

Silencing of Developmental Genes in *Hydra*

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Numerous developmental control genes have been isolated in a variety of organisms by either homology cloning or system-specific strategies. Functional genetic tests, however, are available for only a few model organisms and particularly are missing in a number of animals that occupy key positions for understanding the evolution of development and gene function. Double-stranded RNA-mediated interference (RNAi) opens a way to perform functional studies in such “nongenetic” organisms. Here we show that RNAi can be used to test the function of developmental genes in the cnidarian *Hydra*, a classical model for developmental studies. Introduction of double-stranded RNA corresponding to the head-specific gene *ks1* caused strong depletion of *ks1* transcripts. *ks1* loss-of-function polyps exhibited severe defects in head formation, indicating an important role of *ks1* in *Hydra* head development. Our results demonstrate for the first time efficient gene silencing in *Hydra*. RNAi provides an entry point for a variety of functional studies and a direct approach for analyzing the hierarchy of regulatory genes in *Hydra*, which until now has not been amenable to loss-of-function genetics. © 1999 Academic Press

Key Words: *Hydra*; pattern formation; *ks1*; double-stranded RNA-mediated interference (RNAi).

INTRODUCTION

Understanding evolutionary diversity depends on the ability to functionally compare the genetic basis of development in different organisms. The freshwater polyp *Hydra* is one of the most basal metazoan animals in which development is being studied intensively (Bosch, 1998). Numerous developmental genes have been isolated from *Hydra* by homology cloning. Among them are homeobox genes (Schummer *et al.*, 1992; Shenk *et al.*, 1993; Grens *et al.*, 1996; Gauchat *et al.*, 1998), *bHLH* genes (Grens *et al.*, 1995), winged helix genes (Martinez *et al.*, 1997), T-box genes (Technau and Bode, 1999), *Paired-class* genes (Galliot, 1995; Sun *et al.*, 1997; Galliot *et al.*, 1999), genes encoding protein-tyrosine kinases (Bosch *et al.*, 1989; Steele *et al.*, 1996; Stover and Steele, 1998), and *Ras*-related genes (Bosch *et al.*, 1995). Although these genes are highly conserved on the sequence level, it is not known whether they also function similar to their homologues in more complex metazoa or whether they have been used for different functions throughout evolution. In addition, several genes have been isolated from *Hydra* and exhibit a precisely controlled expression pattern during development, but are not related to genes in higher metazoa. One example is the head-specific gene *ks1*, which is sensitive to patterning signals along the apical–basal body axis and regulated by a complex interaction of inhibitory factors (Weinziger *et al.*,

1994; Endl *et al.*, 1999). Due to the lack of functional genetic tests, direct evidence for the role of a developmental gene in *Hydra* has not been obtained so far.

Recently, introduction of double-stranded RNA (dsRNA) into embryos was found to selectively disrupt the activity of the corresponding gene in a number of organisms (reviewed in Sharp, 1999). The effects of interference are observed in the injected embryos and their progeny (Fire *et al.*, 1998; Kennerdell and Carthew, 1998). Only a few dsRNA molecules are required in each affected cell, and suppression of gene expression can be seen in cells both immediately adjacent to the site of injection and at very distant sites (Sharp, 1999). To determine whether RNAi can be used for functional analysis of developmental genes in *Hydra*, we electroporated dsRNA corresponding to the head-specific gene *ks1* into polyps. Our results demonstrate that RNAi causes efficient *ks1* depletion in intact polyps. *ks1* loss-of-function polyps have defects specifically during head regeneration, but not foot regeneration, indicating that this gene is functionally involved in head development.

MATERIALS AND METHODS

Animals. *Hydra magnipapillata* polyps were cultured according to standard procedures at 18°C.

RNAi. For RNA synthesis, a DNA template was generated by PCR using the T3 and T7 primers and a vector containing the

whole *ks1* coding region. After gel purification, sense RNA and antisense RNA were produced from the PCR template. The PCR product (250 ng) was incubated with 2 μ l 10 \times NTP mixture (10 mM ATP, 10 mM CTP, 10 mM GTP, 10 mM UTP, pH 7.5), 2 μ l 10 \times transcription buffer (0.4 mM Tris-HCl, pH 8.0, 60 mM MgCl₂, 100 mM DTT, 20 mM spermidine), 1 μ l RNase inhibitor (20 units/ml), and 2 μ l RNA polymerase (T3 or T7 RNA polymerase, 20 units/ml) in a total volume of 20 μ l for 2 h at 37°C. All reagents were from Boehringer Mannheim. The DNA template was removed with a 15-min incubation with 2 μ l RNase-free DNase I (10 U/ μ l). After the reaction was stopped with 2 μ l of 0.2 M EDTA (pH 8.0), the RNA was precipitated overnight by adding 2.5 μ l of 4 M LiCl and 75 μ l of 100% ethanol. The RNA was collected by centrifugation for 30 min at 13,000 rpm, and the pellet was washed twice with cold 70% ethanol. After resuspension in 5 μ l DEPC-H₂O, sense and antisense fractions were combined and heated to 65°C for 15 min to eliminate secondary structures. The complementary strands were annealed in 750 mM NaCl, 75 mM sodium citrate by cooling the sample slowly to room temperature. Approximately 20 μ g of dsRNA was obtained using this protocol. Efficient annealing was examined by agarose gel electrophoresis.

Transfection of polyps by electroporation. We previously have demonstrated that reporter gene constructs can be introduced into polyps by electroporation (Brennecke *et al.*, 1998). For transfection of dsRNA in whole polyps, electroporation was carried out as described (Brennecke *et al.*, 1998) with the following modifications. Sixty polyps were placed in chilled electroporation cuvettes with a 0.4-cm gap and washed twice with 800 μ l DEPC-H₂O. Electroporation was carried out in 200 μ l DEPC-H₂O containing 10 μ g dsRNA. To minimize the possibility of degradation, dsRNA was added to the polyps just before electroporation. Whole polyps were pulsed with a Bio-Rad Gene Pulser (Bio-Rad) adjusted to an electric field strength of 0.95 kV/cm and 25- μ F capacitance. One pulse lasted for about 7 to 9 ms. Previous experiments using fluorescently labeled dextran as well as reporter gene constructs have indicated efficient uptake of macromolecules into hydra cells using this procedure (Brennecke *et al.*, 1998). Since electroporation causes cell loss and tissue damage, the polyps were transferred into 10 ml of hydra medium that was supplemented with 20% hyperosmotic dissociation medium (pH 6.9) containing 6 mM CaCl₂, 1.2 mM MgSO₄, 3.6 mM KCl, 12.5 mM *N*-tris-[hydroxymethyl]methyl-2-aminoethanesulfonic acid, 6 mM sodium pyruvate, 6 mM sodium citrate, 6.0 mM glucose, and 50 mg/ml rifampicin. To facilitate recovery, polyps were kept at 10°C for up to 3 days. Twenty-four hours after electroporation the medium was exchanged for standard hydra medium. Four to 6 days after electroporation polyps were fully recovered and used for experimentation.

Molecular techniques. Nucleic acid isolation and Northern blot analysis were carried out following standard procedures. As *ks1*-specific probe for RNA blot analysis we used the cDNA probe described previously (Weinziger *et al.*, 1994). Densitometric analysis of autoradiograms was carried by phosphorimager analysis. Whole-mount *in situ* hybridization was performed as described in Endl *et al.* (1999). Peroxidase staining in foot regenerating polyps was carried out as described (Hoffmeister and Schaller, 1985).

RESULTS AND DISCUSSION

Electroporation of ks1 dsRNA Causes Depletion of ks1 Transcripts in Intact Polyps

To determine whether dsRNA can suppress expression of endogenous genes in *Hydra*, dsRNA corresponding to the

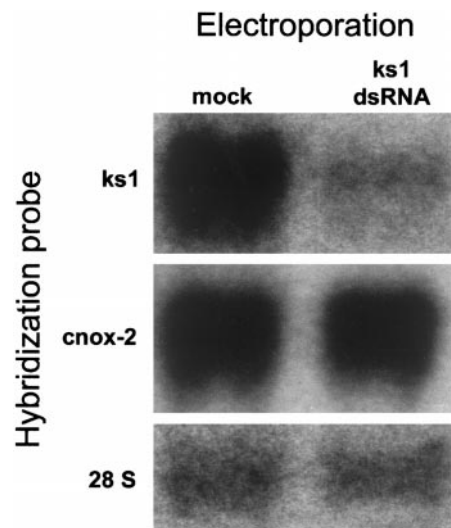


FIG. 1. Effects of *ks1* dsRNA on levels of endogenous mRNAs in intact polyps as analyzed by Northern blotting 6 days after electroporation. While *ks1* transcripts are depleted by *ks1* (RNAi), *Cnox2* transcripts are not affected, indicating gene-specific silencing. Similar results were obtained in three independent experiments. Equal loading is demonstrated by the 28S RNA.

head-specific gene *ks1* was introduced into whole polyps by electroporation. Effects of RNAi on the level of *ks1* transcripts were examined by Northern blot analysis using RNA from 40 animals per sample. Figure 1 shows a drastic reduction of *ks1* mRNA levels 6 days after electroporation. Densitometric analysis indicated depletion of about 90% of the *ks1* mRNA pool (data not shown). Similar results were obtained in three independent experiments. Expression of structurally unrelated genes such as *Cnox-2* was not suppressed by *ks1* (RNAi), indicating gene-specific silencing (Fig. 1).

The strong reduction of *ks1* levels observed by Northern blot analysis was confirmed independently in a separate set of RNAi experiments by *in situ* hybridization. As shown in Figs. 2A and 2B, mock electroporated control animals expressed *ks1* in the head and the base of the tentacles, which is the typical expression pattern (Endl *et al.*, 1999). In polyps electroporated with *ks1* dsRNA, however, *ks1* expression was strongly reduced 6 days after electroporation (Figs. 2C and 2D).

The efficiency of RNAi-mediated *ks1* silencing in *Hydra*, as shown in Figs. 1 and 2, is striking. Essentially all *ks1*-expressing cells appear to be affected, although the dsRNA was introduced by electroporation into presumably only a fraction of the cells. This indicates that the dsRNA must have spread throughout the tissue of the head region and probably the entire animal. A similar "spreading phenomenon" has been observed in plants and nematodes (Sharp, 1999), where RNAi suppression was found in cells

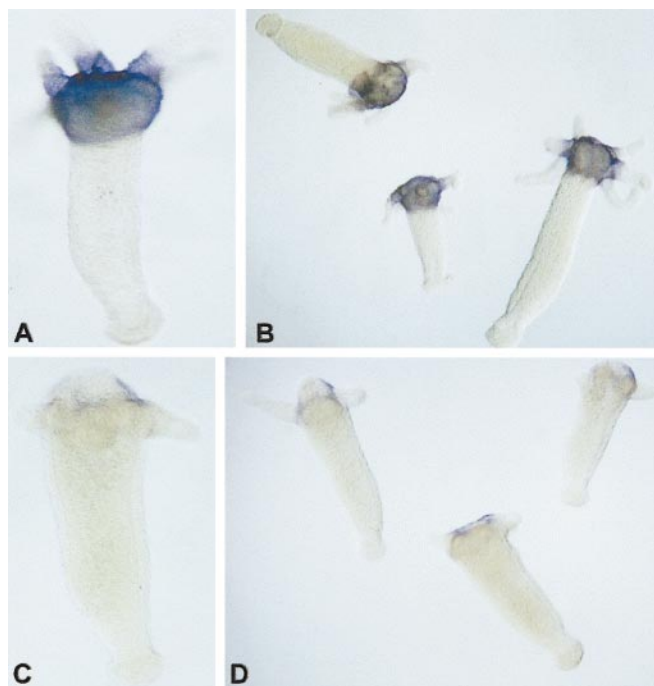


FIG. 2. Loss of *ks1* mRNA in RNAi-treated polyps as revealed by whole-mount *in situ* hybridization. (A and B) Controls (mock electroporated) showing *ks1* transcripts in head and tentacle cells; (C and D) equivalent polyps 6 days after electroporation of dsRNA targeting *ks1*. Polyps are depleted of most of the *ks1* transcripts.

both adjacent to the site of injection and at very distant sites.

***ks1*-Deficient Polyps Have Defects during Head Formation**

Ks1 is a target gene for head-specific signals and is expressed in ectodermal epithelial cells undergoing tentacle-specific differentiation (Weinziger *et al.*, 1994; Endl *et al.*, 1999). To test whether depletion of this gene by RNAi also causes loss-of-function phenotypes, we submitted an independent set of *ks1* depleted polyps to head and foot regeneration experiments. To examine the influence of *ks1* RNAi on head regeneration, polyps were decapitated directly below the tentacles 6 days after electroporation and allowed to regenerate for 36 h as shown schematically in Fig. 3A. *ks1*-deficient polyps had a strongly reduced capacity for head regeneration. The phenotypes varied significantly between individuals, possibly due to variability in the electroporated dose. As shown in Fig. 3B, only 15% ($n = 117$) of *ks1* depleted polyps had a fully developed head compared to 57% ($n = 81$) in control polyps. Moreover, 8% ($n = 117$) of the *ks1* depleted polyps had failed to form any head structures compared to 1% ($n = 81$) in the control. The percentage of polyps developing head structures in-

creased linearly with time after decapitation. At 72 h after onset of regeneration, both control and experimental polyps had fully developed heads. Thus, *ks1* RNA interference seems to delay head regeneration and tentacle formation rather than to disrupt them permanently. To determine whether the defect was head-specific, we examined the influence of *ks1* RNAi on foot regeneration. Feet were removed and allowed to regenerate for 39 h, a time after which foot formation was not yet fully completed. Thereafter, animals were fixed and stained for foot-specific peroxidase activity. No difference was detectable in the appearance of peroxidase-expressing cells in regenerating control ($n = 79$) and *ks1* depleted ($n = 88$) polyps. Thus, introduction of dsRNA into hydra cells appears not to inhibit developmental processes in general but rather causes gene-specific interference. RNAi using other developmental genes besides *ks1* also results in gene-specific depletion and loss-of-function phenotypes (J.U.L. and T.C.G.B., personal observation). Although the precise role of *ks1* remains to be

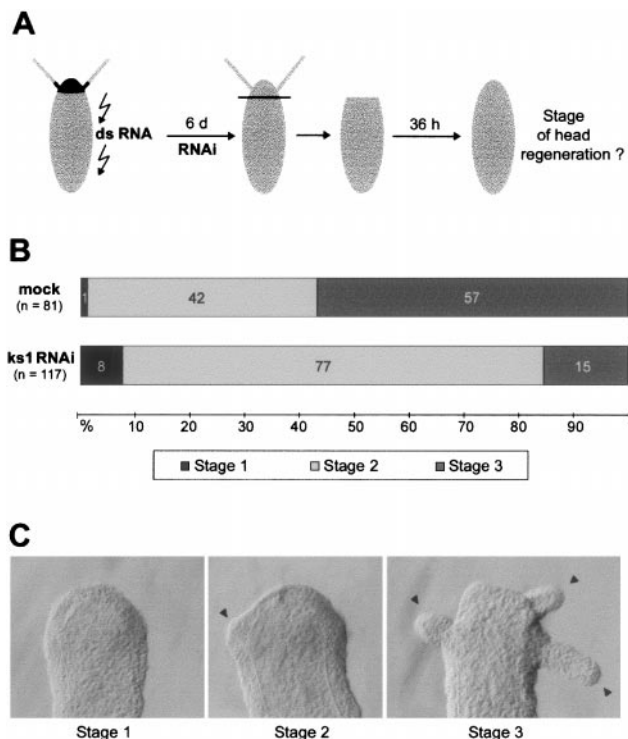


FIG. 3. *ks1*-deficient polyps have defects during head development. (A) Experimental design. Six days following electroporation and 36 h after onset of head regeneration, polyps were morphologically classified as stage 1 (no tentacles), stage 2 (tentacle buds), and stage 3 (fully regenerated head). (B) Histogram showing the percentage of polyps of the various developmental stages. *ks1* RNAi caused a severe delay in the regeneration of head structures. (C) Different morphological stages of head regeneration as observed 36 h after decapitation. Stage 1, having no tentacles; stage 2, having tentacle buds; stage 3, having a fully regenerated head.

identified, the results shown here indicate that this gene is functionally involved in head formation. We previously have suggested (Weinziger *et al.*, 1994) that *ks1* encodes a secreted protein that stays associated with the cell surface of ectodermal epithelial cells due to a large number of charged amino acids. We subsequently have proposed (Bosch, 1998) that the *ks1* gene product, by interaction with other proteins, might be involved in the change of cell shape during differentiation of gastric-specific into tentacle-specific epithelial cells. The defects in tentacle formation caused by *ks1* depletion (Fig. 3) support this idea.

In sum, our results demonstrate that by the use of RNAi, developmental genes can now be functionally analyzed in *Hydra*. Until now one has made use of the variety of tissue manipulations that alter expression patterns to deduce the role of a gene in hydra. Now RNAi provides a direct, and thus, more precise assay. This will be useful for many studies including those involving issues of the evolutionary conservation of gene function.

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