A muscle-specific transgenic reporter line of the sea anemone, *Nematostella vectensis*

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The sea anemone, *Nematostella vectensis*, has become an attractive new model organism for comparative genomics and evolutionary developmental biology. Over the last few years, many genes have been isolated and their expression patterns studied to gain insight into their function. More recently, functional tools have been developed to manipulate gene function; however, most of these approaches rely on microinjection and are limited to early stages of development. Transgenic lines would significantly enhance the tractability of the system. In particular, the study of gene- or tissue-specific promoters would be most useful. Here we report the stable establishment of a transgenic line using the I-SceI meganuclease system to facilitate integration into the genome. We isolated a 1.6-kb fragment of the regulatory upstream region of the *Myosin Heavy Chain1* (*MyHC1*) gene and found that the transgene is specifically expressed in the retractor and tentacle muscles of *Nematostella* polyps, faithfully reproducing the expression of the endogenous *MyHC1* gene. This demonstrates that the 1.6-kb fragment contains all of the regulatory elements necessary to drive correct expression and suggests that retractor and tentacle muscles in *Nematostella* are distinct from other myoepithelial cells. The transgene is transmitted through the germline at high frequency, and G_1 transgenic polyps have only one integration site. The relatively high frequency of transgenesis, in combination with gene- or tissue-specific promoters, will foster experimental possibilities for studying in vivo gene functions in gene regulatory networks and developmental processes in the nonbilaterian sea anemone, *Nematostella vectensis*.

meganuclease | myosin heavy chain | muscle development

The sea anemone, *Nematostella vectensis*, has developed into one of the most attractive model organisms among nonbilaterian animals. It can be easily reared in the laboratory (1), and its spawning can be induced reproducibly (2), providing daily access to thousands of embryos. The expression patterns of numerous genes have been determined, and recently, functional approaches of overexpression and Morpholino oligonucleotide–mediated gene knockdown have been established (3, 4). The genome of *Nematostella* was the first non-bilaterian metazoan to be sequenced (5), which revealed a remarkably slow evolutionary rate, reflected by a high level of conservation of individual gene sequences, exon–intron boundaries, and genomic organization. This corroborates the view that the common ancestor of Cnidaria and Bilateria was genetically complex, and that much of that complexity has been maintained in the sea anemone, *Nematostella* (5, 6). Thus, *Nematostella* is a prime model for comparative developmental biology aimed at reconstructing the last common ancestor of Bilateria and Cnidaria and identifying ancestral gene functions in animals.

A key question is the regulation of muscle cell differentiation in the absence of the third germ layer, the mesoderm. Morpholino-mediated gene knockdowns relies on the microinjection into the zygote and thus can target early, but not late, gene functions, yet muscles start to differentiate only during early metamorphosis. Transgenesis might provide not only a tool to overcome these limitations, but also an excellent way to monitor differentiation and movement of transgenic cell populations in vivo. Recently, stable somatic transgenic lines have been reported for *Hydra* that express GFP under the control of an *actin* promoter in specific cell lineages, depending on the site and timing of integration (7); however, to date no germline transmission has been reported for any cnidarian. Here we report the stable generation and germline transmission of transgenic lines of *Nematostella* expressing fluorescent proteins under the control of a muscle-specific promoter. The transgenic technology allows the dissection of gene promoters of interest and monitoring of the development of the specific cell populations in vivo in a nonbilaterian metazoan model system.

Results and Discussion

Generation of the Transgenesis Vector. In contrast to *Hydra*, in *Nematostella*, the injection of circular or linear plasmid DNA inefficiently integrates into the genome. Usually, integration is strongly facilitated by flanking transposable elements or other sequences that lead to excision and incision into the genome. A successful approach to achieving transgenesis that has proven highly successful in vertebrate and ascidian systems is the use of flanking binding sites for homing endonucleases from yeast, such as the I-SceI meganuclease (8–11). I-SceI recognizes an 18-bp-long nonpalindromic stretch of nucleotides, cuts in a sequence-specific manner, and integrates the inserted sequence into the genome by not-yet fully understood mechanisms (12). We constructed a versatile vector system in which both the promotor sequence and the reporter gene, flanked by inverted I-SceI sites, can be independently exchanged in a single cloning step (Fig. 1*A*). Recombinant I-SceI enzyme efficiently cuts at the inverted recognition sites and releases the insert.

Identification of the *Myosin Heavy Chain1* Gene Promoter. To gain insight into the regulation of muscle cell differentiation in a diploblastic animal, we aimed to identify a muscle-specific gene and isolate its promoter. Toward this end, we searched our EST collection (6) and found a partial cDNA clone of the *Myosin Heavy Chain type II* gene (*MyHC1*). Expression studies by whole-mount in situ hybridization in primary polyps and adult stages demonstrated that this gene is strongly expressed in retractor and tentacle muscles (Fig. 1 *B–D*). Thus, this gene is an excellent marker gene for studying the differentiation of muscle cells. To isolate the DNA sequence regulating the muscle-specific expression, a 1.6-kb genomic fragment upstream of the start codon was amplified from genomic DNA and cloned into the transgenesis vector to drive the expression of the fluorescent reporter protein mCherry (13). This

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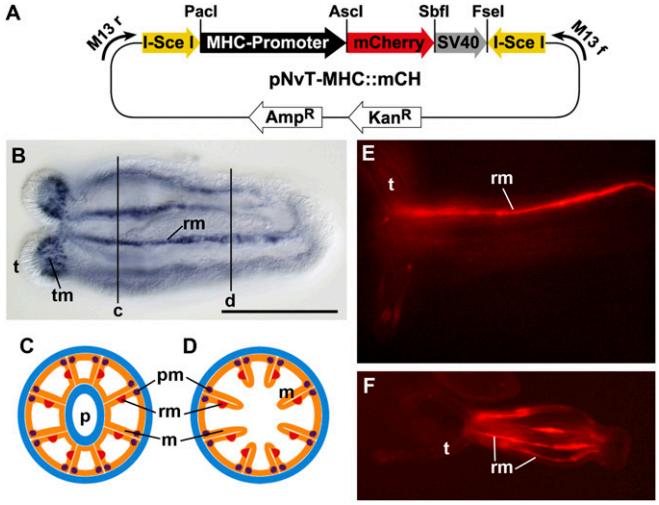

Fig. 1. Generation of transgenesis vector and establishment of G_0 transgenic animals. (*A*) Transgenesis vector consisting of a 1.6-kb fragment of *MyHC1* promoter region upstream of the start codon and an *mCherry* reporter gene, flanked by inverted binding sites of meganuclease I-SceI. (*B*) Whole-mount in situ hybridization detecting *MyHC1* expression in tentacle and retractor muscle cells in all eight mesenteries of a primary polyp. (*C* and *D*) Schematic cross-section through the body of a polyp at pharynx (p) position (*C*) and gastric position, as indicated in *B*. Endoderm is depicted in orange, ectoderm in blue, retractor muscles in red, and parietal muscles in purple. (*E*) mCherry reporter expression (red) in one mesentery of a primary polyp, reflecting a restricted transgenic patch. (*F*) mCherry reporter expression (red) in most mesenteries, demonstrating that large patches of transgenic tissue can be obtained in G_0. *B*, *E*, and *F* show a lateral view, with oral to the left. m, mesentery; *P*, pharynx opening; pm, parietal muscle; rm, retractor muscles; t, tentacle; tm, tentacle muscles. (Scale bar: 200 μm.)

construct exhibited consistent and strong expression and was used in subsequent experiments.

Transgenesis and Expression of Reporter Genes in G_0 Embryos and Primary Polyps. After the injection of a mixture of I-SceI enzyme and the transgenesis vector into zygotes, the onset of mCherry expression could be detected in somatic patches at about 24 h of development. These early patches of strong expression were most likely the result of transient expression from nonintegrated plasmid DNA. mCherry expression subsequently became restricted to smaller clones with clear cellular borders of expression after 5–9 days of development. Because mCherry protein is relatively stable, its transient expression can be detected for several days, longer than that for eGFP. *MyHC1::mCherry* expression in the mesenteries was restricted to longitudinal stripes, reflecting differentiating retractor muscles. In the tentacles, expression was specific to spindle-shaped retractor cells along the proximodistal axis. The number of mCherry-expressing mesenteries corresponded to the size of the initial somatic patch. Interestingly, even relatively small transgenic patches resulted in longitudinal stripes of mCherry expression spanning almost the entire body axis, suggesting that individual muscle cells have very long protrusions (Fig. 1 *E* and *F*).

Germline Transmission and Maintenance of the Transgenic Line in G_1 and G_2 In primary polyps, somatic patches of *MyHC1::mCherry* expression could be found in about 7.5% of the injected embryos (120/1,610) (Table 1) and were restricted to the forming mesenteries and tentacles (Figs. 1 *E* and *F* and 2 *A* and *B*). In *Nematostella*, all germ cells arise within the eight mesenteries in the endoderm; therefore, the chance of germline transmission is highest in animals with large endodermal patches. But because it is improbable that all germ cells within an animal are transgenic,

Table 1. Efficiency of I-SceI–mediated transgenesis of *MyHC::mCherry*

	Total injected *MyHC::mCherry*	%
Injected zygotes	1,610	100
24-h survival	1,442	90
24-h expression	582	40
Recovered primary polyps	539	34
Expressing primary polyps	120	7.5
Germline transmission*		8–40

*Germline transmission is given as the percentage of primary polyps with transgenic patches, which varies greatly because of the mosaicism of the transgenic germline.

only a subset of the gametes from transgenic G_0 individuals will carry the transgene. Consistent with these considerations, we found germline transmission in 8%–40% of all polyps that exhibited a somatic transgenic expression in the endoderm. Thus, the number of transgenic G_1 offspring was highly variable, indicating a mosaic germline (Table 1).

Transgenic G_1 polyps display eight longitudinal stripes of mCherry expression that correspond to the position of the retractor muscles within the eight mesenteries (Fig. 2*A*). Offspring of a cross of somatic transgenic animals with nontransgenic polyps can be easily identified by fluorescent stereomicroscopy (Fig. 2*B*), demonstrating that *MyHC1::mCherry* transgenics can be easily used for muscle mutant screens. Confocal images of tentacles of G_1 *MyHC1::mCherry* primary polyps revealed individual mononuclear muscle cells with long extensions expressing mCherry (Fig. 2*C*). This allows in vivo monitoring of individual contracting muscle cells as well as the differentiation of muscle cells; for instance, continuous peristaltic movements of the body column can be followed in vivo (Movie S1).

Primary polyps need 3–6 months to reach sexual maturity in adult polyps. Whereas expression of the transgene in the mesenteries was maintained throughout the complete life cycle, including sexually mature polyps, expression in the tentacles appeared to be much weaker or was barely detectable. Whether this indicates a shift of muscle differentiation in the tentacle between the primary polyp and the adult polyp is unclear. To study the expression of the transgene in adult polyps in more detail, we prepared cryo cross-sections and performed immunostaining against the expressed mCherry protein. The results confirm that the *MyHC1* promoter drives transgene expression in the retractor muscles of the mesenteries (Fig. 2 *E* and *F*). The endoderm of the body wall and the proximal mesentery also may express the transgene at a low level, detectable only by antibody staining against the stable mCherry protein (Fig. 2*E*).

Transgenesis using transposons often leads to multiple genomic integrations that segregate by subsequent outcrossing, whereas injection of plasmid DNA can lead to concatemeric integration (7, 14, 15). The 18-bp specific binding sequence of I-SceI (16) was not found in the *Nematostella* genome, as in the genome of any vertebrate where the meganuclease has been used for transgenesis (17). Southern blot analyses of single transgenic G_1 polyps were performed to determine the number of integration sites in the genome. Because an antisense probe against the *mCherry* transgene could possibly cross-react with the *GFP*-like genes present in the *Nematostella* genome, we used the endogenous *MyHC1* promoter region as a probe. The Southern blot analyses clearly showed that our transgenic line resulted from a single integration site, and that concatemerization did not occur (Fig. 2*D*). These findings are in line with previous observations in fish showing that meganuclease-mediated transgenesis predominantly results in one or few (i.e., 1–10) integrations (reviewed in ref. 17). Single and non-concatemeric integrations are less prone to subsequent silencing

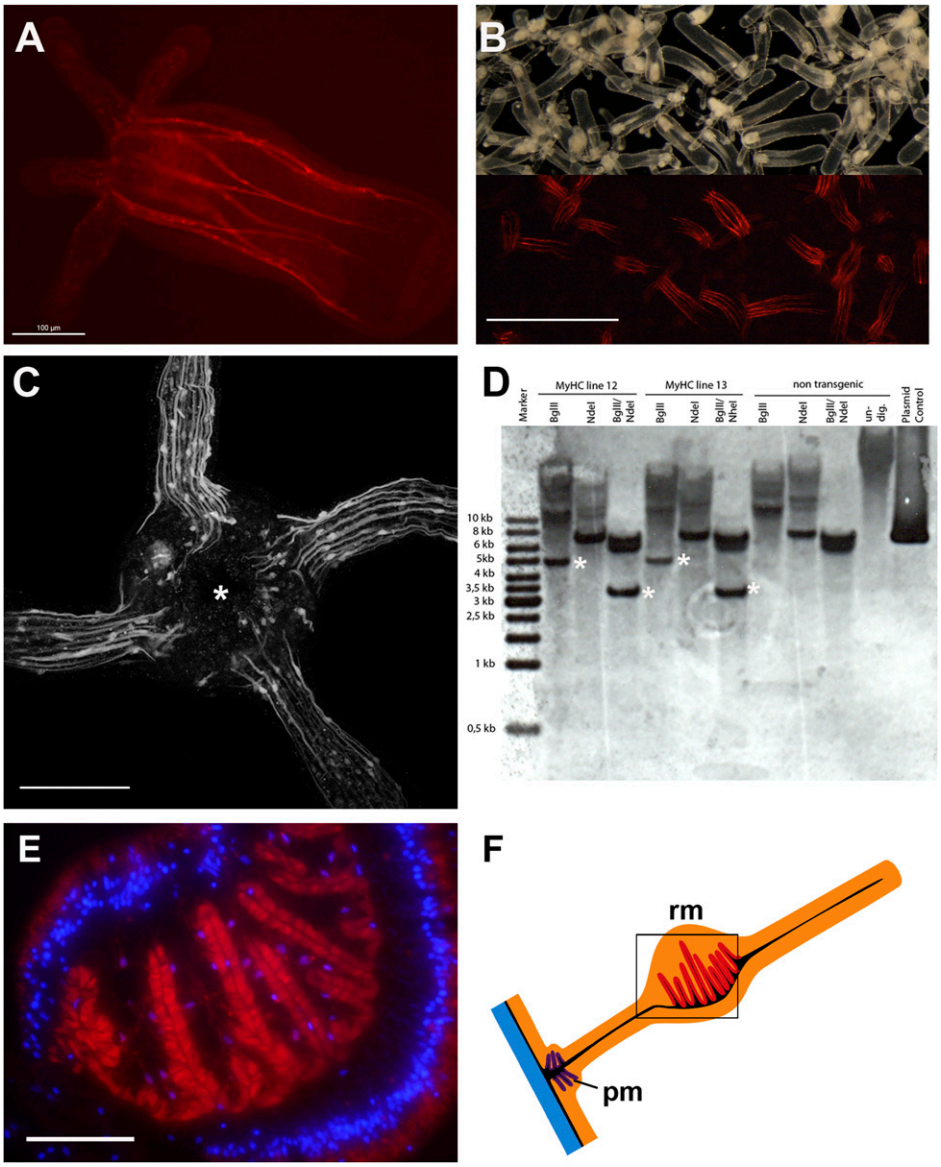

Fig. 2. Germline transmission in transgenic G_1 polyps. (*A*) A G_1 *MyHC1*::*mCherry* transgenic primary polyp showing red mCherry expression in eight mesenteries. (*B*) Live image of offspring of G_0 transgenic polyps with mosaic germline. *Lower*: an epifluorescence light picture of the same sample. G_1 transgenic primary polyps can be easily identified under the epifluorescence stereomicroscope. (*C*) Reconstruction of confocal stacks showing the oral view on the head of a primary polyp with a mouth opening (asterisk) and four tentacles. Note the long extensions of individual mononuclear muscle cells in the tentacles. (*D*) Southern blot analysis of single G_1 transgenic adult polyps and nontransgenic control animals. Both individual polyps have a single integration site for the transgene (marked by asterisks). (*E*) Cryotome cross-section of retractor muscles in the mesentery stained with an antibody against mCherry (red). Nuclear staining was done with DAPI (blue). (*F*) Schematic representation of a mesentery with the retractor muscle on one side in the middle of the mesentery. The color code and abbreviations are as in Fig. 1. [Scale bars: 100 μm (*A*), 800 μm (*B*), 50 μm (*C*), and 50 μm (*E*)].

than high copy numbers generated by plasmid injection (18, 19). Furthermore, the expression levels are somewhat weaker (depending on the promoter used), yet more uniform and reproducible.

In summary, the *MyHC1*::*mCherry* transgenic line faithfully reproduces the expression pattern of the *MyHC1* gene in the retractor and tentacle muscles as detected by in situ hybridization. This shows that the 1.6-kb genomic fragment contains all of the regulatory elements necessary to drive proper expression. Whereas all epithelial cells in Cnidaria have more or less contractile elements at their base and thus are considered myoepithelial cells, the specific expression of mCherry in the retractor muscles reveals that the retractor muscle cells in sea anemones are distinct from other myoepithelial cells of the animal (e.g., circumferential myoepithelial cells in the endoderm).

Monitoring Muscle Differentiation and Reorganization During Head Regeneration. All Cnidaria have a high capacity for regeneration. When an adult polyp is bisected, the remaining part will regenerate the missing part via a morphallactic process (i.e., restructuring of the remaining part through a proportion-regulation mechanism) within a few days. In *Nematostella*, head regeneration requires the formation of a pharynx and new tentacles. The pharynx is an inverted structure, consisting of a thick ectodermal cell layer and a thin endodermal layer in continuation to the endoderm in the mesenteries carrying the retractor muscles (20). We followed head regeneration in *MyHC1*::*mCherry* transgenic animals for 6 days after bisection below the pharynx region and found that immediately after cutting, the muscles retracted from the wound site (Fig. 3*A*). The tissue at the wound site bent inward, in

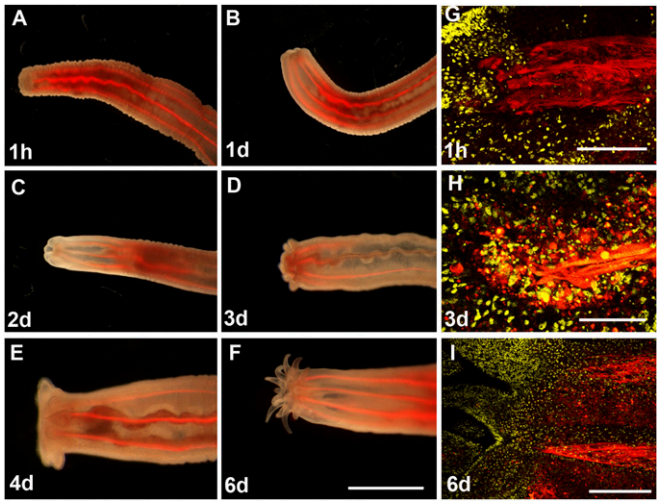

Fig. 3. Muscle reorganization during head regeneration. (*A–F*) A live *MyHC1*::*mCherry* G_1 transgenic polyp regenerating a head after bisection below the pharynx. The ectoderm at the wound bends inward and starts to form a new pharynx. The first tentacle buds appear after 3 days of regeneration. Note that more than 14 tentacles appear at once. (*G–I*) Confocal closeups of the regenerating tip (same orientation as in *A–F*). ToPro-3–stained nuclei are shown in yellow. (*G*) Retractor muscles retract immediately after being cut from the wound. (*H*) At day 3 after bisection, numerous cells expressing the *mCherry* transgene under control of the *MyHC1* promoter accumulate at the wound site. (*I*) After 6 days, mesenteries are attached near the differentiated pharynx close to the base of the tentacles. [Scale bars: 500 μm (*A–F*), 20 μm (*G* and *H*), and 40 μm (*I*).]

preparation for differentiation of the pharynx (Fig. 3 *B–D*). The first tentacle buds appeared about 3 days after bisection (Fig. 3*D*). Notably, unlike primary polyps, which always develop four tentacles, regenerates develop more than 14 tentacles at once (Fig. 3 *D–F*). A more detailed analysis of the regenerating pharynx region by confocal microscopy revealed the accumulation of numerous cells at the regenerating site expressing the *MyHC1*::*mCherry* transgene, suggesting that this region is an active site of new differentiation and reorganization (Fig. 3 *G–I*).

Future Prospects. Here we report the first transgenic line in *Nematostella* and the first germline transmission in a cnidarian using a meganuclease-mediated integration. The identification of a promoter expressed in a specific cell population is a promising example demonstrating that gene- and tissue-specific promoters can be readily identified in *Nematostella*. Such transgenic reporter lines for specific cell populations could be used in in vivo time-lapse studies, laser ablation of one or several transgenic cells, and mutagenesis screens. Current work is focusing on the isolation of other gene-specific promoters and their functional dissection in the endogenous background. Furthermore, the development of ubiquitously expressed or inducible promoters in conjunction with tagged fusion proteins will enhance the repertoire of functional approaches. It also should be feasible to target gene functions late in development or even in adults, which currently are not easily accessible to manipulation. The generation of other transgenic lines specific for neurons or other cell populations will allow the production of multitransgenic lines by genetic crosses and monitoring of the interaction of these cell populations in vivo and in response to environmental signals, stress, and experimental perturbations.

Currently, virtually nothing is known about the complexity of gene regulation in diploblastic animals in comparison with Bilateria. To address this question, basal endogenous promoters must be isolated to identify enhancers by random insertion into the genome. Such basal promoters also will allow the testing of regulatory elements that are conserved between *Nematostella* and different Bilateria. Thus, future applications of the transgenic technology are expected to significantly contribute to the functional aspects of gene regulatory networks and enhance our understanding of the cellular dynamics during morphogenesis and cellular differentiation.

Materials and Methods

Animal Culture. The culture of *Nematostella* was maintained and induction of spawning was carried out as described previously (2).

Isolation of the Myosin Heavy-Chain Promoter. A partial cDNA clone of the *MyHC1* gene was found in an EST collection (6). 5′RACE was used to obtain the 5′ end of the transcript. A fragment of 1,626 bp upstream of the start codon of the *MyHC1* gene was then amplified by PCR from genomic DNA, using primers designed on the base of the assembled genome sequence of *Nematostella* (MyHC5: CCGGATGGGAGCAAAGAAAAACTT; MyHC3: CATCTTGAAACAGTTATTCTAAA) (5). The sequence of the promoter was confirmed by overlap with the 5′ end of the cDNA clone.

Generation of Plasmids for Transgenics. A pCRII-TOPO plasmid (Invitrogen) was used as a backbone for the transgenesis vector. The multicloning site was replaced by a cassette consisting of the *MyHC1* promoter, and the *mCherry* reporter, flanked by inverted I-SceI sites, was inserted. The promoter, reporter gene, and meganuclease sites were separated by single rare cutters (Fig. 1*A*).

Microinjection. Fertilized eggs were prepared for microinjection as described previously (2). Zygotes were injected with 50 ng/μL of DNA, 1× I-SceI buffer, 40 μg/mL of Alexa Dextran (Invitrogen), and 0.2 U/μL of I-SceI (New England Biolabs). The average injected volume was 45 pL. The mix was incubated for 30 min at 37°C before injection. Injected eggs were put into fresh medium and incubated at 20°C overnight. Animals were transferred into fresh medium daily and raised to primary polyps at 20°C in the dark. The first feeding was usually possible after 7–9 days. Animals that still expressed mCherry at day 9 were selected and raised as a pool of animals.

Cryosections and Immunohistochemistry. Adult polyps were relaxed in 7% $MgCl_2$ for 10 min, then cut in smaller pieces and fixed in 4% paraformaldehyde/PBS/0.1%Tween20 for 1 h. After washing in PBS, the specimens were transferred to infiltration solution [20% OCT compound (Sakura), 25% sucrose in PBS, pH 7.4]. After 12–16 h, the specimens were put into 80% OCT compound and 1.25% sucrose for freezing and cutting. Then 12-μm cryosections were incubated with an anti-DsRed (mCherry) antibody (Clontech) for 1 h, followed by anti-rabbit IgG (Alexa Fluor 456; Invitrogen). Nuclear counterstaining was carried out by incubation with ToPro-3 (Invitrogen). Images were obtained with a Leica SP2 confocal microscope and were overlaid in Adobe Photoshop.

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