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Tissue- and Gene-specific Recruitment of Steroid Receptor Coactivator-3 by Thyroid Hormone Receptor during Development* ♦

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Numerous coactivators that bind nuclear hormone receptors have been isolated and characterized *in vitro*. Relatively few studies have addressed the developmental roles of these cofactors *in vivo*. By using the total dependence of amphibian metamorphosis on thyroid hormone (T_3) as a model, we have investigated the role of steroid receptor coactivator 3 (SRC3) in gene activation by thyroid hormone receptor (TR) *in vivo*. First, expression analysis showed that SRC3 was expressed in all tadpole organs analyzed. In addition, during natural as well as T_3-induced metamorphosis, SRC3 was up-regulated in both the tail and intestine, two organs that undergo extensive transformations during metamorphosis and the focus of the current study. We then performed chromatin immunoprecipitation assays to investigate whether SRC3 is recruited to endogenous T_3 target genes *in vivo* in developing tadpoles. Surprisingly, we found that SRC3 was recruited in a gene- and tissue-dependent manner to target genes by TR, both upon T_3 treatment of premetamorphic tadpoles and during natural metamorphosis. In particular, in the tail, SRC3 was not recruited in a T_3-dependent manner to the target TRβA promoter, suggesting either no recruitment or constitutive association. Finally, by using transgenic tadpoles expressing a dominant negative SRC3 (F-dnSRC3), we demonstrated that F-dnSRC3 was recruited in a T_3-dependent manner in both the intestine and tail, blocking the recruitment of endogenous coactivators and histone acetylation. These results suggest that SRC3 is utilized in a gene- and tissue-specific manner by TR during development.

Thyroid hormone (T_3)[1] affects diverse organ functions and metabolism in vertebrates (1–3) and plays critical roles in postembryonic organogenesis and tissue remodeling in vertebrates (1–6). The effects of T_3 are mediated by T_3 receptors (TRs), which are transcription factors belonging to the nuclear receptor superfamily (3, 7–11). TR forms a heterodimer with 9-*cis*-retinoic acid receptor (RXR) and binds to thyroid hormone response elements (TREs) of T_3-responsive promoters to modulate transcription. TR/RXR heterodimers function to repress or activate target gene transcription in the absence or presence of T_3, respectively, by recruiting corepressors or coactivators (3, 12–17).

The best characterized coactivators for TR belong to the SRC or p160 family, comprising three homologous members, SRC1/NCoA-1, SRC2/TIF2/GRIP1, and SRC3/pCIP/ACTR/AIB-1/RAC-3/TRAM-1 (18–26). These proteins share considerable structural homology and are evolutionarily related, being about 40% identical among each other, with extensive similarity at the N-terminal basic helix-loop-helix and PAS dimerization domain (27–29). The central region of SRC proteins contain three leucine rich, L*XX*LL (L, leucine; *X*, any amino acid) motifs, forming short amphipathic α-helices (19, 26, 30–32) and constitute the receptor interaction domain. SRC proteins interact with nuclear receptors directly in a ligand-dependent manner and facilitate transcription via distinct activation domains, AD1 and AD2. AD1, which has two L*XX*LL motifs, can bind to the histone acetyltransferase CBP/p300 (21, 32, 33). SRCs themselves have also been reported to possess weak intrinsic histone acetyltransferase activity (21, 34). AD2 has been reported to interact with chromatin modifying enzymes including methylases such as coactivator-associated arginine methyltransferase-1 (CARM-1) and protein arginine methyltransferase-1 (PRMT-1) (35, 36).

Despite the enormous accumulation of molecular and biochemical information on coactivator-nuclear receptor interactions, the *in vivo* role of SRCs and their physiological significance in nuclear receptor-mediated developmental processes in vertebrates have remained essentially unexplored. Even when gene knock-out studies reveal that cofactor deficiency leads to specific development defects (37–42), the underlying molecular mechanisms are unknown, largely due to the fact that these cofactors are involved in transcriptional regulation by many diverse transcription factors and the difficulty to access and manipulate postembryonic development in mammals.

Amphibian metamorphosis bears strong similarities to postembryonic development in mammals (2, 4, 5) and offers a unique opportunity to study the role of cofactors in nuclear receptor function in vertebrate development. A major advantage of this model is that all tissues/organs require T_3 despite undergoing vastly different transformations during metamorphosis (2, 43). These changes range from the development of adult organs *de novo* from undifferentiated stem cells to the regression of larval-specific organs such as the gills and tail and occur at developmentally distinct stages. All these changes are believed to be due to gene regulation by T_3 through TR (44) and can be easily manipulated by blocking the synthesis of endogenous T_3 or adding physiological concentrations of T_3 to the tadpole rearing water.

We have shown earlier that the mRNAs of TR interacting cofactors, SRC2, SRC3, and p300, are expressed during meta-

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[1] The abbreviations used are: T_3, triiodothyronine; RXR, 9-*cis*-retinoic acid receptor; TR, thyroid hormone receptor; TRE, thyroid hormone response element; ChIP, chromatin immunoprecipitation; RT, reverse transcriptase.

morphosis, among which SRC3 is up-regulated during both natural and T_3-induced metamorphosis, supporting a role for this coactivator (45). In this study, we show that SRC3 is also up-regulated at the protein level during natural as well as T_3-induced metamorphosis. More importantly, by using chromatin immunoprecipitation (ChIP) assay on wild type and transgenic animals, we demonstrated that SRC3 is recruited to T_3-responsive genes in developing animals in a tissue- and gene-dependent manner during both natural and T_3-induced metamorphosis, implicating that SRC3 utilization by TR is affected by tissue and promoter context.

MATERIALS AND METHODS

Animals and Treatment—Wild type tadpoles of the African clawed toad *Xenopus laevis* were obtained from Xenopus I. Inc. (Dexter, MI), and developmental stages were determined according to Nieuwkoop and Faber (46). Stage 54 premetamorphic tadpoles at a density of two tadpoles per liter of deionized water were treated with the indicated amount of T_3 for 2–3 days. The concentrations of T_3 used were according to published protocols. In the experiments conducted, varying the T_3 concentration between 10 and 50 nM did not affect the conclusions. Transgenic tadpoles were generated as described (47).

RNA Isolation and Reverse Transcriptase PCR (RT-PCR)—RNA was isolated using the TRIzol reagent (Invitrogen) per the manufacturer's recommendations. RT-PCRs were carried out using the Superscript One-Step RT-PCR kit (Invitrogen). The expression of the ribosomal protein L8 (rpl8) was used as an internal control (48). The sequences of the primers used were (5′-3′): CGTGGTGCTCCTCTTGCCAAG and GACGACCAGTACGACGAGCAG for rpl8 (48), CACTTAGCAACAGGGATCAGC and CTTGTCCCAGTAGCAATCATC for TH/bZIP (49), ATAGTTAATGCGCCCGAGGGTGGA and CTTTTCTATTCTCTCCACGCTAGC for TRβA (50), GGACATATGAGTGGATTAGGGGAA and CACGGATCCCTACACATCGTCATTAGA for SRC3 (51, 52), CCTGATGCATGCAAAACT and GTTCATCCTGGAAAGCAG for ST3 (53). PCR was also done on RNA samples without reverse transcription as a control for genomic DNA contamination (data not shown). 0.5 μg of total RNA was used in a 25-μl reaction and with the following reaction conditions: 42 °C for 30 min for the RT reaction, followed by 21–25 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s. The resulting products were analyzed on an agarose gel stained with ethidium bromide.

Preparation of Tadpole Tissues for Western Blot Analysis—Tadpoles were sacrificed by decapitation on ice. The dissected organs were sliced into small pieces and homogenized in buffer containing 50 mM Tris-HCl, pH 8.0, 1% SDS, 1 mM dithiothreitol, and protease inhibitor mixture (Roche Diagnostics Corp.). The lysate was centrifuged at 11,000 × *g* for 5 min, and the protein in the supernatant was quantitated by Bradford's assay (Bio-Rad). Equal amounts of protein were loaded on an 8–16% Tris-glycine gel and transferred onto a polyvinylidene difluoride membrane for Western blot analysis.

Chromatin Immunoprecipitation Assays—ChIP assay with tissues from tadpoles was performed as described previously (54, 55) with minor modifications (56). The following antibodies were used in the assay: anti-*Xenopus* TR (57), anti-acetylated histone H4 (Upstate Biotechnology, Lake Placid, NY), anti-FLAG M2-agarose (Sigma), and anti-*Xenopus* SRC3 (47). Immunoprecipitation of dnSRC3 was carried out using anti-FLAG M2-agarose beads (Sigma). Preclearing for the anti-FLAG antibody was done using protein G-Sepharose beads (Amersham Biosciences). After reverse cross-linking, DNA was purified using a PCR purification kit (Qiagen). Quantitative PCR was carried out with ChIP DNA sample in duplicate on an ABI 7000 (Applied Biosystems) using promoter-specific primers and FAM (6-carboxyfluorescein)-labeled Taq-man probes (Applied Biosystems). To perform relative quantitation, six 3-fold serial dilutions from a large batch of ChIP input DNA prepared from intestines prepared especially for the purpose of serving as standards were used for the quantification of the experimental samples. The calculated standard curves ranged in slope from −3.30 to −3.50, where theoretical amplification has a slope of −3.32. Also included was a no template control where double distilled water was added instead of sample DNA as a control for PCR product contamination. Results from the experimental samples were within the range of the standard curve. The primers used for conventional and quantitative PCR were reported previously (44, 56).

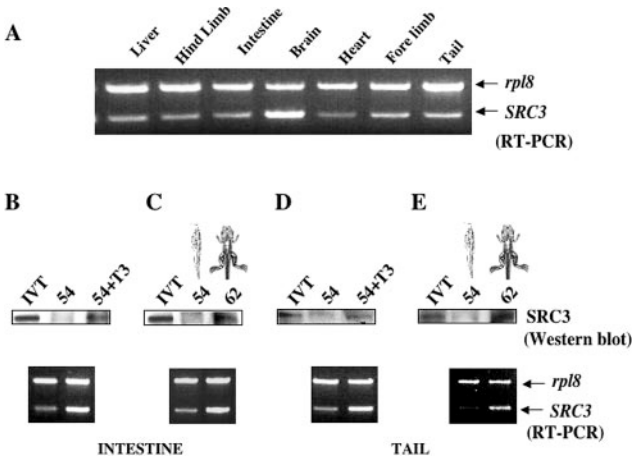

FIG. 1. **Expression and regulation of SRC3 in developing tadpoles.** *A*, expression of SRC3 mRNA in various tissues of stage 62 metamorphic tadpoles as determined by RT-PCR with *rpl8* gene as an internal control. *B* and *C*, up-regulation of SRC3 mRNA and protein levels in the intestine during T3-induced (*B*) and natural (*C*) metamorphosis. For T3-induced metamorphosis, stage 54 premetamorphic tadpoles were treated with 50 nM T3 for 2 days and compared with untreated tadpoles. For natural metamorphosis, premetamorphic tadpoles at stage 54 were compared with tadpoles at stage 62 (climax of metamorphosis), when endogenous T3 levels are high. The *top panels* show the protein expression by Western blot analysis of intestinal protein extract with the SRC3 antibody, and the *bottom panels* show mRNA expression as revealed by RT-PCR with *rpl8* gene as an internal control. *D* and *E*, up-regulation of SRC3 mRNA and protein also occurs in the tail during T3-induced (*D*) and natural (*E*) metamorphosis. The experiments were done as above. *IVT*, *in vitro* transcribed and translated (47).

RESULTS

Expression and T3 Regulation of SRC3 in Developing Animals—We studied the expression of SRC3 using RT-PCR in different organs of *X. laevis* tadpoles. SRC3 was expressed in all organs analyzed, including the intestine, heart, liver, fore limb, hind limb, brain, and tail, with a higher level of expression in the brain (Fig. 1*A*). We next studied the expression of SRC3 protein during T3-induced metamorphosis in the tail and intestine, two organs that undergo dramatic changes during development (2, 43, 58). *X. laevis* tadpoles at stage 54 (46) were treated with T3, which is known to induce the expression of T3-response genes in these organs and initiate metamorphosis (2, 43). The tail and intestine of these animals were dissected out and subjected to Western blotting using anti-SRC3 antibodies to detect the expression of the SRC3 protein. In parallel, total RNA isolated from the two organs was analyzed by RT-PCR to check the expression of the SRC3 transcript. The expression of SRC3 protein was up-regulated in the T3-treated tadpoles in the intestine and tail (Fig. 1, *B* and D, *top panels*), just like SRC3 mRNA (Fig. 1, *B* and *D*, *bottom panels*) (45). Furthermore, during natural metamorphosis, the expression of SRC3 was also found to be up-regulated at the climax of metamorphosis (stage 62) as compared with premetamorphosis (stage 54) in the intestine and tail (Fig. 1, *C* and *E*, *top panels*), again like SRC3 mRNA (Fig. 1, *B* and D, *bottom panels*) (45). These results suggest a role of SRC3 in tissue transformation during both natural and T3-induced metamorphosis.

Gene- and Tissue-dependent Recruitment of SRC3 to T3-responsive Promoters by TR—To study the involvement of SRC3 in T3-mediated transcription *in vivo*, we focused on the intestine and the tail. The metamorphosis of the intestine and the tail have been well characterized (2, 43, 58). These two organs share a number of similarities. Cell death or apoptosis is the major event at early stages of metamorphosis in both

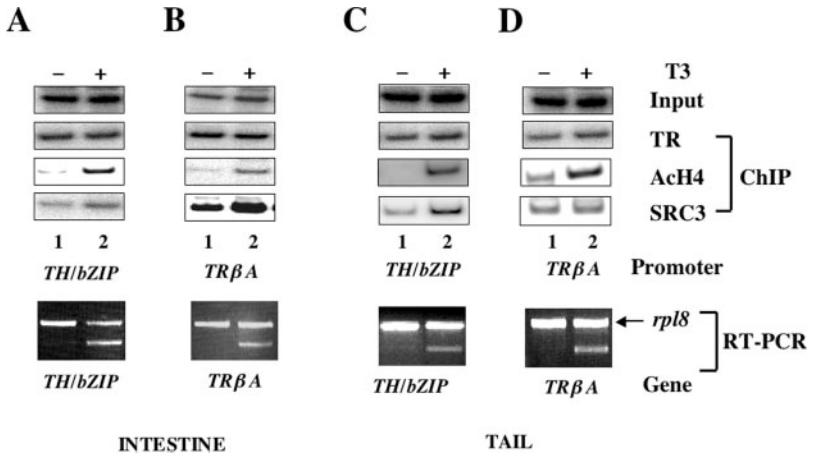

FIG. 2. **T3 induces tissue- and gene-dependent recruitment of SRC3 in premetamorphic tadpoles.** Premetamorphic tadpoles at stage 54 were treated with 10 nM T3 for 2 days. Chromatin from intestine and tail nuclei were immunoprecipitated with antibodies against TR, SRC3, and acetylated histone H4 (*AcH4*) and analyzed by PCR for the presence of TRE regions of two T3 response genes, *TRβA* (*B*, *D*) and *TH/bZIP* (*A*, *C*). Aliquots of the chromatin before immunoprecipitation were used directly for PCR as control (input). Note that T3 treatment increased the SRC3 recruitment to the *TRβA* promoter in the intestine but not in the tail. The data represent one of four independent experiments with different animals, all yielding similar results. The induction of the *TRβA* and *TH/bZIP* genes in both the intestine and tail were confirmed by RT-PCR reactions with *rpl8* gene as an internal control (*bottom panels* of *A–D*).

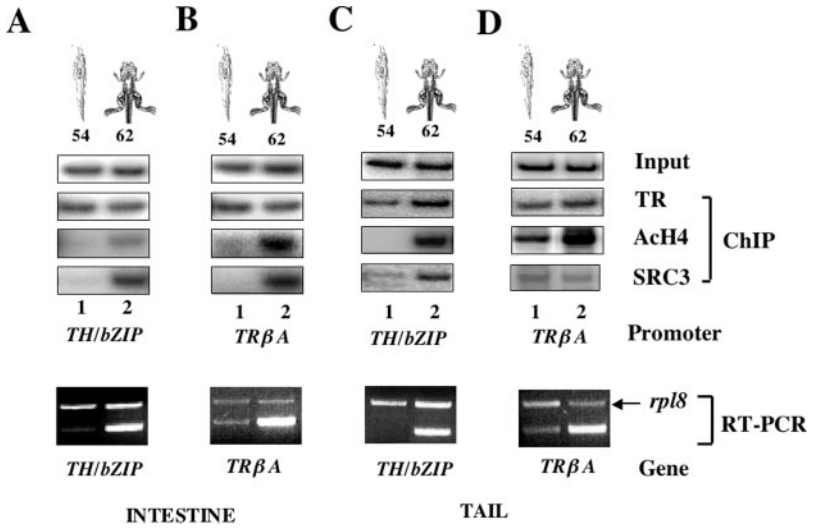

FIG. 3. **Tissue- and gene-dependent recruitment of SRC3 to T3-responsive promoters, *TRβA* and *TH/bZIP*, in the intestine (*A*, *B*) and tail (*C*, *D*) of tadpoles during natural metamorphosis.** Chromatin from intestine and tail nuclei of stage 54 (premetamorphic) and stage 62 (metamorphic) tadpoles were isolated, and ChIP assays were carried out. Again, SRC3 recruitment to the *TRβA* promoter was higher in the intestine but not in the tail during metamorphosis as during T3 treatment (Fig. 2). The data represent one of four independent experiments with different animals, all yielding similar results. The *bottom panels* show the RT-PCR reactions carried out to confirm the up-regulation of the *TRβA* and *TH/bZIP* during natural metamorphosis with *rpl8* gene as an internal control.

organs. A number of T3-response genes are similarly regulated in both organs (58), including the *TRβA* and *TH/bZIP* genes, whose promoters have been shown to contain TREs (49, 59). Thus, we carried out ChIP assays to determine the recruitment of endogenous SRC3 to the T3-responsive promoters, *TRβA* and *TH/bZIP* in the tail and intestine. We treated premetamorphic *X. laevis* tadpoles at stage 54 (46) with T3 for 2 days, which is known to initiate metamorphosis (2, 43). As expected, both *TRβA* and *TH/bZIP* genes were up-regulated in the intestine and tail as shown by RT-PCR analysis of total RNA isolated from the two organs (Fig. 2, *bottom panels*). When the nuclei were isolated from the intestine and tail and subjected to ChIP assays with antibodies against TR, acetylated histone H4 (*AcH4*), and SRC3, we found that as expected, TR was bound constitutively to both promoters, while an increase in levels of acetylated histone H4 was observed with T3 treatment (Fig. 2, *A–D*, *lane 2*), correlating with transcriptional activation and consistent with previous results (60). In the intestine, an increased association of SRC3 was observed at both *TH/bZIP* and *TRβA* promoters upon T3 treatment (*lane 2* of Fig. 2, *A* and *B*). Surprisingly, in the tail, T3 treatment led to an increased recruitment of SRC3 only at the *TH/bZIP* promoter (Fig. 2*C*, *lane 2*) but not the *TRβA* promoter (Fig. 2*D*, *lane 2*).

To investigate whether this tissue- and gene-dependent recruitment of SRC3 is an artifact of precociously metamorphosis induced by T3 or occurs physiologically during metamorphosis, we carried out ChIP assays by using organs isolated from premetamorphic tadpoles or tadpoles at the climax of metamorphosis when endogenous levels of T3 are high. As observed during T3-induced metamorphosis, TR bound the promoters constitutively, and the acetylation levels of histone H4 was much higher at the climax of metamorphosis at both promoters in both organs (*lane 2* of Fig. 3, *A–D*). Furthermore, in the intestine, we found that SRC3 was recruited to both promoters at the climax of metamorphosis (stage 62) but not in premeta-

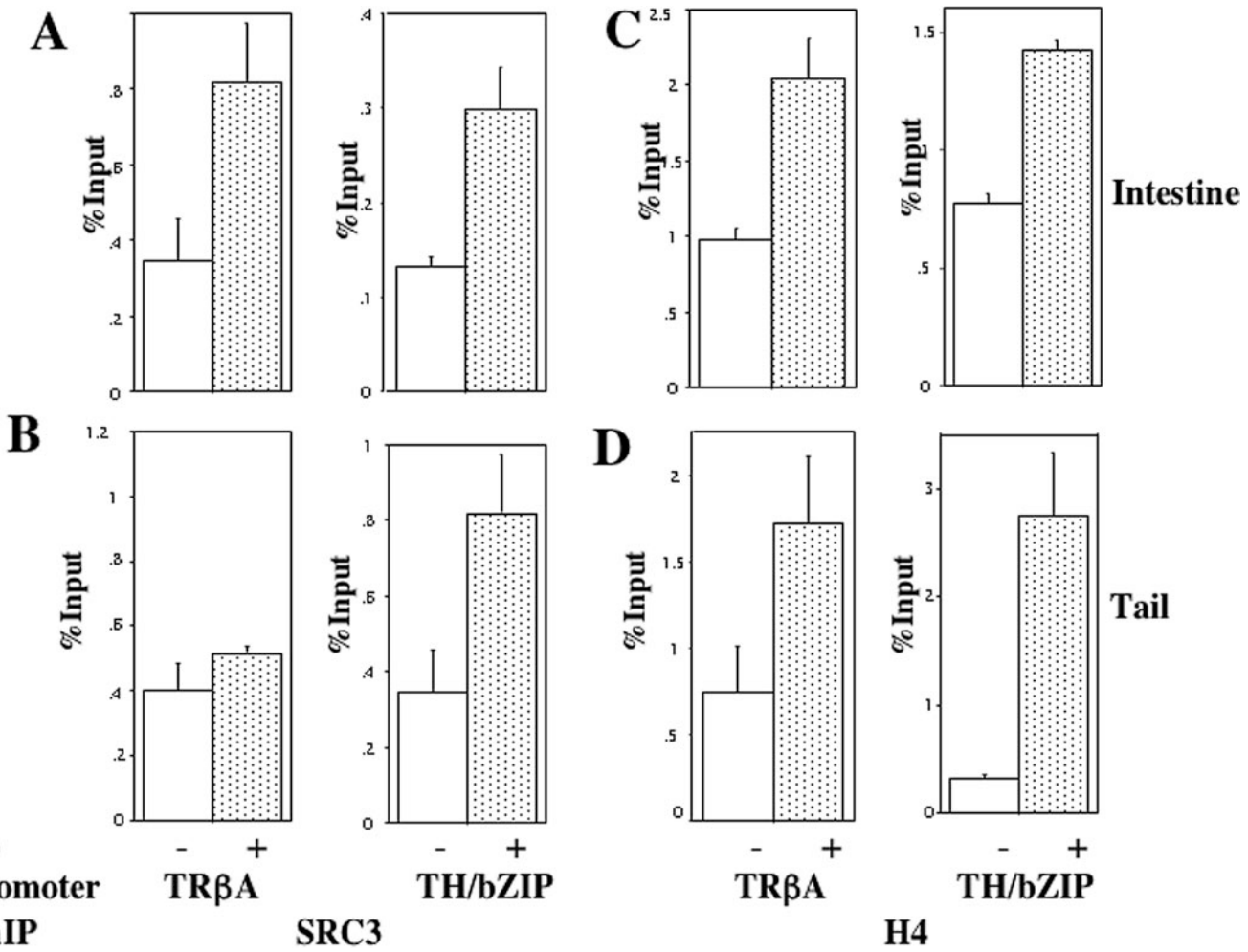

FIG. 4. **Real-time PCR analysis showing differential SRC3 recruitment to T3-dependent promoters during T3-induced metamorphosis.** Premetamorphic tadpoles at stage 54 were treated with 10 nM T3 for 2 days, and chromatin was isolated from the intestine (*A*, *C*) and tail (*B*, *D*) for ChIP assay as in Fig. 2. The TRE regions in the ChIP samples were analyzed using quantitative PCR. Three tadpoles were used per treatment.

morphic tadpoles (stage 54) (Fig. 3, *A* and *B*, *lane 2*). In the tail, SRC3 recruitment to the *TH/bZIP* promoter was low in premetamorphic tadpoles (stage 54) and high at the climax (stage 62) (Fig. 3*C*, compare *lanes 1* and *2*), whereas SRC3 binding to the *TRβA* promoter was similar or even lower at the climax compared with that in premetamorphic tadpoles (Fig. 3*D*, *lanes 1* and *2*).

To confirm the differential recruitment of SRC3, we conducted quantitative real-time PCR on the SRC3 ChIP samples. The results again showed that SRC3 was recruited to both the promoters in intestine in a ligand-dependent manner (Fig. 4*A*) and to the *TH/bZIP* promoter but not the *TRβA* promoter in the tail (Fig. 4*B*). For comparison, the levels of histone H4 acetylation were also analyzed by real-time PCR on the H4 ChIP samples and found to be increased by the T3 treatment in all cases, accompanying gene activation (Fig. 4, *C* and *D*). Real time PCR analysis of ChIP samples from premetamorphic (stage 54) and naturally metamorphosing (stage 62) tadpoles yielded essentially identical results, *i.e.* with increased recruitment of SRC3 to both the promoters in the intestine (Fig. 5*A*) but only *TH/bZIP* promoter in tail (Fig. 5*B*) at the metamorphic climax, and enhanced histone acetylation in all cases (Fig. 5, *C* and *D*). Thus, the results from both natural and T3-induced metamorphosis indicate that even though T3-dependent gene activation through TR involves increased acetylation at both promoters in both organs, SRC3 is utilized in a gene- and tissue-specific manner during the T3 activation process. The agreement between T3-induced and natural metamorphosis further argues that this T3-dependent differential recruitment is physiologically important for proper gene regulation and tissue remodeling.

A Dominant Negative SRC3 (F-dnSRC3) Can Be Recruited by TR in the Presence of T3 and Prevents the Recruitment of Endogenous Coactivators in the Tail—The lack of T3-dependent enhancement of SRC3 recruitment to the *TRβA* promoter in the tail suggests that SRC3 was not recruited to the promoter or constitutively associated with the promoter in the tail, even though the SRC3 ChIP signals at the promoter were at background levels in the tail. To distinguish these two possibilities, we then made use of a dominant negative form of SRC3, F-dnSRC3, comprising only the receptor interacting domain but containing a FLAG tag and nuclear localization signal at the N-terminal end (Fig. 6*A*) (47). We have recently introduced F-dnSRC3 into developing tadpoles through transgenesis by using a double promoter construct (61) that contained the constitutive cytomegalovirus promoter driving the expression of F-dnSRC3 and also harbored GFP under the control of the *γ-crystalline* promoter for color identification of transgenic animals (Fig. 6*B*) (47). This led to constitutive expression of F-dnSRC3 in transgenic animals and inhibition of metamorphosis in most, if not all, organs (47). Furthermore, we showed that in the animal intestine, F-dnSRC3 blocked the regulation of

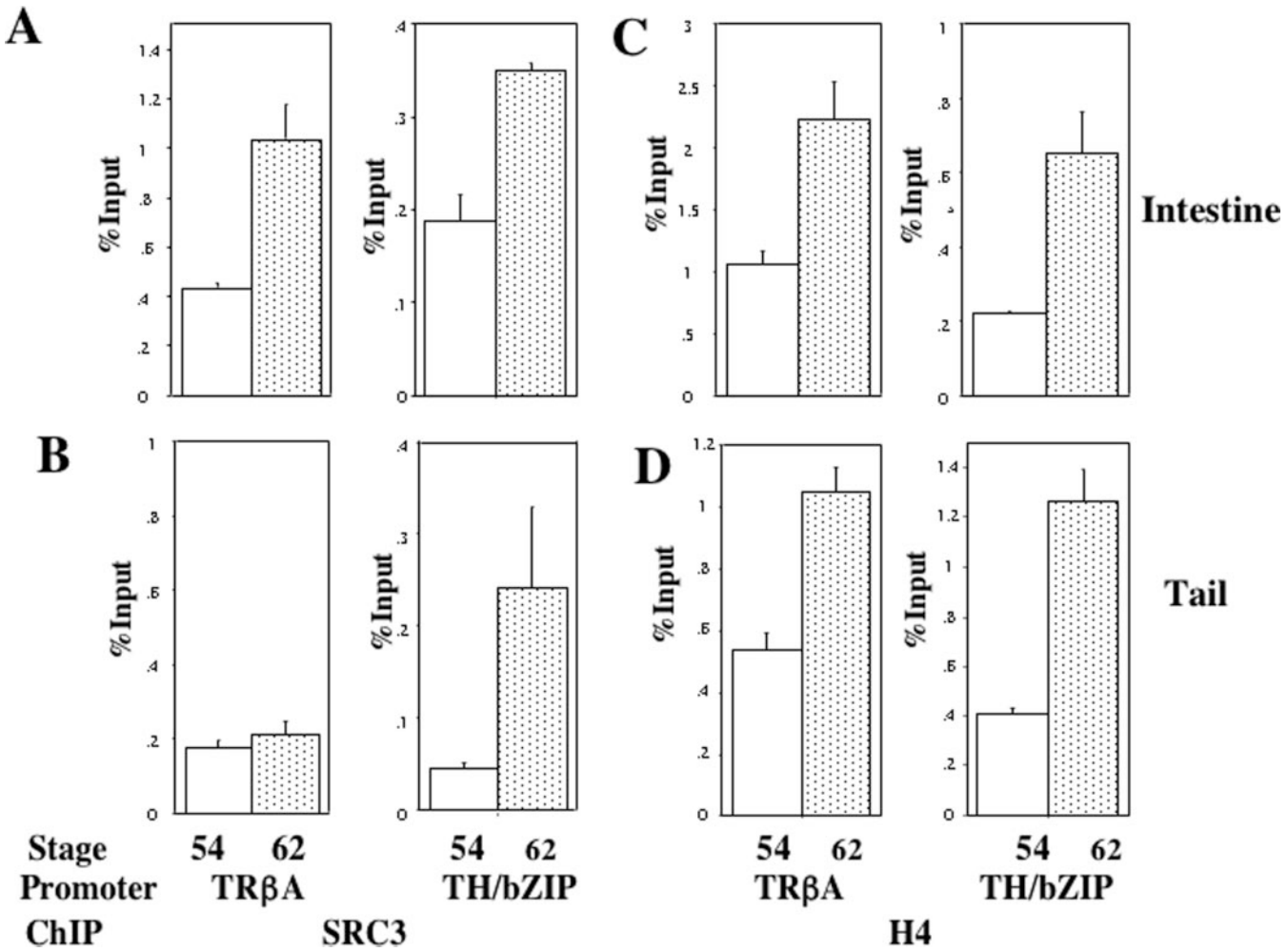

FIG. 5. **Real-time PCR analysis showing differential SRC3 recruitment to T3-dependent promoters during natural metamorphosis.** Chromatin was isolated for ChIP assay from the intestine (*A*, *C*) and tail (*B*, *D*) of premetamorphic tadpoles at stage 54 and metamorphic tadpoles at stage 62. The TRE regions in the ChIP samples were analyzed using quantitative PCR. Three tadpoles were used per treatment.

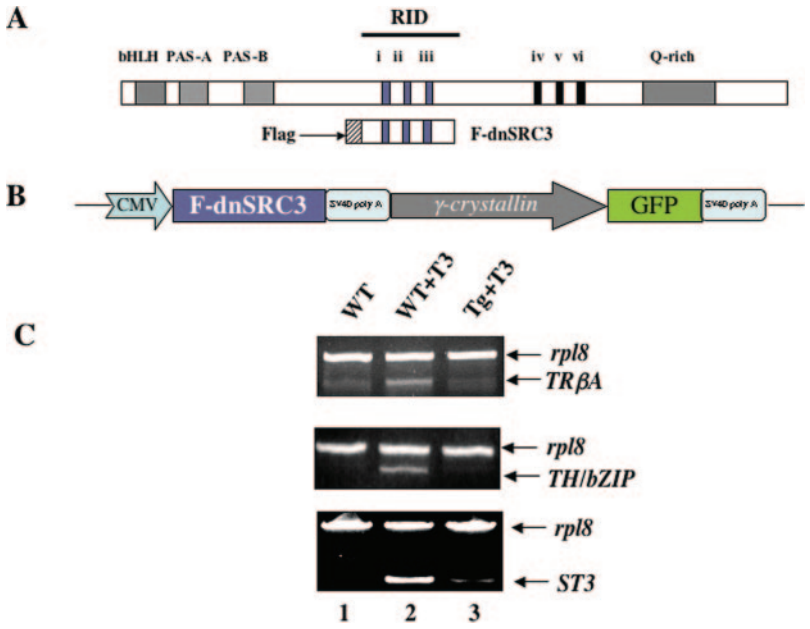

FIG. 6. *A*, schematic representation of the full-length SRC3 illustrating the organization of various domains: *bHLH/PAS*, basic helix-loop-helix and PAS dimerization domains; *RID*, receptor interaction domain. The L*XX*LL motifs present in the protein are numbered from *i–vi*. A glutamine (*Q*)-rich region is present toward the C-terminal end of the protein. *B*, the dominant negative form, dnSRC3 (aa 600–751), which comprises the L*XX*LL motifs *i–iii*, forming the receptor interaction domain and fused to an N-terminal peptide containing the FLAG tag and nuclear localization sequences, is shown below. *C*, transgenic overexpression of F-dnSRC3 impairs gene activation by T3. Wild type (*WT*) and transgenic (*Tg*) tadpoles at stage 54 were treated with or without 5 nM T3 for 3 days. Total RNA was isolated from the tail and RT-PCR was performed to compare expression of the T3-regulated genes *TRβA*, *TH/bZIP*, and *ST3*. The expression of the ribosomal protein L8 gene (*rpl8*) was used as an internal control.

all T3-response genes analyzed and functioned by inhibiting the recruitment of endogenous SRC3 to the target promoters, accompanied by reduced histone acetylation (47).

To determine whether F-dnSRC3 also blocks all T3-dependent gene regulation in the tail even though SRC3 was not recruited in a T3-dependent manner to all tail promoters, we analyzed the expression of three direct T3-responsive genes. RT-PCR analysis revealed that while *TRβA*, *TH/bZIP*, and

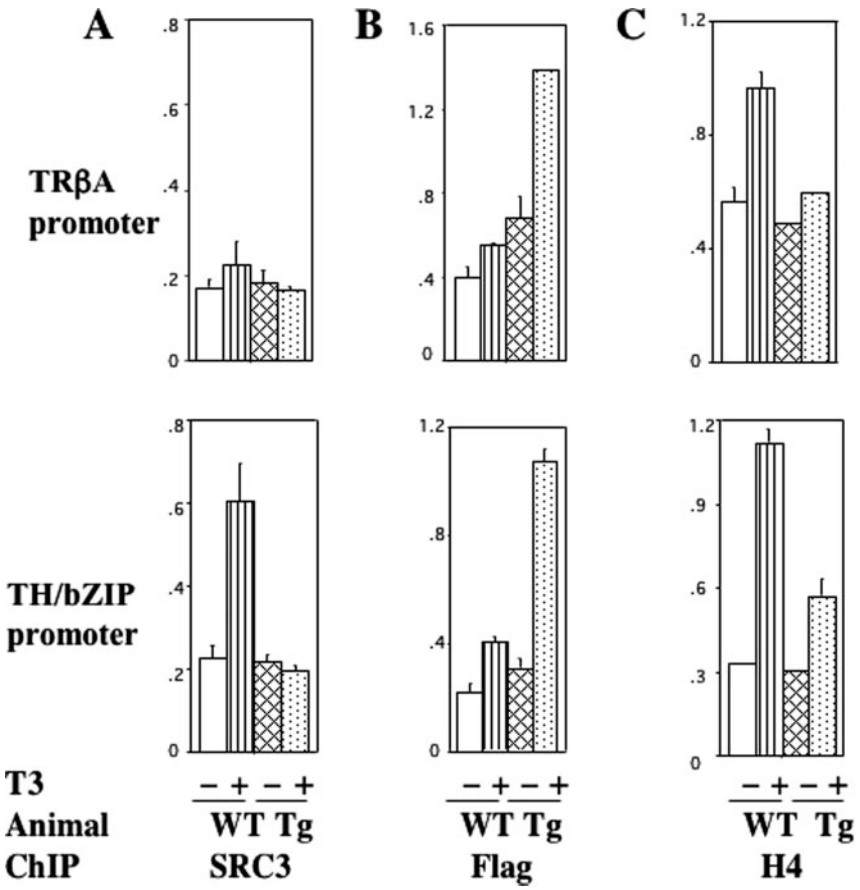

FIG. 7. **Transgenic F-dnSRC3 binds to liganded TR at T3-regulated promoters in the tail and competes out the endogenous coactivators.** Wild type (*WT*) and transgenic (*Tg*) animals at stage 54 were treated with 10 nM T3 for 2 days. Tail nuclei were isolated and ChIP assays performed using anti-SRC3 (for endogenous wild type SRC3) (*A*), anti-FLAG (for F-dnSRC3 transgene) (*B*), and anti-acetylated histone H4 (*AcH4*) (*C*) antibodies. The TRE regions of *TH/bZIP* and *TRβA* promoters were analyzed by real-time PCR after immunoprecipitation with the indicated antibodies. Note the increased binding of F-dnSRC3 and reduced histone acetylation in T3 treated transgenic tadpoles compared with wild type ones at both promoters even though endogenous wild type SRC3 was recruited to *TH/bZIP* but not *TRβA* promoters.

stromelysin-3 (*ST3*) (53), which are known T3-responsive genes, were induced in the tails of premetamorphic tadpoles at stage 54 treated with 5 nM T3 for 3 days (Fig. 6*C* compare *lanes 1* and *2*), their induction was drastically reduced in transgenic tadpoles expressing F-dnSRC3 (Fig. 6*C*, compare *lane 3* with *lane 2*).

To investigate the molecular mechanism of inhibition by F-dnSRC3 in the tail, we determined whether F-dnSRC3 was capable of being recruited to endogenous T3 response genes in the tail in a T3-dependent manner. We treated premetamorphic wild type or F-dnSRC3 transgenic tadpoles with T3 for 2 days and isolated chromatin from the tails. ChIP assays were performed with polyclonal antibodies against SRC3 (recognizing only the endogenous SRC3 as the antigenic peptides used to generate the antibody were not present in F-dnSRC3) or FLAG (recognizing the FLAG epitope in F-dnSRC3). Again, in wild type animals, T3 treatment led to enhanced association of SRC3 to the TH/bZIP promoter but not to the TRβA promoter (Fig. 7*A*). In transgenic animals expressing F-dnSRC3, this T3-induced recruitment of SRC3 to the TH/bZIP promoter was blocked, while no change was observed at the TRβA promoter (Fig. 7*A*). On the other hand, F-dnSRC3 was recruited to both promoters in the tail in transgenic animals treated with T3 (Fig. 7*B*). The ChIP signals for F-dnSRC3 at both promoters in the tail of transgenic animals without T3 treatment were at the background levels as seen in wild type animals treated with or without T3 (Fig. 7*B*), indicating that F-dnSRC3 was recruited to both promoters by TR only in the presence of T3. Analysis of histone H4 acetylation by ChIP assay showed that the T3-induced histone H4 acetylation observed in wild type animals at both promoters were drastically reduced (Fig. 7*C*), accompanying inhibition of gene activation (Fig. 6*C*). These results showed that the receptor interacting domain of SRC3 is capable of interacting with liganded TR bound to the TRβA promoter in the tail (at least when present in F-dnSRC3 and expressed at the levels as controlled by the cytomegalovirus promoter used in transgenesis) and further suggest that little or no endogenous SRC3 was recruited to the TRβA promoter in the tail in the presence or absence of T3 during development.

DISCUSSION

Extensive studies have led to the accumulation of valuable information on the biochemical composition and molecular properties of nuclear receptor coactivator complexes (3, 12–15, 27, 62–68). Cells deficient in cofactors derived from cofactor knock-out mice or due to RNA interference have been used to show the involvement of various cofactors in transcriptional regulation by nuclear hormone receptors (69–75). On the other hand, it has been difficult to investigate the role of cofactors in nuclear receptor function *in vivo* during vertebrate development. Cofactor redundancy and embryonic lethality often interfere with genetic studies in mammals. A further complication is that these cofactors are involved in many different regulatory pathways, making it difficult to determine the role in a particular nuclear hormone receptor pathway even when genetic changes in a cofactor lead to identifiable phenotypes, such as mice deficient in p300, SRC1–3, or TRAP220 (37–42). Thus, few studies have addressed the molecular mechanisms of coactivators in nuclear hormone receptor function *in vivo*, especially during vertebrate development. Here we have used the T3-dependent amphibian metamorphosis as a model to investigate the developmental role of the coactivator SRC3 *in vivo*. Our results suggest that TR recruits SRC3 in a tissue- and gene-dependent manner to regulate gene transcription and effect tissue specific changes during metamorphosis.

Based on TR expression and T3 levels during development, TR has been proposed to have dual functions during frog development, acting as a repressor during the premetamorphosis

lacking measurable levels of T3 and as an activator during metamorphic climax with high levels of endogenous T3 (60). This is supported by the fact that precocious expression of TR together with RXR has been shown to cause distinct embryonic effects depending upon the presence or absence of T3 (76). Furthermore, these developmental effects are accompanied by specific repression or activation in the presence or absence of T3, respectively, of endogenous genes that are known to be regulated by T3 during metamorphosis (76). These results argue that TR/RXR heterodimers repress target genes in the absence of T3 to facilitate tadpole growth during premetamorphosis. Recent studies indicate that unliganded TR represses gene expression in premetamorphic tadpoles at least in part by recruiting corepressors, N-CoR and SMRT, as well as their associated factor TBLR1 to endogenous T3 response gene promoters in premetamorphic tadpoles, leading to local histone deacetylation (56, 77, 78). To study gene activation by TR during metamorphosis, we have previously analyzed the expression of TR-interacting coactivators during metamorphosis (45). We found that the mRNAs of *X. laevis* SRC2, SRC3, and p300 are expressed during metamorphosis (45). Here, we have shown that both the mRNA and protein of SRC3 are up-regulated during both natural and T3-induced metamorphosis, implicating a role for this coactivator in TR function during metamorphosis.

Using ChIP assay, we have examined the recruitment of SRC3 to TR-regulated promoters *in vivo* in the intestine and tail. We chose these two organs because 1) both organs undergo extensive apoptosis as the larval tissues degenerate and 2) consistent with this similarity, a number of genes are regulated similarly in both organs (2, 58). In the tail, we found T3-dependent recruitment of SRC3 to the *TH/bZIP* but not *TRβA* promoter when premetamorphic tadpoles were treated with T3, in agreement with an earlier report (79). More importantly, we extended this observation by showing that this differential recruitment of SRC3 also occurs in the tail during natural metamorphosis, *i.e.* not a non-physiological outcome of T3 treatment of premetamorphic tadpoles. Furthermore, in contrast to the tail, SRC3 is recruited to both *TRβA* and *TH/bZIP* promoters in the intestine in a T3-dependent manner during both natural and T3-induced metamorphosis. This novel discovery that TR utilizes the cofactor in a tissue-dependent manner even for the same gene is unexpected considering that the major event induced by T3 is cell death in both the tail and intestine.

Our studies with the dominant negative SRC3 containing only the TR-interacting domain of *X. laevis* suggest that little or no endogenous SRC3 is recruited to the *TRβA* promoter in the tail throughout development, *i.e.* not constitutively associated with the promoter in the tail. First, the ChIP signals for endogenous SRC3 at the *TRβA* promoter in the tail of premetamorphic wild type animals or animals during either natural or T3-induced metamorphosis are similar to that at the *TH/bZIP* promoter in the tail of premetamorphic wild type animals in the absence of T3. Second and more importantly, although the endogenous SRC3 was not recruited to the *TRβA* promoter in a T3-dependent manner, the overexpressed dominant negative SRC3 was able to be recruited by liganded TR to this promoter and was accompanied by reduced level of histone H4 acetylation, indicating that at least the receptor interacting domain of SRC3 can be recruited by TR to the *TRβA* promoter in the tail. The lack of recruitment of endogenous SRC3 to the *TRβA* promoter in the tail is likely due to competition by other cofactors. The observed inhibition of histone acetylation and TRβA promoter activation by the transgenic F-dnSRC3 is presumably caused by the inhibition of the recruitment of these other coactivators by the overexpressed transgenic F-dnSRC3. When antibodies against *Xenopus* SRC1 or -2 or other coactivators become available, it will be interesting to determine whether they are recruited instead of SRC3 in a T3-dependent manner to activate the *TRβA* promoter in the tail.

In summary, our results provide the first *in vivo* evidence showing that TR is capable of utilizing a cofactor(s) in a gene- and tissue-specific manner during vertebrate development and that coactivator recruitment is absolutely essential for T3-dependent gene activation even though SRC3 was not recruited to all promoters in all tissues. It would be of considerable interest in the future to determine whether and how the promoter context and/or cofactor compositions in different tissues dictate the use of cofactors in gene regulation by TR *in vivo*.

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