Transcriptional regulation of the *Xenopus laevis* stromelysin-3 gene by thyroid hormone is mediated by a DNA element in the first intron.

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**SUMMARY**

The matrix metalloproteinase (MMP) stromelysin-3 (ST3) (MMP11) was first isolated as a breast cancer associated gene and is expressed in diverse human carcinomas and various developmental processes involving apoptosis. The *Xenopus laevis* ST3 is highly upregulated by thyroid hormone (T3) during amphibian metamorphosis and its expression is spatially and temporally correlated with apoptosis in different tissues. Furthermore, it has been shown in *vivo* and in organ cultures to play a critical role in regulating T3-induced epithelial cell death during intestinal metamorphosis. Earlier studies suggest that ST3 is a direct T3 response gene although a thyroid hormone response element (TRE) was not found in the initial analysis of the ST3 promoter. Here, we have identified a strong TRE consisting of two nearly perfect direct repeats of the consensus nuclear hormone receptor binding element AGGTCA separated by 4 bp in the first intron of the *Xenopus* ST3 gene. We show that the heterodimers of T3 receptor (TR) and 9-cis retinoic acid receptor (RXR) bind to the TRE both *in vitro* and *in vivo* in the context of chromatin. Furthermore, T3 induces strong activation of the promoter through the intronic TRE. Interestingly, while the unliganded TR/RXR was able to recruit corepressors to the promoter, it had little repressive effect on the promoter *in vivo*. These results suggest that the intronic TRE mediates the inductive effect of T3 and that promoter context plays an important role in gene repression by unliganded TR.

**INTRODUCTION**

The matrix metalloproteinases (MMPs) are a superfamily of Zn²⁺-dependent extracellular or membrane-bound proteinases (1-3). They are capable of digesting proteinaceous components of the extracellular matrix (ECM) as well as non-ECM proteins (2,4,5), and thus can affect cell fate and behavior through multiple pathways. Extensive analyses have shown that various MMPs are highly expressed in diverse developmental and pathological processes where cell fate change and/or cell migration occurs, consistent with the possible
involvement of MMP in cell function through multiple pathways.

Of particular interest among MMPs is stromelysin-3 (ST3) (MMP11), which was first isolated as a breast cancer associated gene and has since been found to be expressed in diverse human carcinomas (6-9). In addition, high levels of ST3 mRNA are present in a number of developmental processes where extensive cell death and tissue remodeling take place in mammals (6,7,10-13). These results suggest that ST3 participates in vertebrate development as well as carcinogenesis and tumor progression, although mice lacking ST3 were largely normal, likely due to redundancy in MMP activities (14,15).

ST3 is secreted as mature enzymes (16), thus lacking one major mechanism to regulate its activity, i.e., the activation of the proenzymes (zymogens) through the removal of the propeptide seen for most other MMPs (17-21). The transcription of the ST3 gene, therefore, plays a critically important role in regulating the biological function of ST3. This is especially true considering the strictly controlled expression profiles of ST3, whose mRNA is absent in most adult organs in mammals but is at high levels in a cell type specific manner during many developmental and pathological processes (see above).

We are using amphibian metamorphosis as a model to study the regulation and function of ST3 in postembryonic vertebrate development. This process is controlled essentially by a single hormone, the thyroid hormone (T3), but involves drastically different changes in different tissues/organs (22,23). Among the changes are total resorption of tadpole specific organs such as the tail and gills, de novo development of frog-specific organs like the limbs, and remodeling of the vast majority of the organs into their frog forms. Given the complex changes, it is not surprising that a number of MMPs are activated by T3 during metamorphosis in Xenopus laevis and Rana catesbeiana (24-30). Of special interest is the fact that the expression of the Xenopus ST3 but not several other MMPs correlates tightly with larval or tadpole cell death (apoptosis) in different organs during metamorphosis (24,31-34). Furthermore, in situ hybridization analysis showed that like the mammalian ST3, the frog ST3 is also expressed specifically in the fibroblasts, which underlie or surrounds the apoptotic cells during metamorphosis (24,31-34).

To determine how ST3 gene is spatially and temporally regulated by T3 during frog development, we have previously characterized the promoter of the Xenopus ST3 gene (35). We failed to identify any functional T3 response element (TRE) within 1000 bp upstream of the transcription start site even though expression analyses indicates that the ST3 gene is regulated by T3 directly at the transcription level through T3 receptors (TRs) (24,26,27). We report here that identification of a TRE consists of two near perfect direct repeats of AGGTCA separated by 4bp, i.e., a DR4 (direct repeats with a 4 bp spacing) element, located in the large first intron. We show that this TRE binds to the heterodimers of TR and RXR (9-cis retinoic acid receptor) in vitro and in vivo. Furthermore, we demonstrate that it mediates T3-induction of the ST3 promoter in the context of chromatin in vivo, suggesting that it is responsible for the upregulation of the ST3 gene by T3 during metamorphosis.

**MATERIALS AND METHODS**

**Promoter constructs.**

The construct bearing the Hind III fragment of the ST3 promoter (-985+25) driving luciferase gene was described before (35). An Eco RI fragment encompassing the promoter region was subcloned from a lambda genomic clone (35). The subclone was sequenced on both strands and found to contain the sequence from -4340 to +1042, the end of the first exon (35), and an additional 767 bp of the first intron.

The construct pCR-ST3M1-luc (pM1) for transcriptional assay was generated through a PCR-mediated mutagenesis strategy. In brief, a fragment containing the ST3 promoter, the first exon, and the putative TRE (corresponding to DR4 in Figure 1) within the first intron was amplified from Xenopus laevis genomic DNA in the first PCR reaction (PCR 1) using primers
P46 (bearing Acc I, Cla I and Ase I at its 5'-end) and primer P47 (Table 1). A fragment encompassing the junction of the first intron and the second exon of ST3 gene was amplified from *Xenopus laevis* genomic DNA in a separate PCR reaction (PCR 2) using primers P48 and P49 (Table 1). Both PCR products were gel purified, and an aliquot of each was mixed and subjected to another PCR amplification (PCR 3) with primers P46 and P49 to produce a mutated ST3 promoter fragment (ST3M1) through the overlapped region in primers P47 and P48. Similarly, a fragment containing the coding region of firefly luciferase was amplified from pGL3 DNA (Promega) with primers P50 and P51 in PCR 4, and a fragment encompassing the SV40 polyA signal was amplified from pST6ST-C1 (Clontech) with primers P52 and HA4 in PCR 5. Both PCR products were gel purified and mixed together as the template in PCR 6 with primers P50 and HA4 to fuse the SV40 polyA signal to the end of luciferase gene through the overlapped regions in primers P50 and P51. Finally, PCR 7 was set up to amplify the entire expression unit containing the mutated promoter ST3m1 driving the expression of the luciferase, following by SV40 polyA signal (ST3M1-luc) with primers P46 and HA4 by using mixed aliquots of gel purified products of PCR 3 and PCR 6 as the templates through the overlapped region in primers P49 and P50. All the PCR reactions were performed with pfu polymerase (Promega) to minimize mutations during PCR. The final PCR fragment was ligated into pCR Blunt II TOPO vector (Invitrogen), transformed into competent *E.coli* TOP 10 cells, and spread on LB plates containing kanamycin. Clones were screened by colony PCR with primers P48 and P49. Positive clones (pM1) were selected to prepare small amount of DNA and subjected to sequencing to confirm the sequence.

Deletions were introduced into pM1 through PCR-mediated mutagenesis strategy as above, with primers P46, P53, P54 and HA4 to generate pM2, with primers P46, P61, P62 and HA4 to generate pM3, and with primers P46, P63, P64 and HA4 to generate pM4. A fragment encompassing –985 to +499 was also amplified from *Xenopus laevis* genomic DNA with primers P46 and P91, digested with Sal I and gel purified, and inserted into pre-digested (Sal I and Afe I double digestion) pM1 DNA bearing a Sal I and a blunt end to produce the construct pW.

**Transcription assay in Xenopus oocyte.**

The cytoplasm of stage VI oocytes from adult *Xenopus laevis* (Nasco) was injected with 5.75 ng/oocyte mRNAs for Flag-tagged TR and RXR (36). The firefly luciferase reporter under the control of the ST3 promoter (0.33 ng/oocyte) (pW, pM1, 2, 3 and 4) and the control vector phRG-TK (0.03 ng/oocyte) (Promega), which contained the *Renilla* luciferase under the control of the T3-independent TK promoter, were co-injected into the germinal vesicle (nucleus) after mRNA injection. After overnight incubation at 18°C, six oocytes per sample were lysed by pipetting in 90 μl of 1 X lysis buffer from the Dual Luciferase Assay Kit (Promega), and 7.5 μl of the lysate was used for each luciferase assay. Triplicate assays were performed at the same time, and the experiments were repeated three times. The relative expression of firefly luciferase from the reporter plasmid to *Renilla* luciferase from the control plasmid was determined and reported here.

**Gel mobility shift assay.**

After overnight incubation, stage VI oocytes injected with mRNAs for TR and RXR were lysed to prepare oocyte extract for gel mobility shift assay with 32P-labeled double stranded oligonucleotides DR2, DR4, or DR6 in the presence or absence of unlabeled competitor double stranded oligonucleotides as described (37). The oligonucleotides used were (with the direct repeats in bold letters): ST3TRE1A (5’ AGCTGAAAGTCAGTTAAGGTAAG 3’) and ST3TRE1B (5’ AGCTTCTCAACTAAGTGACCTTC 3’) for DR4, ST3TRE2A (5’ AGCTTCAGGGTAACAGGACCTC 3’) and ST3TRE2B (5’ AGCTGGTGCTCGTCCACCTGA 3’) for DR2, and ST3TRE3A (5’ AGCTCGAGGTCAGGGACACAGGTAAGC 3’) and ST3TRE3B (5’...
AGCTGCTACCTGTTCCCTGACCTCG 3’ for DR6.

Chromatin immunoprecipitation (ChIP).

The ChIP assay on oocyte samples for TR binding or the recruitment of corepressors was done as described (36,38). The precipitated DNA was PCR amplified with the following PCR primers: forward 5’-CAG CAC AGA ATC CAG GTA AG-3’; reverse 5’-CAA ACC CTA ACC ACC GCT TA -3’). Note that the forward and reverse primers recognize ST3 promoter and pGL2 vector sequences in pW, respectively, thus amplifying only the plasmid DNA rather than genomic DNA of the oocyte. Amplified DNA was loaded onto a 2% agarose gel and visualized by ethidium bromide staining after electrophoresis. To quantitatively analyze the precipitated DNA, the samples were assayed by quantitative PCR on an ABI 7000 (Applied Biosystems) using FAM-labeled Taq-man probes (Applied Biosystems) (39). The primers and probe for the ST3 TRE region were: forward primer, 5’-ACA CTC ACC CTA CAG CTT GTG-3’; reverse primer, 5’-CCA AGT ACA CAG AGT GCA GGT A-3’; and the probe, 5’-AAG GAT GAG GAG ATG CAT TC-3’ (labeled with reporter dye FAM and reporper quencher NFQ).

RT-PCR

Total RNA from injected oocytes was extracted with TRIzol reagent according to manufacturer’s instructions (Invitrogen, CA). The RNA was treated with RNase-free DNase I (Ambion, Texas) to remove any DNA contamination, and re-purified with TRIzol reagent. RT-PCR was performed by using Superscript One-Step RT-PCR (Invitrogen, CA) by using 0.1 μg of total RNA as described (40). The following primer pair was used in RT-PCR: forward 5’-CCT TCA GCA CAG AAT CCA G -3’ (corresponding to the end of exon 1 of the ST3 gene) and reverse 5’-CTT TTT GGA AAC GAA CAC CAC G-3’ (located in firefly luciferase coding region) for detecting spliced RNA generated from the transcripts of ST3 promoter constructs. When indicated, the primer pair, forward 5’-

RESULTS

A DR4 type TRE is located downstream of the first exon of ST3 gene.

In our previous transfection studies of the ST3 promoter in tissue culture cells, we failed to detect significant T3 induction of the promoter with constructs that included sequences from over 4 kb upstream (to the Eco RI site, Fig. 1A) to about 500 bp downstream (to Hind III site, Fig. 1A) of the transcription start site (35). These results may suggest either that the TRE in the ST3 gene is located upstream of -4000 or downstream of +500, or that the ST3 gene is not a direct T3 response gene despite the apparent resistance of its up-regulation by T3 treatment to protein synthesis inhibition (26,27). Alternatively, a TRE may be present but too far away from the start site to have a significant effect on the promoter activity in the transient transfection assay in tissue culture cells (possibly due to the lack of proper chromatin organization that is normally present in somatic cells). Thus, we sequenced a genomic subclone containing the 5 kb Eco RI fragment flanking the transcription start site as well as the region preceding exon 2 (Fig. 1A-D, GenBank accession number: AF019253). As DNA elements made of two direct repeats or inverted repeats of AGGTCA or highly related sequences are known to be binding sites for nuclear hormone receptors, we searched for such sequences and identified 3 DNA elements made of two near perfect direct repeats of AGGTCA separated by 2, 4, or 6 base pairs (bp) (DR2, DR4, DR6), respectively (Fig. 1B, C). The DR2 and DR6 elements are located upstream of the start site (not shown) and the DR4 is located downstream of the start site within the first.
intron and about 3.3 kb away from intron 1/exon 2 junction (Fig. 1C, D) (35).

Since DNA elements of DR4 type are known to bind to TR/RXR heterodimers strongly, we investigated whether the DR elements in the ST3 gene was capable of binding to TR/RXR. Thus, we microinjected mRNAs for TR and RXR into the frog oocytes and isolated oocyte extract after overnight incubation to allow the synthesis of the receptors. Gel mobility shift assay with the oocyte extracts and \( ^{32}P \)-labeled double stranded oligonucleotides containing the DR2, DR4, and DR6 sequences of the ST3 gene revealed that DR4 bound to TR/RXR heterodimer strongly while no significant binding was detected with the DR2 and DR6 elements (Fig. 2A). To further investigate the binding of these DR elements to TR/RXR, \( ^{32}P \)-labeled DR4 TRE of the ST3 gene was incubated with oocyte extracts in the presence of unlabeled double-stranded oligonucleotide competitors and the resulting mixtures were analyzed by gel mobility shift assay. The results showed that the DR4-TR/RXR complex was not competed by as much as 50 fold excess of a mutated version of the TRE oligonucleotide derived from the T3-inducible promoter of the \textit{Xenopus laevis} TR\( \beta \)A (37) (Fig. 2B, lanes 3-5) or the DR2 or DR6 oligonucleotide (Fig. 2B, lanes 6-11). On the other hand, the complex was competed by unlabeled DR4 oligonucleotide (Fig. 2B, lanes 15-17) as efficiently as by the TRE of the \textit{Xenopus laevis} TR\( \beta \)A promoter (Fig. 2B, lanes 12-14), a well characterized strong TRE of the DR4 type (41), consistent with the fact that both the DR4 TRE and TR\( \beta \)A TRE differ by only one nucleotide from the consensus TRE (two direct repeats of AGGTCA separated by 4 bp). These results suggest that the DR4 sequence of the ST3 gene is likely the element mediating the induction of the promoter by T3 through strong binding to TR/RXR.

The ST3 promoter responds to liganded TR/RXR \textit{in vivo}.

While our previous transfection studies of the ST3 promoter in a frog tissue culture cells failed to show a T3 response even with a construct containing the DR4 sequence (35), it was possibly due to either that the TR/RXR levels in the cells were too low or the lack of proper chromatin structure in the reporter plasmid in transiently transfected cells. Thus, we chose the \textit{Xenopus} oocyte system to study the ST3 promoter. The frog oocyte has a large storage of all factors necessary for early embryo development but has little or no TR and RXR. This allows one to introduce TR and/or RXR by microinjecting their mRNAs to reconstitute a T3 responsive \textit{in vivo} transcription system (37). Furthermore, any reporter plasmid injected into the frog oocyte nucleus will be chromatinized with a regular array of nucleosomes, thus enabling the study of gene regulation by TR/RXR in the context of chromatin (42).

To study the regulation of ST3 promoter in the frog oocyte, we placed the ST3 promoter from –985 to +499, containing the first exon and part of the first intron (+276 to +499) that included the DR4 TRE at +389 to +404, in front of the firefly luciferase reporter gene (construct pW, Fig. 3A). We introduced Flag-tagged TR and RXR into frog oocyte by microinjecting their mRNAs into the cytoplasm. Two hours later, the firefly luciferase reporter plasmid and an internal control plasmid, which contained the \textit{Renilla} luciferase under the control of the T3-independent TK promoter, were coinjected into the oocyte nucleus. After overnight incubation in the presence or absence of T3, the oocytes were collected to assay for the activities of the firefly luciferase over those of the \textit{Renilla} luciferase. The results showed that the expression of TR/RXR in the absence of T3 had little or a small repressive effect on the promoter activity (Fig. 3B) but the addition of T3 led to strong activation of the promoter, consistent with the presence of a strong TRE in the promoter. Furthermore, when total RNA was isolated from the oocytes and subjected to RT-PCR analysis, we found again that TR/RXR had little effect on the reporter firefly luciferase mRNA level in the absence of T3 (compare lane 2 to 1, Fig. 3C) but strongly increased it when T3 is present (compare lane 3 to 1 or 2, Fig. 3C). In contrast, the mRNA level of the internal control \textit{Renilla} luciferase was not affected by either TR/RXR and/or T3 (Fig. 3C). These results indicate that liganded TR/RXR activates the ST3 promoter \textit{in vivo}.
TR binds to the TRE in vivo and recruits corepressors to the ST3 promoter in the absence of T3.

We and others have shown previously that unliganded TR/RXR heterodimers repress TRE-containing promoter in the absence of T3 in the frog oocytes by binding to these promoters and recruiting corepressors N-CoR, SMRT, and their associated cofactor TBLR1 (36,43-45). The lack of significant repression of the ST3 promoter by unliganded TR/RXR prompted us to investigate whether TR/RXR was bound to the ST3 TRE in the absence of T3 and/or recruited corepressors to the promoter. Thus, oocyte were injected with TR/RXR mRNA and the luciferase plasmids. After overnight incubation with or without T3, oocytes were isolated and chromatin immunoprecipitation (ChIP) assay was carried out on the plasmid minichromosome with antibodies against the Flag tag of the TR, endogenous N-CoR or TBLR1. PCR analysis of the precipitated DNA revealed that the anti-Flag antibody brought down little ST3 TRE region of the reporter plasmid in the absence of injected Flag tagged TR and RXR (Fig. 4). Expression of the Flag tagged TR and RXR led to dramatically increased amount of ST3 TRE DNA immunoprecipitated by the anti-Flag antibody both in the absence and presence of T3, indicating that TR/RXR bound to the TRE independently of T3.

When ChIP assay was carried out with anti-N-CoR and TBLR1 antibodies, we found that there was little association of either N-CoR or TBLR1 with the ST3 TRE in the absence of injected TR/RXR (Fig. 4). On the other hand, the expression of TR/RXR led to enhanced binding of N-CoR and TBLR1 to the ST3 TRE in the absence of T3 and the addition of T3 resulted in their dissociation from the TRE. Thus, unliganded TR/RXR bound to the TRE in the absence of T3 and recruited corepressors N-CoR and TBLR1 to the TRE, even though relatively little repression of the promoter was observed.

Intronic TRE is capable of mediating the effects of T3.

During the above studies, we noticed that the absolute activity of the firefly luciferase was very low compared to that we observed with the T3-dependent TRβA promoter driving the firefly luciferase (using the same Renilla luciferase vector as the internal control) (data not shown). This suggests possible translational inhibition by the long 5′-UTR and/or the intronic sequence present in the transcribed RNA. In addition, the low level of the basal promoter activity might make it difficult to observe any repression of the promoter by unliganded TR/RXR. Furthermore, the promoter construct above had the luciferase coding region linked to the intron sequence downstream of the TRE, thus lacking the exon-intron (TRE)-exon organization seen in the genomic ST3 gene. To investigate whether the presence of the TRE in the intronic sequence affects the promoter activity and/or regulation by TR/RXR, we generated several additional promoter constructs. As the first intron is over 3.4 kb in length (35), we removed most of the intron but kept 160 bp of the 5′-end of the intron that also included the TRE and 115 bp of the 3′-end of the intron plus 64 bp of the exon 2 (see Fig. 1C and 1D for sequences) to ensure proper splicing. This resulted in the construct (pM1) that would produce a transcript with 738 bases (from the upstream, major transcription start site at the position -94) (35) of 5′-UTR before splicing and 463 bases after splicing (Fig. 5A). Two additional constructs with shorter 5′-UTR were also made, one with the minor transcription start site at +1 position (35) and part of the exon 1 deleted (pM2) and the other removed part of the exon 1 but kept both transcription start site (pM3). Finally, a deletion construct was made to remove most of the exon 1 and part of the intron before the TRE (pM4). This would produce a transcript that would not undergo splicing but with a 199 base 5′-UTR (pM4, Fig. 5A).

The transcriptional activities of these constructs were assayed as above in the oocytes. The constructs were injected into frog oocytes nuclei and the resulting luciferase activity in the oocytes was assayed after overnight incubation.
The results showed that while the original construct (pW) had a very low level of basal activity (i.e., the activity in the absence of expressed TR/RXR and/or T3 treatment), all new constructs had much higher basal activity (Fig. 5B), suggesting that a long 5'-UTR in the transcript from the pW construct inhibited translation in the oocyte. The results also showed that the deletion of the minor transcription start site at the +1 position (pM2) did not affect the overall promoter activity, in agreement with our earlier finding in tissue culture cells (35).

To investigate whether the RNA from the various constructs underwent appropriate splicing as intended, the RNA was isolated from injected oocytes and subjected to RT-PCR analyses with one primer near the 3'-end of exon 1 and the other near the 5'-end of the luciferase coding region. As shown in Fig. 5C, RT-PCR analysis of the transcript revealed that the RNA from the pW construct gave a band of identical size as that from PCR of the plasmid DNA itself, indicating the lack of splicing. On the other hand, the transcripts from M1-M3 produced both the band identical to that from PCR of the plasmid DNA and a shorter PCR product of expected size from splicing of the primary transcript (M4 was not analyzed since the 3'-end of the exon 1, where the first PCR primer was located, was deleted in this construct). Thus, the primary transcripts underwent the expected splicing. The results further showed that while the luciferase activity from the pW construct was much lower than those from pM1-pM3 (Fig. 5B), the transcript level was similar based on this semi-quantitative RT-PCR analysis (Fig. 5C), supporting the conclusion that 5'-UTR in the transcript from pW inhibited translation of the RNA in the oocyte.

To determine whether the regulation of the promoter by TR/RXR varies among different constructs, the plasmids were injected into the nuclei of oocytes preinjected with mRNAs for TR and RXR as above. After overnight incubation in the presence or absence of T3, the oocytes were harvested for luciferase activity measurement. As shown in Fig. 5D, all promoter constructs except pM4 behaved similarly. That is, the unliganded TR/RXR had little or a small repressive effect on the promoter activity while the addition of T3 led to strong but similar activation of the promoter in all constructs. The exception was the pM4 construct, which had the highest activity in the presence of T3-bound TR/RXR. As the TRE in pM4 is closest to the major transcription start site compared to the other promoter constructs, these results may suggest that TRE location can affect the regulation of the promoter by TR/RXR. Regardless, the results indicate that the location of the TRE in either the intron or exon does not affect the regulation of the promoter by TR/RXR.

**DISCUSSION**

The expression of MMP genes is tightly regulated. In adult vertebrates, MMPs genes are usually not expressed or expressed at low levels in most normal organs/tissues, possibly due to the potential deleterious effects of their enzymatic activity. On the other hand, many MMPs are highly upregulated during development and pathological processes. This makes the regulation of MMP expression a critical aspect of regulating MMP function. The *Xenopus laevis* ST3 was initially isolated as a highly upregulated, direct T3 response gene during amphibian metamorphosis (24,26,27). The tight correlation of its expression with apoptosis in different tissues during metamorphosis (24,31-34) and the functional requirement for ST3 for T3-induced intestinal cell death (46,47) make it critical to understand how T3 regulate the transcription of this important MMP gene during development. Here we provide evidence to show that this upregulation by T3 is mediated by a strong TRE in the first intron of the ST3 gene.

Through sequence analysis, we identified three putative nuclear hormone receptor binding sites consisting of two direct repeats of AGGTCA sequence with 2, 4, 6 bp spacing or DR2, DR4, DR6, respectively. As TRs are known to bind to DR4 type of DNA sequences, it is not surprising that the *Xenopus* TR/RXR binds strongly to the ST3 DR4 but not DR2 or DR6. Furthermore, the ST3 DR4 has only a single bp change from the consensus TRE, suggesting that it is a strong TRE. Consistently, we showed that TR/RXR binds to
this TRE with similar affinity as to another well characterized strong TRE, the DR4 type TRE from the T3-inducible of the *Xenopus laevis* TRβA gene. More importantly, by using chromatin immunoprecipitation assay, we demonstrated that TR/RXR binds to the TRE in the context of chromatin *in vivo* independently of T3 and activates the promoter in the presence of T3. It is worth pointing out that the TR/RXR mRNA levels in the injected oocytes are higher than those in metamorphosing tissues (data not shown), although currently it is difficult to quantify the receptor protein levels. A few lines of evidence argue that TR/RXR would be able to bind to the ST3 TRE in vivo during metamorphosis. First, our oocyte study is very similar to studies in tissue culture cells where TR or other transcription factors are normally overexpressed through transfection and the conclusions in tissue cultures are generally supported by more recent studies on endogenous genes in cell cultures. Second and more importantly, the TRE in the ST3 gene has similar affinity for TR as the TRE in the TRβA gene. We have shown by ChIP assay that endogenous TR is bound to the TRβA TRE in different tadpole organs during development (48), although cell type-specific expression of ST3 gene makes it difficult to do similar ChIP assays on animal tissues at the present.

It is interesting to note that the ST3 promoter was not repressed significantly by unliganded TR/RXR *in vivo*. Under the same conditions, the T3-inducible promoter of TRβA gene, which has a nearly identical DR4 TRE with similar binding affinity to TR/RXR as the TRE in ST3 gene and also located downstream of the transcription start site (41,42), is repressed dramatically by unliganded TR/RXR (36,42). A major difference between the two promoters is that the TRE in the ST3 gene is located in the first intron while the TRβA TRE is in the first exon. In addition, the TRE in the ST3 gene is further away from the transcription start site. However, our mutational and deletion analyses indicate that the location of the TRE in the intron or exon and the distance of the TRE to the start site have no effect on the inability of the unliganded TR/RXR to repress the ST3 promoter. This suggests that promoter context may play a critical role in the ability of unliganded TR/RXR repress transcription. Our result here is also consistent with our earlier studies in developing animals. There, we overexpressed TR/RXR in developing *Xenopus laevis* embryos through microinjection of their mRNAs into fertilized eggs. When the developing embryos were treated with T3, the endogenous ST3 gene was highly upregulated whereas we failed to observe significant repression of the gene in the absence of T3 (49). These data together indicate that unliganded TR/RXR has limited, if any, ability to repress the ST3 gene *in vivo* in the absence of T3.

Like in *Xenopus laevis*, the ST3 gene is also tightly regulated temperately and spatially in mammals. Both the frog and mammalian genes are specifically expressed in fibroblastic cells during developmental and/or pathological processes. This raises the possibility of the presence of conserved regulator sequences in the ST3 genes. Indeed, a DR4 type of TRE has been found at about 550 bp upstream of the transcription start site in mouse ST3 gene (50). In addition, both mouse and human ST3 genes contain DR1 and/or DR2 type of retinoic acid response elements (RAREs) and both are activated by retinoic acid (50,51). Interestingly, a near perfect DR2 RARE (with only a single bp difference from the consensus DR2 RAREs made of two direct repeats of AGGTCA with 2 bp spacing, Fig. 1B) is present upstream of the transcription start site of the *Xenopus* ST3 promoter. This suggests that retinoic acid may also regulate ST3 expression in *Xenopus laevis*. Since retinoic acid receptors are known to be present during *Xenopus laevis* embryogenesis when ST3 is highly expressed, especially during the period shortly before hatching (24,37,52), it is tempting to speculate that retinoic acid may regulate ST3 expression during *Xenopus laevis* embryogenesis.

In summary, through sequence analysis, we have identified several DNA elements consisting of two direct repeats of AGGTCA, the core sequence of many nuclear hormone response elements. We have shown that the DR4 element functions as a strong TRE *in vivo* in the context of chromatin both when present in the
exon or in the intron, where it is located in the genome. Our ChIP assay directly demonstrated that TR binds to the DR4 \textit{in vivo}. Although unliganded TR can recruit corepressors to the promoter, it has little effect on the transcription of the promoter in the frog oocyte model system. This result is similar to that we observed previously in developing animals, suggesting the promoter context affects gene repression by unliganded TR. While currently it is unknown if the DR6 element can be recognized by any transcription factors, the DR2 element is likely to function as an RARE, possibly allowing retinoic acid to regulate this promoter during \textit{Xenopus} embryogenesis. It would be interesting to investigate this possibility directly both in developing animals and in model systems in the future.

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FIGURE LEGENDS

Figure 1. Organization of the ST3 promoter region and location of the putative hormone response elements.

A. Schematic diagram of ST3 gene. There are 3 hormone response elements (DR2, DR4, and DR6) flanking the two transcription start sites (arrows) with the major one at -94 and minor one at +1 (35), among which DR6 and DR2 are upstream of the transcription start sites while DR4 is in the first intron of the ST3 gene. E: EcoR I recognition site; H: Hind III recognition site; E1 and E2: the first and second exon, respectively.

B. Sequences of the putative hormone response elements. Bold letters represent the half site of each element in the direct repeats, and the faded letters represent the spacer sequences. The numbers are the relative position to the transcription start site (+1).

C. Sequences of the promoter region, exon 1, and 5'-end of intron 1 of Xenopus laevis ST3 gene. The two transcription start sites are indicated by arrows and the sequence of the first exon is underlined. Boxed sequences are putative TATA boxes and italic bold letters represent the DR4 element. The GAGA factor binding sites known to be important for promoter function (35) are indicated by stars.

D. Sequences around the junction of intron 1 and exon 2. The first nucleotide of the exon 2 is denoted as +1E2, and the last nucleotide of the first intron is denoted as -1I. The exon sequence is underlined.

Figure 2. DR4 but not DR2 or DR6 element in ST3 gene binds to TR/RXR complex in gel shift assay.

A. Labeled DR2, DR4, and DR6 elements were incubated with oocyte extract containing over expressed TR/RXR and the resulting mixtures was analyzed on a non-denaturing polyacrylamide gel. Note that only DR4 formed a stable complex with TR/RXR.

B. Specific competition for TR/RXR binding by ST3 DR4 element. Labeled ST3 DR4 element was incubated with TR/RXR extract in the presence of 0, 5, 25 or 50 fold excess of indicated unlabeled competitors. The resulting mixture was analyzed as above. Note that only upper band representing the DR4-TR/RXR complex as seen in (A) was shown here. TRβ TRE and TRβ mTRE were wild type and mutant TRE (DR4 type) from the T3 regulated Xenopus laevis TRβA gene promoter, respectively (41).

Figure 3. T3 upregulates the ST3 promoter containing the DR4 element in vivo in the context of chromatin.

A. Schematic diagram of the reporter construct pW with the firefly luciferase reporter gene under the control of the ST3 promoter containing the putative TRE. The arrows indicate the two transcription start sites. E1: exon 1.

B. TR/RXR heterodimer activates ST3 promoter in the presence of T3. The pW construct was coinjected with the internal control plasmid phRG-TK driving the expression of Renilla luciferase into the nuclei of the oocytes with or without prior microinjection of mRNAs for Flag-tagged TR and RXR (F-TR/RXR) into the cytoplasm. The oocytes were incubated at 18 °C overnight in the presence or absence of 100 nM T3 and then subjected to dual luciferase assay. The firefly luciferase activity (F) over the Renilla luciferase activity (R) for each sample, i.e., F/R, was measured and plotted here with the F/R for the oocytes without TR/RXR mRNA microinjection or T3 incubation set to 1 (the actual firefly luciferase activity ranged from 10000 to 60000 in different oocyte samples with the background of about 200).

C. RT-PCR demonstrates the upregulation of the reporter mRNA (firefly luciferase, F-luc) by TR/RXR plus T3. Some of the oocytes as in (B) were subjected to total RNA isolation. The RNA was made DNA-free and analyzed by RT-PCR for the mRNA levels of F-luc driven by the ST3 promoter. A pair of primers specific for the Renilla luciferase mRNA (R-luc) driven by the coinjected internal control plasmid were included in these RT-PCR reactions. Plasmid DNA pW (lane 4) and phRG-TK (lane 5) were used in PCR reactions as positive controls for R-luc and F-luc PCR. Note that while the R-luc transcripts from the T3-independent TK promoter were similar in the presence or absence of TR/RXR
and/or T3 (lane 1-3), the F-luc mRNA driven the ST3 promoter was upregulated in the presence of T3 and TR/RXR (compare lane 3 to lanes 1 and 2).

**Figure 4.** TR/RXR binds to the DR4 element *in vivo* and recruits corepressors to the promoter in the absence of T3.

**A.** Oocytes were injected with or without Flag-tagged TR (F-TR)/RXR and the reporter plasmids and incubated with or without T3 as in Fig. 3. The oocytes were then subjected to ChIP assay using antibodies against the Flag tag in TR, endogenous N-CoR, or TBLR1. The immunoprecipitates was subjected to PCR with a primer pair flanking the DR4 region to determine the presence of the DR4 TRE sequence. The PCR products were analyzed by agarose gel electrophoresis and visualized with ethidium bromide staining. Oocytes were injected and incubated with or without T3. Aliquots of DNA prior to antibody immunoprecipitation were amplified as the "Input" control to show the amounts of DNA in different samples.

**B.** Quantitative PCR analysis of the ChIP samples as shown in (A). The immunoprecipitated and the "Input" control DNA as shown in A was analyzed by real-time PCR. The ratio of the precipitated DNA by each antibody to the corresponding "Input" control is shown here with the ratio in lane 1 set to 1. The results shown here represent the sum of the data from three independent experiments carried out on different days. Note that TR bound to the promoter constitutively (lanes 2 and 3) and recruited N-CoR and TBLR1 in the absence of T3 (lane 2). The addition of T3 dissociated the corepressors (lane 3).

**Figure 5.** Intronic TRE mediates T3-regulation of the ST3 promoter.

**A.** Schematic diagram of mutant ST3 promoter constructs. Deletions were introduced into the first intron and/or first exon of the ST3 gene to produce pM1, pM2 and pM3, all of which contained regions flanking the intron 1/exon 2 junction to allow proper splicing of the primary transcripts. The construct pM4 had an internal deletion in exon 1 and truncation of the first intron. The transcripts from constructs pW and pM4 should not undergo splicing. The total 5'-UTR nucleotide lengths (nt) (from the major start site at -94) before and after (if applicable) splicing are shown. N/A, not applicable.

**B.** The 5'-UTR from pW inhibits translation. The constructs in A were co-injected with phRG-TK DNA into the nuclei of the oocytes. The oocytes were incubated at 18 °C incubator overnight and were isolated for dual luciferase assay. The firefly luciferase activity over Renilla luciferase (F/R) from the construct pW was denoted as 1, and the F/R ratios from other constructs were normalized accordingly. Note that all mutated promoter constructs had much higher activity than pW, suggesting that the long 5'-UTR from pW inhibited translation.

**C.** The primary transcripts from pM1, pM2, and pM3 undergo proper splicing in the oocyte. The plasmids pW, pM1, pM2, and pM3 were injected into oocyte nucleus. The oocytes were incubated at 18 °C. Total RNA was then isolated from the oocytes and subjected to RT-PCR analysis. The plasmid DNA for each construct was also amplified for comparison. The bands designated as S and US correspond to spliced and unspliced RNA transcripts, respectively. D: DNA template; R: RNA template.

**D.** All promoter constructs containing TRE respond to T3 similarly. The promoter constructs were co-injected with phRG-TK DNA into the nuclei of the oocytes with or without prior microinjection of Flag-tagged TR (F-TR)/RXR mRNAs into the cytoplasm. The oocytes were incubated at 18 °C overnight in the presence or absence of 100 nM T3 and then isolated for dual luciferase assay. The ratio of the firefly luciferase activity (F) over that of the Renilla luciferase (R) for each sample was normalized against that from the oocytes without F-TR/RXR mRNA microinjection or T3 incubation for each promoter construct separately. Note that for all constructs, TR/RXR had little or a small repressive effect in the absence of T3 but activated strongly and similarly in the presence of T3. The exception was pM4, which had about 4 fold higher activity, possibly due to the fact that the TRE in this construct is the closest to the transcription start sites.
Table 1: Primers used in preparing promoter constructs

| Primer | Sequence (5’ to 3’) |
|--------|---------------------|
| P46    | 5’ GTC GAC ATC GAT TAA TGC TTA AAG TTA TTA GTG ATC CAG G 3’ |
| P47    | 5’ GGT AGG GCC TGG GTT TTC AAG TGT ATC TAC C 3’ |
| P48    | 5’ GGT AGA TAC ACT TGA AAA CCC AGG CCC TAC CGC ACA TAT AGA ATA GA 3’ |
| P49    | 5’ ACC GGT AGC GCT CCT TTA TAT GAG GAT AAT GTA ATG 3’ |
| P50    | 5’ CAT TAC ATT ATC CTC ATA TAA AGG AGC GCT ACC GGT ACT GTT GGT AAA GCC ACC A 3’ |
| P51    | 5’ GAA TTC AGA TCT TAC ACG GCG ATC TTT CCG CCC TTC 3’ |
| P52    | 5’ CGG AAA GAT CGC GTG AGA TCT GAA TTC TGA TCA TAA TCA GCC ATA CCA CAT 3’ |
| HA4    | 5’ CTA TAG AAT GCA GTG AAA AAA ATG C 3’ |
| P53    | 5’ TGC TGA AGG TAC TCC TCT CTG CCT TTT GTT CTC 3’ |
| P54    | 5’ GAG AAC AAA AGG CAG AGA GGA GTA CCT TCA GCA CAG AAT CCA GGT AAG 3’ |
| P61    | 5’ TGC TGA AGG GCG GAG GAG GAG CTG TCC GGT GCT 3’ |
| P62    | 5’ GAC AGC TCC TCC GCC CTT CAG CAC AGA ATC CAG GTA AG 3’ |
| P63    | 5’ CTA TCT ACC TCA AAT CTC ACC TTA ACT GAC CTT CAG GCT GAA CCC CCC TA GCG GAG GAG CTG TCC GGT GCT 3’ |
| P64    | 5’ TAA GGT GAG ATT TGA GGT AGA TAC AGC GCT ACC GGT ACT GTT GGT AAA GCC ACC A 3’ |
| P91    | 5’ GCT TCG TAG TCC TTT GTT CCA 3’ |

Note: Italic letters indicate restriction enzyme recognition sequences, and bold letters indicate DR4 sequences (Fig. 1B, complementary sequences of DR4 in p63 and partial of DR4 in p64).
B

-3614  -3597
DR6: AGGTCAGGGAACAGGTAA
      +389  +404
DR4: AGGTCAGTTAAGGTGA
      -597  -584
DR2: AGGTGAACAGGACA

Fig. 1
C

Sequence of promoter and exon 1/intron 1 junction:

-300 AGATTTCACA CCTGAATCAC CAACAAAGAA ACCATTCAG ATGATAGAGT TTTGTTCTTT -241
-240 TTACTGCCAC TGTGGTTTAA TTTGTCTTTA AGGAAGGAGA CCATGCTTTT TCTCCACCTC -181
-180 CCAGCTGTAC CCCTCTCCTC AGTCACCCCC TTTCTCCTTC CCAAACTACC AGCACGCTGT -121
-120 GCGCGCTCAG AGATGGTTGTT GTCACTCCAG GCCCTAAAAG TGAAGAGGGA GAGAGAGAGA -61

-60          GAGAGAGAGA ACAAAGGCA GAGAGAGA ACAAAGGCA GCTGGATTCT CATGCACACT

1           GACAAGCACC GGACAGCCTC TCCGCGCGCACACACACAGA GCAAGCAGGG AGAGAGCCAG

61          AGCCACAGAC AAGACACCT ACACAGACTC ACAGGGAAAA GACTTAGTGA AAGGACTTGT

121         GTAATACCTT GCAGTGAGGA AACACTTGTA GCCATGTAT CACACTCACC CTAAGCTTG

181         TGAGATGCA TCTCCTCATC TCTGTGACTG CACTGTGTTT ACTTGGAGCT CACTCAGCCC

241         CTCGTCCTA TAAGGTCTGT CAGCACAGAA TCCAG

301         ACTTGAAAAC CCAGTCTCAG GTGAGATTG AGGGGTAAG

361         GAGCCTGA AAG GTGAGTTAAG GTGAGATTG AGGGGTAAG

421         ACTTGGAAAAC CCAGGTTCAA GTGAGATTG AGGGGTAAG

481         AAGGACTACA TCAAGCTT

Figure 1C
Sequence of the intron 1/exon 2 junction:

-296I     CTCCAT CCTATTCTA TTACCTTGCA TCTGTGCTA GAGTAAGGCC AAGTACAGGC
-240I     AGCAGTTAAA ATTCCGACC TGAGAGATTA GAATAAAAA GAATGCAA A TTGTCTTGGA
-180I     ATATTCCTTT CCTTTCTACA TAAATACTAA ATGTATTTTT ATGGTGACCT ACCACTTTTC
-120I     ATCACCCCTA CGCAGCATAT AGAATAGATA ATAAACAAGTG GAGGTCTTTTC AAATATGAAT
-60I      CATAAGGTAA TCATAATATA AAACCTCTAC CCTTCATAA CCAGATTGT TTTATTCACAG
1E2       GAAAAGCCTC AGAAAGATCA TGCGAGGTTG CAGTTTAATT CATTACATTA TCCTCATATA
61E2      AAGGACTAGC T

Figure 1D
Fig. 2
Fig. 2
A

B

Fig. 3
Fig. 3
Fig. 4
Fig. 4
Fig. 5
Fig. 5
Fig. 5
Fig. 5
Figure S1: Comparison of TR mRNA levels in the intestine of metamorphosing tadpoles and the oocytes with or without F-TR mRNA injection. The intestines were dissected from tadpoles at premetamorphic stage 54 (lane 1) and during metamorphosis (stage 60, the climax of metamorphosis, lane 2), respectively, and subjected to RNA isolation. Oocytes without (lane 3) or with (lane 4) F-TR mRNA injection were collected 2 hours after mRNA microinjection and groups of 8 oocytes each were used for RNA isolation. The RNA was extracted with TRIzol reagent (Invitrogen), made DNA-free with RNA-free DNase I treatment, and re-extracted with TRIzol reagent. 0.5 µg (oocyte RNA) or 2.5 µg (intestinal RNA) of each RNA sample was used for reverse-transcription in 50 µl with a High Capacity Reverse Transcription Kit (Applied Biosystems), and 4 µl of the product were subjected real-time PCR analysis for TR transcripts. The value for the endogenous TR mRNA in oocytes (lane 3) was set to 1.
Transcriptional regulation of the *Xenopus laevis* stromelysin-3 gene by thyroid hormone is mediated by a DNA element in the first intron

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