RNA Production Systems, Promega). RNAs were complementary to contiguous cDNA sequences spanning from nucleotides: 360–1016 for ds-*ntl* (GenBank Accession No. S57147); 28–749 for ds-*flh* (GenBank Accession No. L48017); 966–1320 and 1–2409 (full-length) for ds-*pax2.1* (GenBank Accession No. X63961); 3155–3555 for ds-*lacZ* (Genbank Accession No. U02451). All RNA products were extracted with phenol/chloroform, ethanol precipitated and stored as precipitates at −80°C until immediately before use. Sense and antisense *pax2.1* RNAs were dissolved in nuclease-free water and mixed at equimolar quantities in annealing solution (5 mM KCl) to a final concentration of 0.45 $\mu$M each. 10 $\mu$l aliquots of the mixture were incubated at 70°C for 10 min, at 37°C for 30 min, and then phenol/chloroform extracted, precipitated and stored at −80°C. RNA produced from the double T7-PCR templates self-annealed during synthesis, hence no annealing reaction was necessary. Immediately before injection, RNA precipitates were dissolved in injection buffer (0.25 M KCl). All RNA products were tested by native agarose gels electrophoresis in TAE, and stained with ethidium bromide.

*Injections.* Adult fish were kept at 28.5°C on a natural 14-h light/10-h dark cycle and all embryos were obtained from natural matings (12). All embryos were injected at the 1–2 cell stage with 15 pg RNA/embryo (injection solution 50 ng/$\mu$l) and 60 pg RNA/embryo (injection solution 200 ng/$\mu$l). Embryos were incubated at 28.5°C in E3 medium (12).

*Phenotypic analysis.* Phenotypes were evaluated at 24–28 hpf. Features analysed included for *ntl:* disrupted notochord, abnormal somites and reduced tail (13, 14); *flh:* disrupted notochord and fused somites (15); *pax 2.1:* disrupted midbrain-hindbrain boundary (16). The general defects included gastrulation defects, bent tail, and reduction in convergence extension.

*X-gal staining.* For embryos injected with the $\beta$-galactosidase expression construct CMV-$\beta$ (50 ng/$\mu$l), X-gal staining was performed at 24 hpf as previously described (12).

*Whole mount in situ hybridisation.* Whole mount in situ hybridisation with *ntl* was carried out using digoxigenin (DIG)-labelled DNA probes as described previously (17) but at a higher annealing temperature (50°C). Embryos were harvested at 6 hpf, 10 hpf and 14 hpf.

*RT-PCR.* Embryos were injected with *pax2.1*-dsRNA complementary to cDNA position 966–1320. To analyse the effect of *pax2.1*-dsRNA on endogenous *pax2.1* mRNA, RNA was isolated from an equal number, 40, of injected and control embryos at 11 hpf and 26 hpf, using TRIZOL Reagent (GIBCO-BRL), phenol/chloroform extraction and ethanol precipitation. Reverse transcription (RT) was performed using *pax2.1* specific primer Pax2.1prd3′ (5′-AGAGGTGTTCCTGTGCCGTCGGAG-3′), 2 $\mu$g total RNA and M-MuLV reverse transcriptase (Promega). 2 $\mu$l of the cDNA reaction was used in each PCR reaction. PCR was performed with the primer Pax2.1-5′ (5′-TGCTATCCCATGGATATTCAC TGCA-3′) and the primer used in the RT reaction, generating a 479 bp product corresponding to cDNA segment 618–1096. PCRs were run 35 cycles at 94°C for 30 s, at 55°C for 30 s and at 72°C for 1 min, with a final extension at 72°C for 7 min. PCR products were analysed by electrophoresis in 2% agarose and subsequently visualised with ethidium bromide using the Gel Documentation System and Grab-IT (Ultra Violet Products), and quantified using Gelbase Pro (Ultra Violet Products). RT-PCR with primers specific for zebrafish *prosaposine,* a ubiquitously expressed gene (H.C. Seo, unpubl. res.), was used as an internal standard to normalise differences in template amounts used in the *pax2.1* RT-PCR.

*Northern blot hybridisation.* Northern blot was performed following the protocols of Sambrook *et al.* (18) and Ready-To-Go DNA Labelling Beads (Pharmacia). Total RNA was isolated from embryos at different developmental stages as described for RT-PCR (above). 10 $\mu$g RNA was loaded per lane on a 1% agarose/0.7 M formaldehyde gel, and separated by electrophoresis in 1 × MOPS buffer for 3 h at 60 mA. The RNA was transferred to a nylon membrane (Hybond-XL, Amersham Pharmacia Biotech) by capillary blotting in 10 × SSC, and fixed to the membrane by UV light exposure. Ethidium bromide staining of gel and filter to visualise rRNAs was done as a control for equal loading in different lanes (not shown). Hybridisation with [$\alpha$-$^{32}$P]dCTP labelled *pax2.1* specific probe (both strands) was done at 68°C overnight, followed by several washes at a maximum stringency of 0.1 × SSC/0.1% SDS at 65°C. Hybridised probes were visualised using the FLA-2000 Phosphoimager and Image Reader V1.3E (Fuji Photofilm), and quantified using Image Gauge V3.0 (Fuji Photofilm).

## RESULTS AND DISCUSSION

To determine whether dsRNA-mediated interference can occur in zebrafish, we assayed the effects of microinjecting dsRNA fragments corresponding to sequences in several cloned genes. In such experiments it may be difficult to make correct interpretations from the observed phenotypic abnormalities since microinjection of large amounts of RNA in zebrafish embryos has been shown to produce several unspecific defects (19). To distinguish between these different types of defects we used dsRNA derived from genes with known mutant phenotypes. Currently, only a few mutant loci in zebrafish are known to be allelic to any of the cloned genes. The majority of these genetically characterised genes are expressed maternally and/or initiates expression before the onset of gastrulation at $\sim 5\frac{1}{2}$ h postfertilisation (hpf). This early expression suggested to us that it would be possible to inhibit their function by dsRNA injection into 1–2 cell zebrafish embryos. Therefore, we included two of the genes belonging to this category, *floating head* (*flh*) and *no tail* (*ntl*), in our study of how dsRNA treatment may affect embryonic development. Partly these genes were also selected because of their easily recognisable mutant phenotypes. Hence, *ntl* mutants lack tail, notochord and have abnormal somite patterning (13, 14). Similarly, in *flh* mutants the notochord does not form and somites are fused medially under the neural tube (15). Both these genes encode transcription factors and initiate transcription about one h before the onset of gastrulation.

To assess the ability of microinjected dsRNA to interfere with gene function at later developmental stages we also analysed whether it was possible to produce specific defects for the *pax2.1/no isthmus* (*noi*) gene. Expression of this gene is known to be initiated at an early neurula stage (~9 hpf) (11), and the most obvious mutant defect is a deletion of brain tissues in the midbrain-hindbrain boundary region which includes the isthmus, tectum and cerebellum (16).

For all three genes that were tested, injection of dsRNA yielded some embryos with specific defects (Fig.

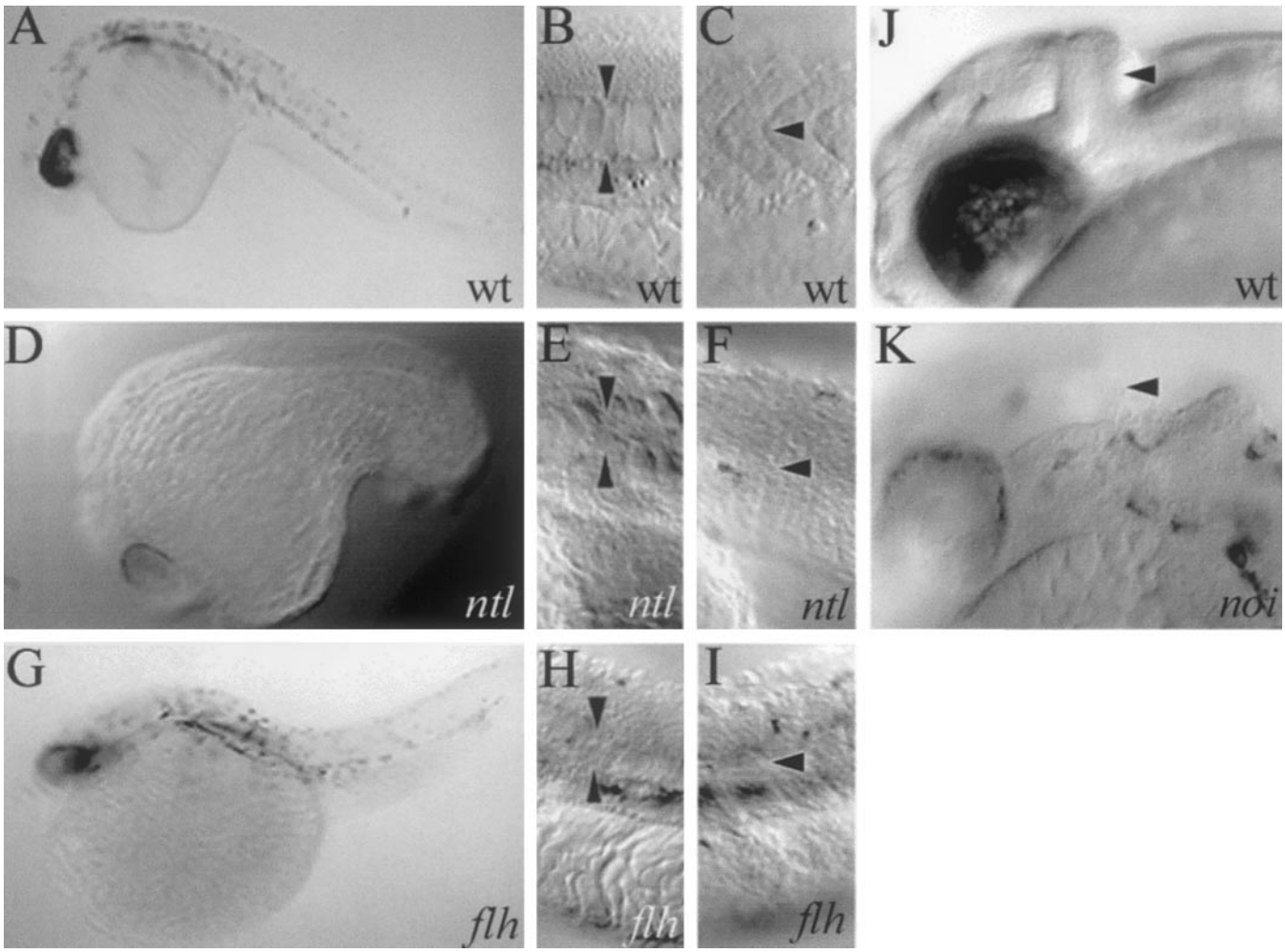


**FIG. 1.** Gene-specific defects caused by dsRNA treatment. The phenotypes of dsRNA injected embryos are compared with the typical morphological features of wild type (wt) embryos (A, B, C, J). Embryos injected with ds-*ntl* show abnormalities in the tail (D), notochord (E) and somites (F). The defects induced by ds-*flh* treatment include a characteristic bending of the tail (G), disrupted notochord (H) and fused somites (I). The *pax2.1*-dsRNA injected embryos show loss of tissue and normal morphological features at the midbrain-hindbrain boundary (K). Arrowheads mark equivalent regions that are affected in the injected embryos.

1). However, the individual genes showed variable degrees of induced phenotypes. Hence, injection of *ntl*- and *flh*-dsRNA caused complete absence of notochord in some embryos whereas others where not affected or showed only local defects within this axial structure (Fig. 1). These observations are similar to the results reported for *Drosophila* where both complete and localised phenotypes were induced by dsRNA (3). One major difference is that a significant proportion of the microinjected zebrafish embryos shows non-specific defects (e.g. bent tail, gastrulation defects). This effect is apparently not gene or sequence specific since also injection of ds-*lacZ* produced the same general defects at high frequencies (Table 1).

The induction of both types of morphological defects showed a clear dependence on dsRNA concentration. The smallest amount of dsRNA tested (~15 pg/embryo) induced specific phenotypes in less than 10% of the embryos and similar frequencies were observed for non-specific defects (Table 1). Four times more dsRNA (~60 pg/embryo) generally increased the frequencies of both types of defects to 20% or more (Table 1). A further increase in the amount of dsRNA to about 150 pg/embryo caused non-specific abnormalities in a majority of the zebrafish embryos (not shown). This high incidence was combined with high lethality at early developmental stages and prevented identification of individuals with specific phenotypes. When compared to results reported for *Drosophila* the amounts of dsRNA required for induction of specific defects in zebrafish were about 100 times higher (3).

One unresolved issue concerns the optimal length of the dsRNA fragments used for genetic interference experiments. Studies on different genes in various or-

**TABLE 1**

Induction of Gene-Specific Phenotypes by dsRNA Injection

| Gene | Segment, cDNA pos. | RNA, pg/embryo | # injected animals | Specific phenotype (%) | General defects (%) |
|---|---|---|---|---|---|
| *ntl* | 360–1016 | 15 | 193 | 2% | 0.5% |
| | 360–1016 | 60 | 163 | 27% | 21% |
| *flh* | 28–749 | 15 | 155 | 7% | 4% |
| | 28–749 | 60 | 106 | 23% | 36% |
| *pax 2.1* | 966–1320 | 15 | 114 | 5% | 16% |
| | 966–1320 | 60 | 188 | 31% | 39% |
| | 1–2409 | 60 | 79 | 19% | 19% |
| | 1–2409 (sense) | 60 | 76 | 0% | 3% |
| | 1–2409 (antisense) | 60 | 184 | 2% | 2% |
| *lacZ* | 3155–3555 | 15 | 83 | 0% | 1% |
| | | 60 | 94 | 0% | 32% |

*Note.* Embryos injected with different amounts of dsRNA or ssRNA were analysed by phenotypic observation at 20–28 hpf. The data shown were the results of several individual injection experiments. Injection of ds-*lacZ* was performed as a negative control to monitor the percentage of general defects. Classification of embryos with specific phenotypes was based on the following features: disrupted notochord, abnormal somites, and reduced tail (*ntl*); disrupted notochord and fused somites (*flh*); and disrupted midbrain-hindbrain boundary (*pax2.1/noi*). The general defects included gastrulation defects, bent tail, and reduction in convergence-extension.

ganisms suggest that there may be no general answer to this question. Our experiments with dsRNA fragments of two different lengths from *noi/pax2.1* also do not provide a basis for firm conclusions (Table 1). Although we observed a significantly higher frequency for the short fragment (31%) than the full-length fragment (19%) this could possibly reflect a difference in the number of molecules present since the total amounts of injected dsRNA were identical.

To make a better judgement of the value of dsRNA-mediated interference as a tool for gene function analysis in zebrafish it is necessary to compare with other methods. In this connection we investigated the effects of injecting single-stranded RNA (ssRNA) for *pax2.1*. The results showed that full-length antisense RNA induced specific defects at a much lower frequency (2%) than dsRNA (Table 1). However, further investigations are required to determine whether this 10-fold difference in efficiency is generally valid for zebrafish. Supporting our assumption that injection of antisense RNA is less efficient in producing loss-of-function phenotypes in zebrafish, only one case of successful application of this method has been reported (20). In other organisms a considerable variation has been observed in the efficiency of antisense RNA-mediated interference. Surprisingly, recent experimental evidence in *C. elegans* indicates that most of the effects produced by antisense RNA are actually mediated by dsRNA present at low levels in the *in vitro* RNA preparations (1, 7). Another possible explanation for the higher efficiency of dsRNA may be its resistance against degradation. This could partly also be the reason why general defects are produced more frequently by *pax2.1*-dsRNA than antisense *pax2.1* RNA (Table 1). Consistent with this we observed that in the stages between 11 hpf and 26 hpf, less than 50% of injected *pax2.1*-dsRNA is degraded (Fig. 4A).

Studies on both *C. elegans* and *T. brucei* have shown that the mechanism of dsRNA-mediated interference involves specific degradation of endogenous mRNA (5, 6). We have performed several experiments to investigate whether such mRNA degradation also occurs in dsRNA-injected zebrafish embryos. Co-injection of a *lacZ* expressing construct (CMV-$\beta$) with ds-*lacZ* lead to a strong reduction in the number of embryos with $\beta$-gal expression when compared to embryos injected only with CMV-$\beta$ (Fig. 2). This effect was not observed when ds-*ntl* was co-injected with CMV-$\beta$, suggesting that the reduction in expression depended on sequence specificity. Our RT-PCR assays for *pax2.1* demonstrated more directly how dsRNA can influence the level of endogenous mRNA. The estimated levels of RT-PCR products

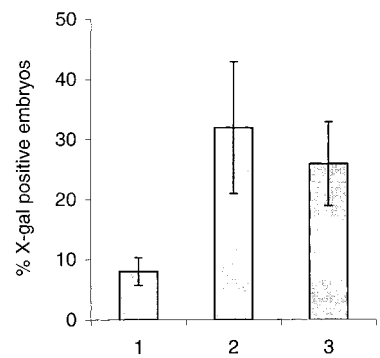


**FIG. 2.** Co-injection of ds-*lacZ* affects expression of a $\beta$-galactosidase expressing construct (CMV-$\beta$) in zebrafish embryos. Following injections at the 1-2 cell stage, embryos were stained for X-gal activity at 24 hpf. Embryos with general defects were discarded from the experiments. All injected embryos with positive X-gal staining were classified as positive. Results are shown for injection of 1: CMV-$\beta$ + ds-*lacZ*, 2: CMV-$\beta$ + ds-*ntl* (negative control) and 3: CMV-$\beta$. The numbers of injected embryos for each of these three experiments were: 128, 42, and 165, respectively.

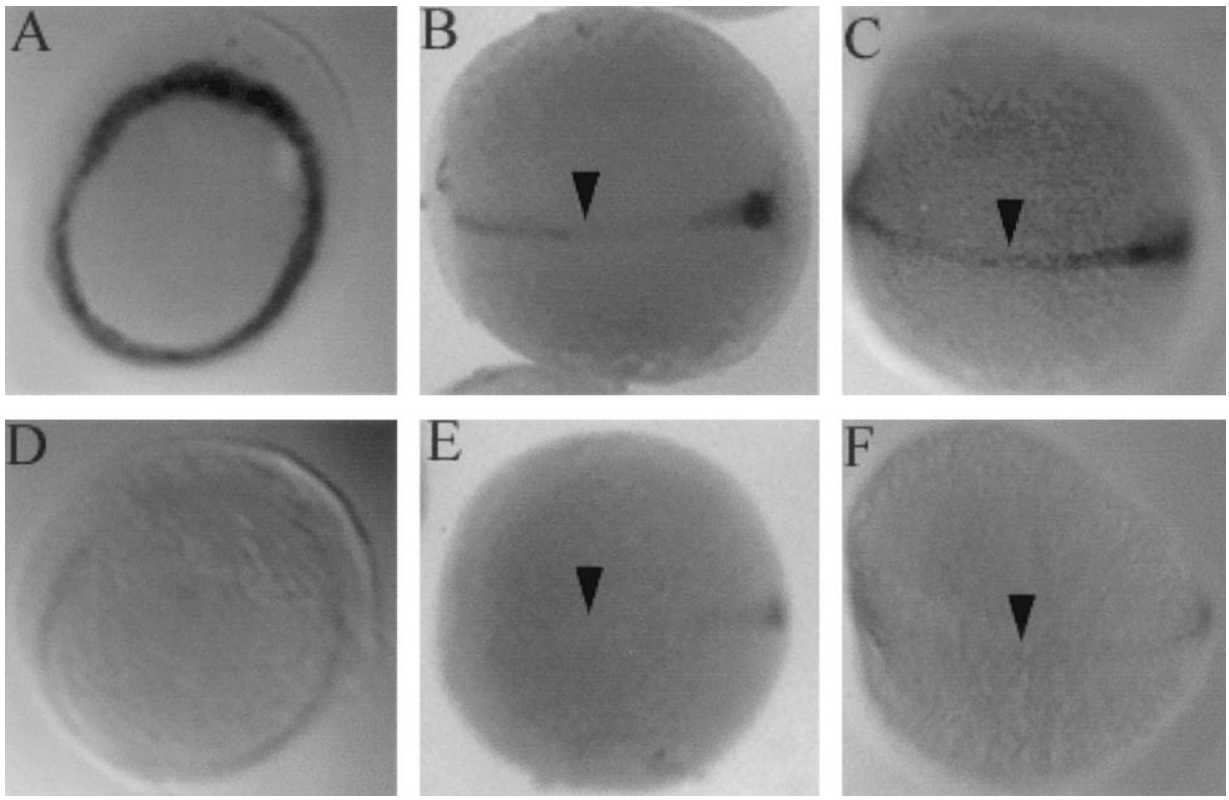


**FIG. 3.** Microinjection of ds-*ntl* affects distribution of the endogenous mRNA. The spatiotemporal distribution of *ntl* mRNA in zebrafish embryos was analysed by in situ hybridisation. Wild type (A–C) and injected (D–F) embryos of different stages, 6 hpf (A, D), 10 hpf (B, E), and 14 hpf (C, F), are compared. Ventral views are shown for the 6 hpf embryos. The other embryos are viewed from the dorsal side with anterior to the left. Arrowheads mark equivalent positions of the notochord.

derived from RNA extracted from *pax2.1*-dsRNA injected embryos showed a significant reduction (~70%) relative to the buffer-injected embryos at 11 hpf (Fig 4B). The reduction was less significant at 26 hpf (~30%) (Fig. 4B), which is consistent with the decrease of injected *pax2.1*-dsRNA seen at 26 hpf (Fig. 4A). We made further investigations by in situ hybridisation analysis to see if the mRNA levels of all the cells within a particular expression domain were affected. In the case of the *ntl* gene, which by 6 hpf during normal development is strongly expressed within a circumferential domain corresponding to the blastoderm marginal zone, most of the *ntl*-dsRNA injected embryos showed a reduction in staining intensity of more than 50% (not shown). This effect was generally observed throughout the entire expression domain (Fig. 3D), suggesting that the dsRNA is uniformly distributed to all the cells of the embryo at this developmental stage.

The observed reduction of endogenous mRNA most likely reflects a mechanism involving degradation and this ultimately leads to specific developmental defects. However, the proportion of injected embryos that shows specific abnormalities is relatively low (Table 1) and within this group many of the individuals only have localised defects. This limited phenotypic expression could be explained if reduction of the endogenous mRNA concentration below a critical threshold level must occur to affect normal development. Hence injected embryos with almost complete elimination of the targeted mRNA will be strongly affected whereas local/partial defects may be produced in other individuals that have higher and/or more variable levels.

It is also necessary to take into consideration the possible effects of temporal changes. Our analyses of the endogenous levels of *ntl* and *pax2.1* transcripts showed that the reduction is less extensive at later developmental stages (Fig. 3E, 3F, and 4B). Therefore, unless the targeted gene plays a critical role during the earlier stages specific defects may not be induced. In other cases where the gene function is required during a longer period the induced defect(s) may only partially mimic a complete loss-of-function phenotype.

In conclusion, we have demonstrated that dsRNA can mediate genetic interference in zebrafish embryos by a mechanism in which the level of the targeted endogenous mRNA is strongly diminished. In contrast to the results reported for *C. elegans* and *Drosophila* the induction of defects in zebrafish clearly depends on

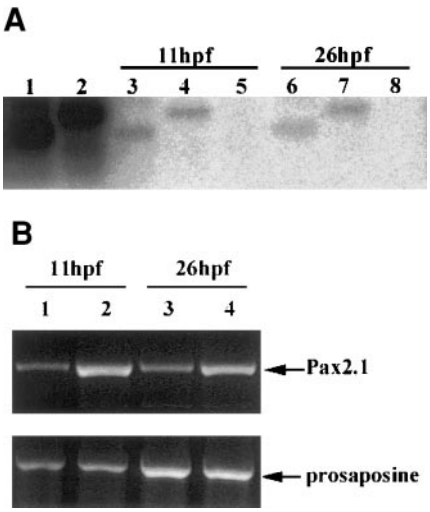


**FIG. 4.** Changes in the levels of ds-*pax2.1* (A) and endogenous *pax2.1* mRNA (B) following microinjection. (A) Stability of ds-*pax2.1* after injection into zebrafish embryos. Embryos were injected with two different ds-*pax2.1*, complementary to cDNA positions 966-1320 (I) and 1845-2363 (II), respectively, and RNA was isolated at the times indicated above the lanes. Following separation on an agarose/formaldehyde gel, dsRNA fragments were visualised by Northern blotting and hybridisation to a *pax2.1* specific probe (both strands). Lanes 1 and 2, dsRNA-I and -II (positive controls), respectively; lanes 3 and 6, embryos injected with dsRNA-I; lanes 4 and 7, embryos injected with dsRNA-II; lanes 5 and 8, embryos injected with buffer (negative controls). (B) Reduction of the *pax2.1* mRNA level after injection of dsRNA-I. Upper panel shows RT-PCR products from *pax2.1* mRNA, while the lower panel shows RT-PCR products of *prosaposine* which was used as an internal control for equal amounts of mRNA used in the RT-PCR reactions. Lanes 1 and 3, RNA isolated from embryos injected with dsRNA-I; lanes 2 and 4, RNA isolated from embryos injected with buffer.

the use of larger amounts of dsRNA. It also remains to determine whether dsRNA can be used to inhibit gene functions at later developmental stages. Despite these possible limitations dsRNA-mediated interference is likely to become an important supplement to the methods that are already available for analysing embryonic development in zebrafish.

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