Up-Regulation of Muscle-Specific Transcription Factors During Embryonic Somitogenesis of Zebrafish (Danio rerio) by Knock-Down of Myostatin-1

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Myostatin, a secreted growth and differentiation factor (GDF-8) belongs to transforming growth factor (TGF-β) superfamily and is shown to be essential for proper regulation of skeletal muscle mass (McPherron et al., 1997). It has been demonstrated that, during skeletal muscle development, myostatin expression is restricted initially to the myotome compartment of developing somites and continues to be limited to the myogenic lineage at the later stage of development and in adult animals (McPherron et al., 1997). Moreover, mice carrying a targeted deletion of the gene encoding...
myostatin have a dramatic and widespread increase in muscle mass, suggesting that myostatin acts as a negative regulator of muscle growth. Furthermore, a similar type of developmental expression pattern of myostatin was observed in cattle (Kambadur et al., 1997), pigs (Shaoquan et al., 1998), and chicken (Kocamis et al., 1999). In mammals, myostatin mRNA is expressed to a higher level in skeletal muscle and to lower levels in the adipose tissue (Chomezynski, 1993), mammary gland (Roberts et al., 2000), and cardiac muscle (Sharma et al., 1999). However, in fish, myostatin transcript was detected in other tissues such as brain, eye, intestine, gill filaments, gonads, and kidney (Rodgers et al., 2001; Maccatrozzo et al., 2001a,b; Roberts and Goetz, 2001). Although the functional role of myostatin in regulating the muscle growth has been well documented by genetic models, the actual mechanism remains unknown.

During development, skeletal muscle cells arise from the pluripotential mesenchymal precursors, which are committed to the myogenic lineage. The myogenic differentiation is under the control of a family of muscle-specific transcription factors, which includes MyoD, Myogenin (Edmondson and Olson, 1989), and Myf5 (Rhodes and Konieczny, 1989). These muscle-specific genes convert nonmuscle cells to a myogenic lineage (Thayer et al., 1989). Among MRFs, MyoD and Myf5 are involved in the determination of myogenic lineage and initiating myogenic differentiation. Myogenin and MRF4 serve to the process of terminal differentiation (Perry and Rudnick, 2000). Once activated, Myo-D and Myf-5 induce the expression of Myogenin and transcription factors from the family of myocyte enhancer factors (MEF-2). Muscle-specific genes such as mck, myosin light chain, myogenin, and desmin have multiple E boxes, their promoters that act cooperatively to regulate the gene transcription (Rao et al., 1996).

IGF-1 is the primary mediator of many responses regulated by the growth hormone throughout the body (Butler and LeRoith, 2001). It has long been recognized that IGF-1 and IGF-2 are important for pre- and postnatal development of skeletal muscle (Fiorini et al., 1991). Knock-down or knock-out models have shown promise for the production of animals. Keeping all these strategies in mind, the present investigation was aimed to see the expression pattern of myostatin and the role of myostatin on MRFs, MSP, and IGFs in normal and knock-down fish, which may help us to understand the functional role of myostatin in the physiology of muscle formation.

RESULTS
Expression of Myostatin During Embryonic Development

The expression of myostatin gene during embryonic and early larval stages of zebrafish was examined by RT-PCR and the products myostatin and MAX (internal control) genes with 496 and 479 bp, respectively, were subjected to 3% agarose gel electrophoresis. Myostatin transcripts, detected from one-cell stage onward, demonstrate the maternal expression (Fig. 1A). This finding was further confirmed by sequencing and Southern blot hybridization (Fig. 1B).

To study the expression pattern of myostatin gene, we performed in situ hybridization using sense (data

Myostatin gene expression in different tissues. A: Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. The upper panel, MSTN; lower panel, Max. Lane 1, liver; 2, heart; 3, gut; 4, gill; 5, ovary; 6, testis; 7, kidney; 8, muscle; 9, eye; and 10, brain. B: Southern blot analysis. Lanes 1–10 are the same as in A. C: Results of semiquantitative RT-PCR.
not shown) and antisense riboprobe in the developing embryos (Fig. 2) followed by histologic observation (Fig. 2). In zygote, the nononylky cytoplasm begins to stream toward the animal pole and form a cytoplasmic cap called blastodisk (Lewis and Roosen-Runge, 1943; Devoto et al., 1996). The blastodisk showed a signal due to the presence of myostatin transcripts (Fig. 2A). The signal was maintained through the cleavage period (Fig. 2B,D) and clearly detected in the highly flattened cells of blastula (Fig. 2E). In general, the myostatin gene expression was ubiquitous (Fig. 2F,G). At 24 hr, strong signals could be observed in the eye, retina, brain (midbrain, hindbrain, and cerebellum), and the somites (Fig. 2J), which was maintained through 48 and 72 hr (Fig. 2K,L). A strong signal was also shown in a cross- and sagittal section of the muscles (Fig. 2H,M). In the transverse section of the eye and brain (Fig. 2I,N,O), the myostatin signal was detected in the optic vesicles, retina, and in the hind- as well as midbrain regions. The signal was also observed in the gut epithelia and lumen (Fig. 2P).

**Differential Expression of the Myostatin Gene**

To analyze the expression of the myostatin gene in various tissues such as brain, eye, ovary, testis, liver, muscle, gill, gut, kidney, and heart, RT-PCR was performed. The gene had a wide distribution of expression in all the 10 tested tissues. However, no expression could be observed in liver, heart, gut, testis, and ovary for 25 cycles (data not shown), whereas we observed signals in gut, testis, ovary, kidney, muscle, brain, and eye for 30 cycles (data not shown); after 35 cycles, we observed the signal in all the tested tissues (Fig. 3A,B). In addition, its expression levels varied greatly among different tissues. Myostatin was strongly expressed in muscle, brain, and eye; intermediately in gut testis, ovary, and kidney; and weakly expressed in gill, heart, and liver (Fig. 3A). Semi quantitative RT-PCR was also carried out (Fig. 3C). This was again confirmed by Southern blot hybridization (Fig. 3B).

Myostatin transcript with a size of 2.7 kb was detected in all the tested tissues (Fig. 4). The expression was very high in muscle; intermediate in brain, eye, and kidney; weak in testis, ovary, and gut heart; and undetectable in liver and gill (Fig. 4).

**Effect of Myostatin Knock-Down on Muscle Growth**

To understand the regulatory role of myostatin, we injected the control morpholino, and morpholino specific for myostatin into the embryos and observed the phenotypic difference in stages 18 and 20 hours post-fertilization (hpf). At 20 hpf, the morpholino-treated embryos showed faster growth and its size was similar to the size of 30 hpf controls (Fig. 5A,B). Eighty percent of morpholino-injected embryos (>1,000 numbers, at different doses) showed similar phenotypic difference.

To confirm, whether the observed phenotypic effect was due to morpholino, we performed Western blot hybridization. The results showed that there was an inhibition of myostatin expression in morpholino knock-down fish when compared with control (Fig. 6). The molecular weight of processed myostatin was 27 kDa. Gonzalenz-Cadavid et al. (1998) have reported that the monomer form of human myostatin protein (26 kDa) is glycosylated. In contrast to this, McPherron et al. (1997) have reported the molecular weight of recombinant, processed, and precursor forms of myostatin varied from 12- to 52-kDa proteins, respectively.

**Up-Regulation of Muscle Marker Genes by Myostatin**

The effect of myostatin knock-down on the IGF-1 and IGF-2 musclespecific genes (Desmin and Mck) and musclespecific transcription factors (MyoD, Myogenin, and Myf5) was examined by RT-PCR, and the products were analyzed by 3% agarose gel electrophoresis. The results revealed that the expression of MyoD, Myogenin, and Mck were significantly up-regulated in the knockdown fish than the control at 10, 12, and 22 hpf (Fig. 7A,B). The IGF-2 transcription showed mild response to myostatin knock-down, whereas no effect was found in IGF-1, Myf5, and Desmin (Fig. 7A).

Whole-mount in situ hybridization was carried out for morpholino-injected and control morpholino-injected embryos (Fig. 8). The embryos were hybridized with Mck (A-F).
Myogenin (G–L), and MyoD (M–R) probes. At 10 hpf, the morpholino-injected embryos showed a very strong signal, and at 12–13 hpf, apart from the signal, there was an increase in the number of somites. A total of 11–12 somites were seen in treated embryos, whereas in controls, only 7–8 somites were observed. At or before 22 hpf, all the somites were well developed and the signal was also very high. Figure 9A,B showed changes in the size of the somites. In the morpholino-injected embryos, the size of the somites was significantly increased compared to controls.

![Image](image_url)

**Fig. 5.** Phenotypic effects of myostatin antisense morpholino injection include an increase in the size and growth of fish injected with morpholino. A: Control (20 hours postfertilization). B: Morpholino-injected embryos. Original magnification, ×20.

![Image](image_url)

**Fig. 6.** Decreased expression of myostatin in antisense morpholino-injected embryos. Protein extraction from 72 hours postfertilization embryos injected approximately 10 ng of the morpholino were separated by electrophoresis, transferred to a nylon membrane and analysed by Western blot. M, marker; 1, control morpholino injected; 2, morpholino injected. β-Actin was used as an internal control.

![Image](image_url)

**Fig. 7.** A: Reverse transcriptase-polymerase chain reaction analysis at different time points after injection with myostatin. B: Results of semiquantitative RT-PCR: Lane 1, 10 hpf morpholino injected (10h mor); 2, 10 hpf control morpholino injected (10h con); 3, 12 hpf morpholino injected (12h morpholino); 4, 12 hpf control morpholino injected (12h con); 5, 22 hpf morpholino injected (22h mor); 6, 22 hpf control morpholino injected (22h con). MAX was amplified from the same samples used as an internal control.
**Fig. 8.** Whole-mount in situ hybridization was carried out for morpholino-injected and control morpholino-injected embryos. A–R: Embryos were hybridized with Mck (A–F), Myogenin-D (G–L), and MyoD (M–R). At 10 hours postfertilization (hpf) in the morpholino-injected embryos, the signal is very high and at 12–13 hpf, apart from the signal, there was an increase in the number of somites. In the control fish, 7–8 somites were observed, but in morpholino-injected ones, 11–12 somites were seen. A, G, and M are 10 hpf control morpholino-injected embryos; B, H, and N are morpholino-injected 10 hpf embryos. C, I, and O are 12–13 hpf control morpholino-injected embryos; D, J, and P are morpholino-injected 12–13 hpf embryos. E, K, and Q are 22 hpf control morpholino-injected embryos; and F, L, and R are 22 hpf morpholino-injected embryos.

**Fig. 9.** Measurement of the somites in \(\times 40\) original magnification. (length \(L\), width \(W\), gap \(G\) between two somites, and total length \(TL\)). Values given in brackets are without magnification. A: Control 12–13 hours postfertilization embryo. B: Morpholino-injected embryo.

**Fig. 10.** Rescue experiment. A: Control embryos. B: Morpholino-injected embryos. C: Rescued embryos.
jected embryos, the total length of the myotome compartment was $9.4 \pm 0.1$ cm, and in the controls, the length was $7.8 \pm 0.5$ cm. The length and width of one somite was $1.1 \pm 0.3$ cm each, but the control was $0.9 \pm 0.1$ and $0.5 \pm 0.3$ cm, respectively. In control, the gap between two somites was $0.6 \pm 0.2$ cm, whereas almost no gap was seen in morpholino-injected embryos. To understand the specificity of myostatin morpholino, a rescue experiment was performed. At high dose of capped RNA (9.5 ng/embryo), all the embryos were rescued without any phenotypic changes (Figs. 1A,B, 3A,B). We performed Southern blot hybridization as a further confirmation (Figs. 1B, 3B). This result suggests that the role of myostatin in fish is not only limited to skeletal muscle growth inhibition but also contributes to the homeostatic growth control of other tissues. Furthermore, it seems that myostatin might potentially regulate some specific physiological processes that are unrelated to skeletal muscle growth. For instance, myostatin expression is found in gill filament, indicating a possible role on osmoregulation; in brain, it may coordinate the neuronal growth and development; and in eye may also be involved in the development of longitudinal and circular muscle growth. It is also expressed in the testis and ovary, indicating its possible roles in reproductive tissues. Previously, it was thought that the myostatin regulation is restricted to the development of skeletal muscle only. However, a recent report revealed the detection of myostatin mRNA and protein in cardiac muscle (Sharma et al., 1999) and in various tissues (Roberts et al., 2000; Ostbye et al., 2001; Rodgers et al., 2001; Rescan et al., 2001; Kocabas et al., 2002). Moreover, TGF-β superfamily members along with some isoforms are found in a wide variety of tissues (Millan et al., 1991). So it is considered that the isoforms may have different roles in gene regulation during tissue development and growth. Although the functional aspect remains unclear based on the earlier studies, the myostatin protein is assumed to have diverse roles in the zebrafish development.

The Northern blot results also revealed wide distribution of myostatin gene in all the tissues tested (Fig. 4). Although the expression was ubiquitous, the level was not uniform and was found to be either high or very low in some muscle groups, as in mouse myostatin (McPherron et al., 1999). This variability may be due to the distribution of different fiber types within individual muscles as the abundance of myostatin message, which is directly correlated with the presence of myosin heavy chain isoform IIb (McPherron et al., 1999).

**DISCUSSION**

**Expression of Myostatin**

The major role of myostatin is to regulate the muscle growth negatively (McPherron et al., 1997). As expected, myostatin was expressed throughout the development (Kambadur et al., 1997). It had been reported that the myo-related genes would be activated from six to seven somite stages (Weingberg et al., 1996; Xu et al., 2000). Similarly, the expression of myostatin was mild from the one-cell stage to 16 hr and significantly increased at 24 hr stage onward (Fig. 1A,B). The development of fish skeletal muscle occurs rapidly during the earlier stages (Devoto et al., 1996) and the onset of myostatin expression coincides with early myogenesis.

Our results clearly show a wide distribution of myostatin expression in various tissues apart from skeletal muscle. In higher animals, myostatin expresses only in skeletal muscle and in mouse myostatin was detected as early as 9.5 days post coitus. But in fish, we observed that myostatin expression was ubiquitous and is maternal (Figs. 1A,B, 3A,B). We performed Southern blot hybridization as a further confirmation (Figs. 1B, 3B). This result suggests that the role of myostatin in fish is not only limited to skeletal muscle growth inhibition but also contributes to the homeostatic growth control of other tissues. Furthermore, it seems that myostatin might potentially regulate some specific physiological processes that are unrelated to skeletal muscle growth. For instance, myostatin expression is found in gill filament, indicating a possible role on osmoregulation; in brain, it may coordinate the neuronal growth and development; and in eye may also be involved in the development of longitudinal and circular muscle growth. It is also expressed in the testis and ovary, indicating its possible roles in reproductive tissues. Previously, it was thought that the myostatin regulation is restricted to the development of skeletal muscle only. However, a recent report revealed the detection of myostatin mRNA and protein in cardiac muscle (Sharma et al., 1999) and in various tissues (Roberts et al., 2000; Ostbye et al., 2001; Rodgers et al., 2001; Rescan et al., 2001; Kocabas et al., 2002). Moreover, TGF-β superfamily members along with some isoforms are found in a wide variety of tissues (Millan et al., 1991). So it is considered that the isoforms may have different roles in gene regulation during tissue development and growth. Although the functional aspect remains unclear based on the earlier studies, the myostatin protein is assumed to have diverse roles in the zebrafish development.

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**Knock-Down of Myostatin**

Skeletal myogenesis or myogenic differentiation involves a hierarchical regulatory mechanism for the control of growth and differentiation of myoblast. As myogenesis involves highly ordered, temporally separable elements and coordinated gene expression, knock-down of a specific gene in vivo by morpholino could offer information about this coordination. In the present study, the knock-down of myostatin by morpholino enhanced the expression of muscle-specific transcription factors (MyoD and Myogenin), muscle-specific gene (Mck), and IGF-2 (Fig. 7A,B). The overexpression of myostatin, inhibited the expression of the myoD, myf5, myogenin, and mck was found in C2C12 cell line (Rios et

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**TABLE 1. Dose-Response of Phenotype, Rescued by Coinjection With Myostatin Morpholino and Capped RNA of Myostatin**

<table>
<thead>
<tr>
<th>Dosage of mRNAa (ng/embryo)</th>
<th>Total No. injected</th>
<th>No. of survived embryos</th>
<th>No. of embryos showed phenotypic effect (enlargement)</th>
<th>Rescued embryos</th>
<th>% of rescue</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>150</td>
<td>128</td>
<td>116 (90.6%)</td>
<td>12</td>
<td>9.3</td>
</tr>
<tr>
<td>3.5</td>
<td>150</td>
<td>134</td>
<td>78 (58.2%)</td>
<td>56</td>
<td>41.7</td>
</tr>
<tr>
<td>6.5</td>
<td>150</td>
<td>127</td>
<td>24 (18.8%)</td>
<td>103</td>
<td>81</td>
</tr>
<tr>
<td>9.5</td>
<td>150</td>
<td>142</td>
<td>0 (0%)</td>
<td>142</td>
<td>100</td>
</tr>
</tbody>
</table>

aThe mRNA was coinjected with 10 ng of morpholino in all treatments.
whether the phenotypic changes be investigated. To determine for normal skeletal muscle remains to (McPherron et al., 1997), its physiological role and double-muscle cattle (McPherron gene knock-out mice with skeletal muscle hypertrophy in 1986). Although lack of myostatin protein is known to be associated with the TGF-β superfamily, along with myostatin, the factors belonging to the TGF-β superfamily, which inhibit both proliferation and of myostatin, thus suggesting a negative role of myostatin in the regulation of differentiation process in vivo. Figure 9 shows an increase in size of the somites. Generally, under normal conditions, somites develop first, preceding muscle formation, and from the somites, all the skeletal muscles of the vertebrate body and some head muscles are derived (Christ and Ordahl, 1995). This study was carried out during early embryonic stages, so only an enlargement of somites could be observed. There were no changes in the level of Myf5, Desmin, and IGF-1 (Fig. 7A), and this finding may be due to the factors belonging to the TGF-β superfamily, along with myostatin, which inhibit both proliferation and differentiation (Massague et al., 1986). Although lack of myostatin protein is known to be associated with skeletal muscle hypertrophy in McPherron gene knock-out mice and double-muscle cattle (McPherron et al., 1997), its physiological role for normal skeletal muscle remains to be investigated. To determine whether the phenotypic changes can be attributed to loss of function of myostatin or some other effects, we carried out rescue experiment by injecting myostatin capped RNA. At high dose (9.5 ng of capped RNA per embryo), no phenotypic effect was observed. In conclusion, this study was particularly performed to understand the mechanism of muscle growth. Up-regulation of muscle related genes in the knock-down embryos suggested that myostatin inhibit differentiation by down-regulating the muscle-specific transcription factors. In addition, the maternal expression and the role of myostatin is not restricted to muscle, but it might also possess some other functions that may be related to brain and eye development and it deserves further elucidation. Furthermore, dominant negative mutant and knock-down experiments may reveal essential information regarding functional aspects related to cell cycle and apoptosis.

EXPERIMENTAL PROCEDURES

Animals and Tissue Collection

Zebrafish used in these studies were obtained from Dr. Barne’s laboratory. The fish were maintained as brain, eye, ovary, testis, liver, muscle, gill, gut, kidney, and heart were dissected out and frozen in liquid nitrogen immediately.

Embryo Harvest

Fertilized eggs were maintained in the freshwater tank at 28°C, and the developing embryos were collected at the 1-cell stage (zygotic period), 32-cell stage (cleavage period), dome stage (blastula period), bud stage (gastrula period), 14-somite stage (segmentation period), prim-5 stage 24 hr (pharyngula period), 2 days (hatching period), and early embryonic period (3 and 4 days). Developmental stages were classified (Westerfield, 1994), and the embryos were observed under the microscope.

RT-PCR

Total RNA was isolated from different tissues by using TRIZOL (Boehringer Mannheim), according to the manufacturer’s instruction. First-strand cDNA was synthesized in a 20-μl RT reaction from 5 μg of total RNA by using Thermoscript RT-PCR system (Invitrogen). PCR was performed with 2 μl of the RT reaction. The primers were designed to PCR amplify the 496 bp of myostatin (AF019626) and Max 476 bp cDNA, are presented in Table 2. The PCR program was 94°C, 30 sec, 55°C for 30 sec, and 72°C for 1 min and performed 35 cycles. The final extension time was 7 min at 72°C. The PCR products were subjected to 3% gel electrophoresis and transferred to a nylon membrane (Amersham Bioscience).

Southern Blot Hybridization

The probes were prepared from the 496-bp PCR product (above). Briefly, the PCR products were eluted and purified by QiAquick gel extraction kit (Qiagen, Hilden, Germany). The probe was labeled by α-P32 and the hybridization was carried out overnight at 42°C. The myostatin cDNA was labeled by Rediprime II labeling kit (Amersham Bioscience). After hybridization, the blot was washed one time in 0.2× wash buffer (0.2× standard saline citrate (SSC), 0.1% sodium dodecyl sulfate (SDS)) at room temperature, then in 0.1× wash buffer (0.1× SSC, 0.1% SDS) at 42, 50, and 60°C, respectively, for 30 min, and the membrane was exposed to x-ray film for 12 hr.

Northern Blot Hybridization

Northern blot was performed essentially as described by Sambrook et al. (1989). Briefly 20 μg of RNA was fractionated in 1.2% formaldehyde gel electrophoresis, and RNA was transferred to Hybond N+ membrane (Amersham Bioscience) by capillary transfer by using 10× SSC. The membrane was prehybridized with UltraHyb (Ambion) followed by hybridization with α-P32-labeled myostatin cDNA probe at 42°C overnight. The membrane was washed 20 min at room temperature in 1× SSC, 0.1% SDS, followed by three
24 to 48 hr to x-ray film at 20°C. The membrane was washed, blocked by net buffer at 4°C overnight, and incubated with 1:5,000 dilution of primary antibody with 1:3,000 polyclonal to human myostatin (ab996) in Table 2. Some of the whole-mount embryos at 48 hr were selected for preparing cryosections. The membrane was washed, and activity was detected using an ECL kit according to manufacturer’s protocol. Western Blotting Protein extract was collected by using lysis buffer and was separated by SDS polyacrylamide gel electrophoresis (12%) and transferred to a nitrocellulose membrane. The membrane was blocked by net buffer at 4°C overnight and then incubated with rabbit polyclonal to human myostatin (ab996) primary antibody with 1:3,000 dilutions. This product was washed and incubated with 1:5,000 dilution of peroxidase-labeled anti-rabbit antibody. The membrane was washed, and activity was detected using an ECL kit according to manufacturer’s protocol.

Whole-Mount In Situ Hybridization Whole-mount in situ hybridization was performed with digoxigenin (DIG) -labeled RNA probes (Westerfield, 1994). The probes were prepared by a set of primers, designed based on the myostatin coding region. The primers were 5'-ATG CAT TTT ACA CAG GTT ATT TCT CTA AGT A-3' (forward); 5'-CGA TGG TAG ACC GCT GTG CTC GCT CAT GA-3' (reverse). The 1,110-bp fragment was subcloned into Zero Blunt Topo PCR cloning Kit (Invitrogen) containing T7 and SP6 promoters. By using SP6 and 17 RNA polymerase, the DIG-labeled sense and antisense RNA probes were synthesized. Some of the whole-mount embryos at 48 hr were selected for preparing cryosections.

Morpholino Injection Morpholino antisense oligonucleotides were designed as follows. 5'-TGC ATG TTC CAA GGC GTG CTA AAG G-3' and purchased from Gene-Tools (Corvallis, OR). Zebrafish embryos at one- to four-cell stages were injected with an approximately 2 nl volume of morpholino oligonucleotide (10 ng) by using the microinjector (Nikon SMZ-U, Japan). Embryos were collected at 10, 12, and 22 hpf and fixed in 4% paraformaldehyde, overnight at 4°C. The fixed embryos were dechorionated, washed three times with phosphate buffered saline, and stored in methanol at −20°C. Total RNA isolated from the embryos were subjected to RT-PCR using the primers presented in Table 2.

Whole-mount in situ hybridization was performed by using DIG-labeled antisense RNA probe. The probes were prepared by a set of primers from the coding region of Mck, Myogenin, and MyoD (Table 2). Riboprobes were made from DNA templates, which were linearized and transcribed with T7 RNA polymerase.

For the rescue experiment, we used the full-length coding region of myostatin. The plasmids used for in situ hybridization was linearized with XbaI followed by phenol:chloroform extraction. Capped RNA was transcribed from the linearized templates using the mMessage mMachine kit (Ambion Austin, TX).

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