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Metamorphic T_3-response genes have specific co-regulator requirements

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Thyroid hormone receptors (TRs) have several regulatory functions in vertebrates. In the absence of thyroid hormone (T_3; triiodothyronine), apo-TRs associate with co-repressors to repress transcription, whereas in the presence of T_3, holo-TRs engage transcriptional coactivators. Although many studies have addressed the molecular mechanisms of T_3 action, it is not known how specific physiological responses arise. We used T_3-dependent amphibian metamorphosis to analyse how TRs interact with particular co-regulators to differentially regulate gene expression during development. Using chromatin immunoprecipitation to study tissue from pre-metamorphic tadpoles, we found that TRs are physically associated with T_3-responsive promoters, whether or not T_3 is present. Addition of T_3 results in histone H4 acetylation specifically on T_3-response genes. Most importantly, we show that individual T_3-response genes have distinct co-regulator requirements, the T_3-dependent co-repressor-to-coactivator switch being gene-specific for both co-regulator categories.

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INTRODUCTION

The nuclear receptor superfamily, which includes the thyroid hormone receptors (TRs), which are encoded by the *TRa* (NR1A1; Nuclear Receptors Nomenclature Committee, 1999) and *TRb* (NR1A2) loci, affects vertebrate development, cell homeostasis and physiology. TRs bind as heterodimers with 9-*cis*-retinoic-acid receptor (RXR) to target genes through *cis*-acting DNA sequences known as T_3 (thyroid hormone; triiodothyronine)-response elements (T_3REs). In the absence of T_3, apo-TRs repress basal transcription; in the presence of T_3, holo-TRs relieve repression and activate transcription. The repression-to-activation switch involves changes in co-repressor to coactivator complexes, which are partnered by apo-TR and holo-TR, respectively (Glass & Rosenfeld, 2000). Given the diversity among co-regulators, their tissue-specific distribution and their variable expression levels, it is possible that many modes of gene regulation by TRs could involve different combinations of co-regulators on particular gene subsets or in specific physiological contexts.

We used amphibian metamorphosis to investigate TR action in a physiological context. Metamorphosis is controlled by T_3 (Tata, 1993). As in mammals, both TR-α and TR-β exist in amphibians such as *Xenopus laevis* (Yaoita *et al*., 1990). The *TRb* gene is expressed at low levels before metamorphosis, and is upregulated by T_3 during metamorphosis as a T_3 direct-response gene (Ranjan *et al*., 1994). By contrast, TR-α is activated after completion of embryogenesis, but well before the production of endogenous T_3. A working model of T_3-dependent gene regulation during pre-metamorphosis is that apo-TRs repress the T_3 direct-response genes and, later, the combination of T_3 and TRs permits the activation of T_3 direct-response genes, thus inducing metamorphosis (Sachs & Shi, 2000).

We used chromatin immunoprecipitation (ChIP) to analyse histone H4 acetylation and recruitment of TR and co-regulators on T_3-response-gene promoters. We focused on four well-characterized co-regulators that are required for TR action (Glass & Rosenfeld, 2000): the nuclear co-repressor NCoR, the histone deacetylase (HDAC) Rpd3, steroid receptor coactivator 3 (SRC3), and the co-activator p300. Four important results were obtained: first, we demonstrated constitutive TR binding to T_3 direct-response gene promoters *in vivo* in pre-metamorphic tadpoles; second, we showed that there is induction by T_3 treatment of histone H4 acetylation on T_3-response gene promoters; third, we showed that there is gene-specific recruitment of co-regulators at T_3-response gene promoters; and finally, we showed that there is a gene-specific switch from co-repressor to coactivator recruitment after treatment with T_3.

RESULTS

Constitutive TR binding on T_3-response-gene promoters

We first analysed the expression patterns of several control and T_3-response genes in tadpole tails after T_3 treatment (for 48 h). We chose tail tissue because it is the best-characterized organ that undergoes extensive remodelling during metamorphosis, disappearing completely due to apoptosis and showing strong

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upregulation of T_3-response genes. The tail is composed of connective tissue, blood vessels, spinal cord, notochord and muscles. As skeletal muscle predominates, it provides a relatively homogeneous tissue for studying specific changes in gene regulation and chromatin remodelling. Treatment with T_3 for 48 h was carried out to mimic the physiological conditions of metamorphosis as closely as possible and to obtain maximum activation of T_3 direct-response genes (Furlow & Brown, 1999). Total RNA was extracted from tail tissue and used for RT–PCR (PCR after reverse transcription) analysis. *TRa* was selected as the only TR gene that is expressed at all tadpole stages; *TRb* and *TH/bZIP* (a basic leucine-zipper TH-response gene) are the only two *Xenopus* T_3 direct-response genes with sequenced and characterized promoters. *MyoD* was chosen because it is a muscle-cell-specific gene. *Intestinal fatty acid binding protein* (*IFABP*) is an intestinal epithelial-cell-specific gene. *IFABP* provided a good negative control for these experiments, as it is not expressed in the tail. Finally, *elongation factor 1a* (*EF1a*) and *ribosomal protein L8* (*Rpl8*) were used as controls, as they are housekeeping genes. T_3 treatment significantly increased the messenger RNA levels of *TRb*, *TH/bZIP* and *MyoD* (Fig. 1A), but did not affect the levels of *TRa*, *EF1a* or *Rpl8*. As expected, *IFABP* was not expressed (Fig. 1A). These expression patterns are consistent with earlier data that were based on northern blots (Wang & Brown, 1993) and RNase protection assays (Kawahara *et al*., 1991). Our results provide the first evidence that *MyoD* is a T_3-response gene in *Xenopus* tadpoles.

To obtain direct information about whether these T_3-induced changes in gene expression correlate with the binding of TR to chromatin, we analysed TR binding *in vivo* to the promoters of *TRb*, *TH/bZIP*, *MyoD*, *IFABP* and *EF1a* using ChIP assays. Antibodies that recognize both TR-α and TR-β were used to immunoprecipitate formaldehyde-crosslinked, fragmented chromatin from nuclei that were isolated from tadpole tails and either treated with T_3 or left untreated. The TR-bound DNA fragments were then analysed by semi-quantitative PCR (Fig. 1B). Primers flanking the T_3REs were used for the *TRb* and *TH/bZIP* promoters. For the *TRb* promoter, we distinguished two T_3RE-containing sequences ('promoter region 1', which has one T_3RE at position +266, and 'promoter region 2', which has three putative T_3REs between positions –800 and –500; Urnov & Wolffe, 2001). Primers corresponding to the 300 bp immediately upstream of the transcription start site were used for the *MyoDa* promoter (Leibham *et al*., 1994). This promoter has never been studied for regulation by T_3. However, sequence analysis revealed two imperfect putative T_3REs (data not shown). Finally, for the *EF1a* promoter (Johnson & Krieg, 1995) and the *IFABP* promoter (Gao *et al*., 1998), which are not regulated by T_3, we chose primers in the 500-bp region upstream of the transcription start site. As shown in Fig. 1B, TR was constitutively present on the *TRb*, *TH/bZIP* and *MyoD* promoters, but was absent from T_3-insensitive promoters (*EF1a* and *IFABP*). Furthermore, T_3 does not have any effect on TR binding (Fig. 1B). These results indicate that, first, as previously described (Sachs & Shi, 2000), apo-TR and holo-TR bind T_3REs in chromatin *in vivo*, and second, that TR binds *in vivo* to both of the T_3RE-containing sequences in the *TRb* promoter. Moreover, our results highlight the fact that the levels of TR occupancy correlate with the levels of gene expression. Finally, the presence of TR on the *MyoD* promoter suggests that *MyoD* might be a T_3 direct-response gene. However, more data will be necessary to confirm this.

Histone acetylation correlates with gene regulation

We next investigated histone acetylation of promoters. Co-repressor complexes have HDAC activity, and many coactivators have intrinsic histone acetyl transferase (HAT) activity, which suggests that TRs might regulate transcription by modification of local histone

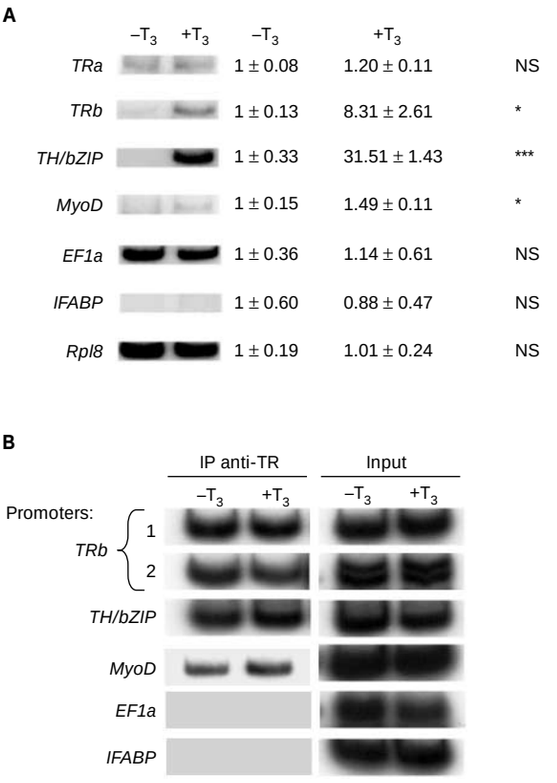

Fig. 1 | Effects of T_3 on transcription and DNA binding by thyroid hormone receptor at T_3-response genes in pre-metamorphic stage NF55 tadpole tail. (**A**) T_3 induces transcription of T_3-response genes. Tadpoles were treated for 48 h with 10 nM T_3. Total RNA was extracted from tail tissue and used for RT–PCR (PCR after reverse transcription) analysis of *thyroid hormone receptor b* (*TRb*), *TH/bZIP* (a basic leucine-zipper TH-response gene), *MyoD*, *intestinal fatty acid binding protein* (*IFABP*), *elongation factor 1a* (*EF1a*) and *ribosomal protein L8* (*Rpl8*) expression. The internal control was *Rpl8*. The results were also quantified by phosphoimager scanning. The average values ± s.e.m. of three independent experiments are expressed as multiples of induction, where 1 is equal to expression in the absence of T_3 (control level). For each sample, densitometry readings were normalized against the value for *Rpl8* RNA (except for the *Rpl8* data, which were not normalized). Statistical significance as compared with untreated animals is indicated as NS (not significant), * ($p < 0.05$) or *** ($p < 0.001$). (**B**) T_3 does not affect TR binding to T_3 response elements. Chromatin isolated from tails of T_3-treated tadpoles (10 nM T_3 for 48 h) was immunoprecipitated (IP) with antibodies against TR and analysed by PCR. Aliquots of the chromatin taken before immunoprecipitation were used directly for PCR as a control (input). For *TRb* promoters, we distinguished two sequences containing T_3REs (sequence 1 at position +266 and sequence 2 at positions –800 to –500). All experiments were carried out at least three times. T_3, thyroid hormone (triiodothyronine).

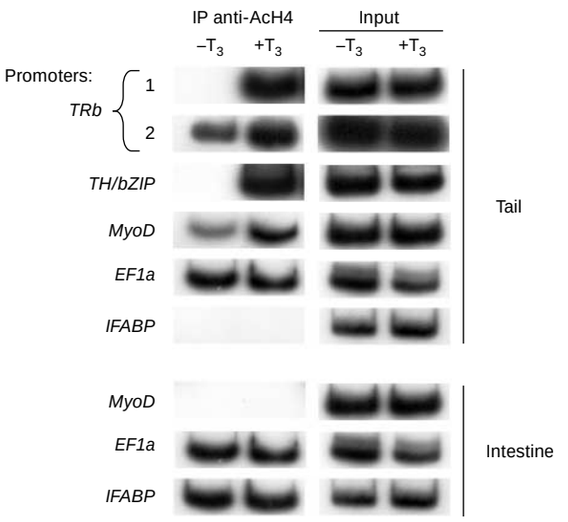

Fig. 2 | T_3 treatment increases histone H4 acetylation specifically at the T_3-response elements of T_3-response genes in pre-metamorphic tadpoles. Chromatin isolated from tail or intestine of T_3-treated stage NF55 tadpoles (treated with 10 nM T_3 for 48 h) was immunoprecipitated (IP) with antibodies against acetylated histone H4 (AcH4) and analysed by PCR, as described for Fig. 1. Each experiment was carried out at least twice. *EF1a*, *elongation factor 1a*; *IFABP*, *intestinal fatty acid binding protein*; T_3, thyroid hormone (triiodothyronine); *TH/bZIP*, a basic leucine-zipper Th-response gene; *TRb*, *thyroid hormone receptor b*.

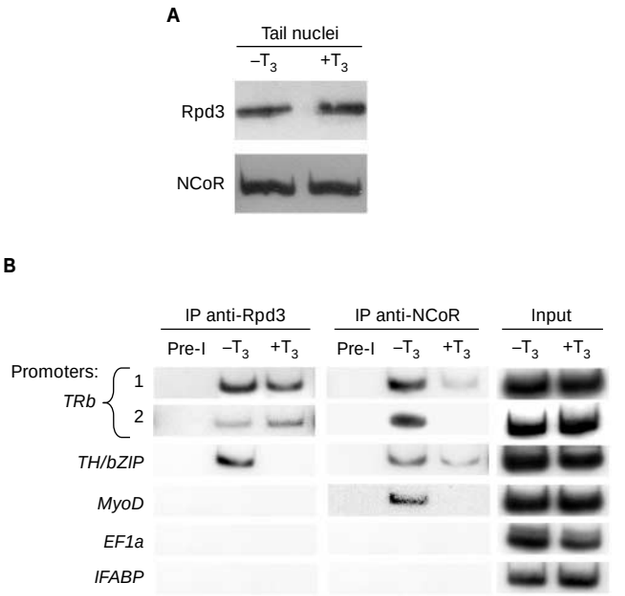

Fig. 3 | Effects of T_3 on Rpd3 and NCoR co-repressor expression and recruitment on T_3-response elements of T_3-response genes in pre-metamorphic tadpoles. (**A**) Rpd3 and NCoR protein levels in tail nuclei are not affected by T_3 treatment. Western blot analysis of protein extracts from the tail nuclei of tadpoles treated with 10 nM T_3 for 48 h. (**B**) Chromatin isolated from tails of T_3-treated tadpoles (treated with 10 nM T_3 for 48 h) was immunoprecipitated (IP) with antibodies against Rpd3 or NCoR and analysed by PCR, as described for Fig. 1. Pre-immune serum (Pre-I) was used as a control for antibody specificity. The data represent one of several independent experiments with identical results. *EF1a*, *elongation factor 1a*; *IFABP*, *intestinal fatty acid binding protein*; NCoR, nuclear co-repressor; T_3, thyroid hormone (triiodothyronine); *TH/bZIP*, a basic leucine-zipper Th-response gene; *TRb*, *thyroid hormone receptor b*.

acetylation levels (Wolffe, 1997). As shown in Fig. 2, using a ChIP assay with an antibody specific to acetylated histone H4 (AcH4), we showed that histone H4 acetylation increased on *TRb* promoter regions 1 and 2 and on the *TH/bZIP* and *MyoD* promoters, but not on the *EF1a* and *IFABP* promoters. Comparison of Fig. 1A with Fig. 2 shows that the levels of histone H4 acetylation correlate with the levels of gene expression. As a control for the specificity of local histone acetylation, we analysed the acetylation levels of the *MyoD*, *EF1a* and *IFABP* promoters in intestine. As expected, as *MyoD* is not expressed in intestine, its promoter chromatin did not contain AcH4 (Fig. 2). However, given that *IFABP* and *EF1a* are highly expressed in this tissue, it was not surprising to find that chromatin from their promoters contained AcH4 (Fig. 2). Finally, for *TRb*, which is strongly repressed in the absence of T_3, AcH4 was detected at region 2 of the *TRb* promoter in the absence of T_3, whereas there was no detectable AcH4 at region 1 (Fig. 2, compare *TRb* lanes 1 and 2). However, both regions contained AcH4 when T_3 was present.

Co-repressor recruitment to T_3-response-gene promoters

Apo-TR is known to interact with co-repressor complexes containing HDAC, whereas holo-TR is known to interact with coactivator complexes containing HATs. We examined the recruitment and T_3-dependent release of two co-repressors, NCoR (Sachs *et al*., 2002) and Rpd3 (Wong *et al*., 1998). After confirming that NCoR and Rpd3 are expressed in tail (Fig. 3A), a ChIP assay was performed using polyclonal antibodies to Rpd3, the only characterized *Xenopus* HDAC (Wong *et al*., 1998), and to *Xenopus* NCoR, a co-repressor that seems to function through mechanisms involving HDACs. As shown in Fig. 3B, Rpd3 is recruited to the *TRb* promoter (regions 1 and 2) in a T_3-independent manner. However, Rpd3 is recruited to the *TH/bZIP* promoter only in the absence of T_3 (Fig. 3B), and is never recruited to the *MyoD*, *EF1a* and *IFABP* promoters (Fig. 3B). We found that NCoR recruitment to the *TRb* (regions 1 and 2), *TH/bZIP* and *MyoD* promoters decreased after T_3 treatment (Fig. 3B). Noticeably, NCoR is never present on the *EF1a* and *IFABP* promoters (Fig. 3B). Thus, all the T_3-response genes that were studied recruit NCoR only in the absence of T_3. By contrast, the recruitment of Rpd3 is gene-specific, and is not always affected by T_3 treatment.

Coactivator recruitment to T_3-response-gene promoters

Finally, we examined, in tadpole tail, the effects of T_3 on the recruitment of the TR receptor coactivators SRC3 (Kim *et al*., 1998) and p300 (Fujii *et al*., 1998), which have HAT activity. After verifying that these coactivator proteins are expressed in the tail at

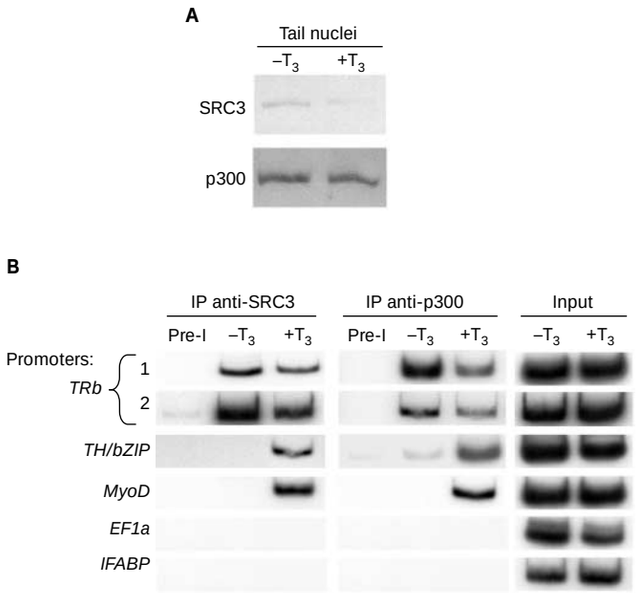

Fig. 4 | Effects of T_3 on steroid receptor coactivator 3 and p300 coactivator expression and recruitment on T_3-response elements of T_3-response genes in pre-metamorphic tadpoles. (**A**) Steroid receptor coactivator 3 (SRC3) and p300 protein levels in tail nuclei are not affected by T_3 treatment. Western blot analysis of protein extracts fron tail nuclei of tadpoles treated with 10 nM T_3 for 48 h. (**B**) Chromatin isolated from tails of T_3-treated tadpoles (treated with 10 nM T_3 for 48 h) was immunoprecipitated (IP) with antibodies against SRC3 or p300 and analysed by PCR, as described for Fig. 1. Pre-immune serum (Pre-I) was used as a control for antibody specificity. All experiments were carried out at least three times. *EF1a*, *elongation factor 1a*; *IFABP*, *intestinal fatty acid binding protein*; T_3, thyroid hormone (triiodothyronine); *TH/bZIP*, a basic leucine-zipper Th-response gene; *TRb*, *thyroid hormone receptor b*.

significant levels (Fig. 4A), we analysed whether they were present on the promoters of the genes studied. Three different situations were found: SRC3 and p300 are continually present on the promoter of *TRb* (regions 1 and 2); these coactivators are only present on *TH/bZIP* and *MyoD* promoters if T_3 is present. In the case of the *EF1a* and *IFABP* promoters, these coactivators are never present (Fig. 4B).

DISCUSSION

We exploited the absolute dependence of amphibian metamorphosis on T_3 and TRs to analyse successive stages of TR/co-regulator association during a physiologically defined sequence of events.

Recruitment of NCoR with and without Rpd3

The first model of transcriptional repression by apo-TR, proposed by Wolffe (1997), described the recruitment of a multiprotein complex that included NCoR and Rpd3. Recently, on the basis of results from the TR regulation of the *Xenopus TRb* promoter, this model was refined to conclude that apo-TR specifically recruits an NCoR–HDAC3 complex, and not an NCoR–Rpd3 complex (Li *et al*., 2002a). Nevertheless, Rpd3 is constitutively associated with chromatin and contributes to chromatin deacetylation in a non-targeted manner (Li *et al*., 2002a). Here, by examining three T_3-response-gene promoters, we show that NCoR is present at all promoters in the absence of T_3, but that NCoR is absent from the same promoters after T_3 treatment. However, Rpd3 recruitment is gene-specific. Rpd3 is associated with the *TRb* promoter whether T_3 is present or not, thus confirming the finding of Li and collaborators (2002a). However, Rpd3 is recruited to the *TH/bZIP* promoter only in the absence of T_3, and is never recruited to the *MyoD* promoter. However, we did observe the presence of NCoR and Rpd3 on the *TH/bZIP* promoter, showing that such complexes can be recruited by apo-TR. Thus, it is still possible that NCoR–Rpd3 or NCoR–HDAC3 might function through targeting to different T_3 direct-response gene promoters.

Ligand-dependent co-repressor to coactivator switches

It is thought at present that TRs repress transcription by recruiting co-repressors and activate transcription by recruiting coactivators. Our results from the *TH/bZIP* and *MyoD* promoters are consistent with this model. By contrast, at the *TRb* promoter, Rpd3, SCR3 and p300 are present simultaneously both in the presence and absence of T_3, emphasizing that NCoR release is a key event in the activation of *TRb* transcription. This finding supports an earlier hypothesis that *TRb* expression results from the relief of repression, rather than from activation by holo-TR (Collingwood *et al*., 1999).

The direct interaction of coactivators and co-repressors in a single regulatory unit has been described before. Examples of these are NCoR and SRC3 (Li *et al*., 2002b), and NCoR and CBP/p300 (Saleh *et al*., 2000). Direct interactions such as these provide an integral control mechanism that affects the timing of repression and activation. Indeed, the induction of *TRb* expression precedes *TH/bZIP* expression (Furlow & Brown, 1999). These more rapid kinetics could be due to the simultaneous association of co-repressors and coactivators on the *TRb* promoter. In such a complex, the presence of co-repressors inhibits transcription, and their release rapidly activates transcription. In this context, it would be interesting to compare the occupancy of the various promoters at various times after T_3 treatment. However, when using an *in vivo* approach, one has to take into account the fact that all the cells are not simultaneously T_3 responsive.

A growing body of evidence suggests that co-regulators are themselves highly regulated by covalent modification. Such modifications not only alter protein function, but can also confer specificity to ubiquitous factors. The acetylation of SRC3 by p300 has been shown to induce its release from the holo-ER form of the oestrogen receptor to attenuate transcriptional activation by oestradiol (Chen *et al*., 1999). In addition, covalent modification of CBP/p300 induces changes in HAT activity, substrate specificity, protein–protein interactions and stability (Gamble & Freedman, 2002). For example, on binding of NCoR and CBP to the homeobox heterodimer pbx–hox, protein kinase A stimulation of CBP has been found to facilitate the switch from transcriptional repression to activation in this system (Saleh *et al*., 2000).

Transcriptional activation might also involve chromatin structure. Binding by apo-TR to the *TRb* promoter T_3RE is potentiated by assembly of the DNA into a mature array of transitionally positioned nucleosomes, and holo-TR disrupts this array, creating a lower-affinity template for itself (Urnov & Wolffe, 2001). Another

Table 1 | Primers used in this study

Gene	Reverse primer	Forward primer	Reference
RT–PCR			
TRa	5′-CTCTATCTTGTCCGTGCAGAT-3′	5′-ATGGCTTCCATGCCGGATGGG-3′	Yaoita *et al*. (1990)
TRb	5′-CTTTTCTATTCTCTCCACGCTAGC-3′	5′-TAGTTAATGCGCCCGAGGGTGGA-3′	Yaoita *et al*. (1990)
TH/bZIP	5′-CTTAAACCTCAGCTTATGTGGAAG-3′	5′-ACTGGGAAAAGAGGCGCAAGAAC-3′	GenBank U37375
MyoD	5′-GACAGTTGAGTGCAGGTA-3′	5′-GGACTCAGATGCCTCAAG-3′	GenBank X16106
EF1a	5′-TGAGGAAGAGAGCGAACC-3′	5′-TGCACAGTTGGCGCAGTG-3′	Johnson & Krieg, 1995
IFABP	5′-GCCTCTCTTGAAAATCCTTTTG-3′	5′-GGAACTTGGAAGGTTGACAGA-3′	GenBank L19946
Rpl8	5′-GACGACCAGTACGACGA-3′	5′-AAAGAGAAACTGCTGGC-3′	GenBank U00920
Promoter			
TRb 1	5′-GACAGTCAGAGGAACTG-3′	5′-GTAAGCTGCCTGTGTCTATA-3′	Ranjan *et al*. (1994)
TRb 2	5′-GACTATGGCATGTTACAGC-3′	5′-TAGATGCTGCACCTGCTC-3′	Ranjan *et al*. (1994)
TH/bZIP	5′-CTCCCAACCCTACAGAGTTCA-3′	5′-TCTCCCTGTTGTGTATAATGG-3′	Furlow & Brown (1999)
MyoD	5′- CCTCTGCCCTCACTCTAT-3′	5′-AGTTCCTTGGATCGTTCG-3′	Leibham *et al*. (1994)
EF1a	5′-TGAGGAAGAGAGCGAACC-3′	5′-TGCACAGTTGGCGCAGTG-3′	Johnson & Krieg (1995)
IFABP	5′-GGCCACAAGATCTACTCG-3′	5′-ATAGCAGCAGGTGGTTGCG-3′	Gao *et al*. (1998)

EF1a, *elongation factor 1a*; *IFABP*, *intestinal fatty acid binding protein*; *Rpl8*, *ribosomal protein L8*; T_3, thyroid hormone (triiodothyronine); *TH/bZIP*, a basic leucine-zipper TH-response gene; *TR*, *thyroid hormone receptor*.

possibility is the recruitment of other types of coactivator complexes by T_3 treatment. Indeed, TR can recruit SRC–p300 and Mediator complexes in at least two sequential steps. SRC and p300 are recruited first and rapidly induce histone acetylation, followed by the recruitment of the Mediator complex (Sharma & Fondell, 2002). However, sequential models are usually cyclic, with windows of time in the range of minutes (Shang *et al*. 2000; Sharma & Fondell, 2002), and these complexes are all required for correct gene transcription (Huang *et al*., 2003).

Our results suggest that during metamorphosis, combinatorial associations of TR, co-repressor and/or coactivator molecules that are influenced by cell history and promoter context provide the specificity of the response of genes to T_3. This study underlines the importance of tissue specificity with regard to promoter occupancy. We are now analysing this problem in tissues such as intestine and brain that show different metamorphic organizational responses to those of tail tissue. Finally, promoter specificity for co-regulator requirements is a particularly interesting phenomenon, as the different gene regulatory mechanisms revealed in this study might be correlated with the multiple T_3-induced cellular responses that underlie tissue remodelling during metamorphosis.

METHODS

Animals. *Xenopus laevis* tadpoles were staged in accordance with the method of Nieuwkopp & Faber (NF staging; 1956). For T_3 treatment, stage NF55 tadpoles were kept for 48 h in 5 l of dechlorinated tap water with 10 nM 3,5,3' triiodothyronine (T_3; Sigma). Tadpoles were sacrificed by decapitation after anaesthesia. Animal care was carried out in accordance with institutional guidelines.

RT–PCR. Tissues were stored at 4 °C in RNAlater (Ambion). RNA extractions and RT–PCR were carried out as described in Sachs *et al*. (2002). The primers used are shown in Table 1. To define for each gene the optimal number of PCR cycles for quantitative analysis, 10–24 cycles were carried out (data not shown). The numbers of cycles chosen were as follows: *EF1a*, 14 cycles; *TH/bZIP*, 18 cycles; *TRa* and *MyoD*, 20 cycles; *TRb* and *IFABP*, 22 cycles. PCR products were loaded onto acrylamide gels (6%) in 1 × TBE buffer and visualized by autoradiography. Phosphoimager scanning (Molecular Dynamics) was used to quantify each of the PCR products. A Student's *t*-test was used to assess statistical differences between means.

Antibodies. Rabbit polyclonal antibodies against *Xenopus* Rpd3 and *Xenopus* NCoR have been described previously (Vermaak *et al*., 1999; Sachs *et al*., 2002). Rabbit polyclonal antibodies against *Xenopus* SRC3 were generated against two synthetic peptides (amino acids 1–20, MSGLGENSLDPLASETRKRK, and amino acids 62–77, DNFNVKPDKCAILKETVR) and those against *Xenopus* p300 were generated against two synthetic peptides (amino acids 1033–1049, KSEPVELEEKKEEVKTE, and amino acids 1487–1506, KPRLQEWYKKMLDKSVSER).

ChIP assays. ChIP assays were carried out as described by Sachs & Shi (2000). 5 μl of anti-AcH4 antiserum (Upstate Biotechnology) or 8 μl of antibodies against the *Xenopus* proteins TR, Rpd3, NCoR, p300 and SRC3 were used for immunoprecipitation. Pre-immune serum was used as a control for antibody specificity. Immunoprecipitated DNA was analysed by semi-quantitative PCR, as described in Sachs & Shi (2000). The primers used are shown in Table 1. 10 μl of PCR product was resolved on a 6% acrylamide–TBE gel, and bands were visualized by autoradiography.

Protein isolation and western blotting. After the isolation of tail nuclei (Sachs & Shi, 2000), protein extraction and western blotting were carried out as described in Sachs *et al*. (2001). One modification to this method was the time of transfer to nitrocellulose membranes (Bio-Rad), which was 1 h for Rpd3 and SRC3 and overnight for NCoR and p300.

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