A role for nucleosome assembly in both silencing and activation of the *Xenopus* TRβA gene by the thyroid hormone receptor

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We have assembled the thyroid hormone-inducible promoter of the *Xenopus* thyroid hormone receptor (TR)βA gene into chromatin using replication-coupled and -independent assembly pathways in vivo. We establish that heterodimers of TR and 9-cis retinoic acid receptors (RXR) can bind to their recognition sites within chromatin both in vivo and in vitro and alternately repress or activate transcription dependent on the absence or presence of thyroid hormone. Maximal transcriptional repression requires the presence of unliganded TR/RXR heterodimers during replication-coupled chromatin assembly. We demonstrate an increase in transcription directed by the TRβA promoter of over two orders of magnitude in vivo, following the addition of thyroid hormone. This increase in transcription involves the relief of the repressed state that is established by the unliganded TR/RXR heterodimer during replication-coupled chromatin assembly. The association of thyroid hormone with the chromatin-bound TR/RXR heterodimer leads to the disruption of local chromatin structure in a transcription-independent process. Thus, chromatin structure has multiple roles in the regulation of TRβA gene expression in vivo: The TR/RXR heterodimer recognizes the response element within chromatin, TR/RXR makes use of the chromatin assembly process to silence transcription more efficiently, and TR/RXR directs the disruption of local chromatin structure in response to thyroid hormone.

*Key Words:* Nucleosome assembly; *Xenopus*; thyroid hormone receptor gene; transcription

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The thyroid hormone receptor (TR) controls a wide variety of biological responses involved in growth, development, and differentiation (Tsai and O’Malley 1994; Yen and Chin 1994). The TR is a member of the steroid/nuclear receptor superfamily (Evans 1988; Beato 1989); these proteins associate with DNA as homo- or heterodimers and contain ligand-inducible transcription activation domains (Forman and Samuels 1990). Although TR can regulate gene expression as a homodimer, in vivo TR readily forms heterodimers with other members of the nuclear receptor superfamily, especially the receptors for 9-cis retinoic acid, RXR [Yu et al. 1991; Kliewer et al. 1992; Zhang et al. 1992].

The activity of TR is influenced by the presence or absence of thyroid hormone [Thompson and Evans 1989; Glass et al. 1989; Sap et al. 1989; Banialhad et al. 1990, 1992], by heterodimerization [Heyman et al. 1992; Leid et al. 1992], and the exact DNA recognition sequence [Glass et al. 1988; Naar et al. 1991; Umeson o et al. 1991; Perlmann et al. 1993]. TR has the capacity to both activate transcription in the presence of thyroid hormone and repress transcription in the absence of hormone [Brent et al. 1989; Damm et al. 1989; Glass et al. 1989; Graupner et al. 1989; Sap et al. 1989; Banialhad et al. 1990, 1992].

Repression of transcription might operate by either passive or active mechanisms. TR might passively occlude binding sites for transcriptional activators or basal factors [Glass et al. 1989]; it might also passively repress transcription through the formation of inactive heterodimers [Glass et al. 1989; Forman and Samuels 1990]. Active repressive mechanisms could require TR to act directly through inhibitory interactions with the general initiation factors, with activators, or with cofactors that communicate between general initiation factors and activators [Banialhad et al. 1993; Fondell et al. 1993]. Alternatively, TR could actively repress transcription through indirect mechanisms such as recruiting repressive factors or directing the assembly of a repressive chromatin structure. Roeder and colleagues have demonstrated that unliganded TR alone can function as an active transcriptional repressor in vitro [Fondell et al. 1993]. In their in vitro system, TR inhibits transcription at an early step in preinitiation complex assembly, because preassembled complexes are refractory to inhibition.

Whatever the mechanism by which TR activates or represses transcription, a prerequisite is that this nuclear factor needs to be bound to thyroid response elements (TREs) in the absence or presence of thyroid hormone.
This requires TR to gain access to TREs within chromatin. Early studies by Samuels and colleagues (1980) established that the TR is constitutively associated with chromatin in vivo (Casanova et al. 1984). Chromatin structure has the capacity to prevent trans-acting factors gaining access to their recognition elements (Bogenhagen et al. 1982; Gottesfeld and Bloomer 1982; Knezetic and Luse 1986; Workman and Roeder 1987; for review, see Wolfe 1992). Molecular mechanisms that allow transcription factors to bind to DNA in spite of nucleosome assembly include translational positioning of the histone octamer with respect to DNA sequence. Such positioning can direct repressive histone–DNA interactions away from transcription factor binding sites (Thomas and Elgin 1988; Schild et al. 1993). Alternatively, rotational positioning of DNA can occur on the surface of the histone octamer such that recognition sites for transcription factors are exposed toward solution (Perlmann and Wrang 1988; Pine et al. 1990a,b, Li and Wrang 1995). It is also possible that molecular machines might exist that disrupt local chromatin structure (Côté et al. 1994; Imbalzano et al. 1994; Tsukiyama et al. 1994; Varga-Weisz et al. 1995).

In this paper we have made use of the assembly of minichromosomes within the Xenopus oocyte nucleus to examine the role of chromatin in both transcriptional silencing and activation of the Xenopus TRβA promoter. Transcription from this promoter is under the control of thyroid hormone and the TR (Kanamori and Brown 1992, Shi and Brown 1992, Ranjan et al. 1994), which exists as a heterodimer of TR and RXR (Bugge et al. 1992, Leid et al. 1992, Marks et al. 1992, Zhang et al. 1992). Microinjection of either single-stranded or double-stranded DNA templates into the Xenopus oocyte nucleus offers the opportunity to examine the influence on gene regulation of chromatin assembly pathways that are either coupled or uncoupled to DNA synthesis (Almouzni and Wolfe 1993). The staged injection of mRNA encoding transcriptional regulatory proteins and of template DNA offers the potential for examining the mechanisms of transcription factor-mediated transcriptional activation of promoters within a chromatin environment (Almouzni and Wolfe 1993). In particular, it is possible to discriminate between preemptive mechanisms in which transcription factors bind during chromatin assembly to activate transcription and postrepressive mechanisms in which transcription factors gain access to their recognition elements after they have been assembled into mature chromatin structures.

We find that TR/RXR heterodimers bind constitutively within the minichromosome independent of whether the receptor is synthesized before or after chromatin assembly. We demonstrate that the coupling of chromatin assembly to the replication process (Almouzni and Wolfe 1993) augments transcriptional repression by unliganded TR/RXR without influencing the final level of transcriptional activity in the presence of thyroid hormone. The addition of thyroid hormone to the chromatin-bound receptor leads to the disruption of chromatin structure. Finally, we establish that rotational positioning of the TRE on the surface of the histone octamer allows the specific association of the TR/RXR heterodimer in vitro. We propose a molecular mechanism by which the TR/RXR heterodimer can constitutively bind to DNA within chromatin to either silence or activate transcription.

Results

Regulation of the TRβA promoter by TR/RXR heterodimers in Xenopus oocyte nuclei

We initially examined the transcriptional activity of the TRβA promoter following microinjection of either double- or single-stranded templates into Xenopus oocyte nuclei. Previous work has established that the single-stranded template will be rapidly converted into double-stranded form by complementary strand synthesis (Almouzni and Wolfe 1993). Chromatin assembly is coupled to this DNA synthetic event. “Replication”-coupled assembly represses basal transcription more efficiently than the slow assembly of nucleosomes on double-stranded DNA (dsDNA) (Almouzni et al. 1990a,b, 1991, Almouzni and Wolfe 1993; Kaufman and Botchan 1994). An earlier analysis of the TRβA promoter had established that a functional TRE was located within the promoter but had not determined the exact start site of transcription (Ranjan et al. 1994). We therefore mapped the major start site of transcription within the TRβA templates microinjected into the Xenopus oocyte nuclei. Transcription from the TRβA promoter in the oocyte nucleus initiated 264 bp upstream of the previously defined TRE element (Fig. 1). Transcriptional efficiency was not influenced by the presence or absence of thyroid hormone [T3] at concentrations [50 nM] adequate to induce ligand-dependent transcription either within tissue culture cells or during normal development (Leloup and Bucaglia 1977; Yaota and Brown 1990, Kawahara et al. 1991, Elichei and Brown 1994; Ranjan et al. 1994). This is because of the absence of detectable TR in oocytes (see Fig. 2; Elichei and Brown 1994). We find that microinjection of single-stranded DNA (ssDNA) leads to synthesis of a dsDNA template (Fig. 1C) that has the same transcriptional start site and T3 independence but is much reduced in transcriptional efficiency (>15-fold) relative to dsDNA injected into the oocyte nucleus (Fig. 1B, cf. dsDNA with ssDNA). Controls indicated that ssDNA was efficiently converted to dsDNA following microinjection into the oocyte nucleus (Fig. 1C). Micrococcal nuclease digests (Fig. 1D) reveal that both the templates microinjected as ssDNA or as dsDNA are assembled into nucleosomes. The nucleosomal repeat is less clear for the microinjected dsDNA (Fig. 1D, lanes 1–4) than for the microinjected ssDNA (Fig. 1D, lanes 5–8). We suggest, in agreement with previous results (Almouzni et al. 1990b, 1991, Almouzni and Wolfe 1993), that replication-coupled chromatin assembly on the Xenopus TRβA promoter more efficiently represses basal transcriptional activity.
Figure 1. Efficient T3-independent transcription of double-stranded but not single-stranded TRpA promoter when injected into Xenopus oocytes. (A) Structure of the TRpA promoter construct. The transcriptional start site in oocytes is designated as +1 (see B). The TRE identified previously (Ranjan et al. 1994) is located at +264 (underlined). Also indicated are the relative positions of primer I (+107) and CAT primer (+397), which were used for primer extension analysis. (B) Replica-coupled chromatin assembly effectively represses the transcription from the TRpA promoter. Forty oocytes were injected into the nuclei with double-stranded [2.3 ng/oocyte] or single-stranded [1.15 ng/oocyte] pTRpA templates. The injected oocytes were then divided into two groups with equal number of oocytes and incubated at 18°C overnight with (+) or without (−) 50 nM of T3. The transcription from the TRpA promoter was analyzed by primer extension using primer I. DNA sequencing markers using the TRpA promoter and end-labeled primer I indicates the +1 as the start site. (C) Recovery of DNA after either ssDNA or dsDNA are injected into oocytes. DNAs recovered from the groups of oocytes in B were resolved by a 1% agarose gel, blotted, and hybridized with random primer-labeled pTRpA. [Lane 1] Uninjected ssDNA; [lane 2] uninjected dsDNA. Double-stranded form I (closed circle supercoiled), form II (nicked circular), and ssDNA (ss) are indicated. (D) Chromatin assembly through replication-independent and replication-coupled pathways. Naked DNA (lanes a, b) or chromatinized DNA from oocytes injected with dsDNA or ssDNA as indicated were digested with increasing amounts of micrococcal nuclease (MNase) (Δ) and analyzed by Southern blotting using a random primed probe containing the TRpA promoter and vector DNA, after resolution on a 1.5% agarose gel. Markers indicate mono-, di-, and trinucleosomes (1, 2, and 3) visible in the microinjected ssDNA after replication and chromatinization.

We then examined the consequences for TRpA promoter activity of synthesizing TRp and RXRa in the Xenopus oocyte following the injection of mRNA encoding both proteins (Yaoita et al. 1990; Blumberg et al. 1992). Neither endogenous TR nor RXR proteins are detected by immunoblotting of protein extracts from Xenopus oocytes [Fig. 2A, control]. Injection of mRNA encoding TRp or RXRa leads to the appearance of immunoblotting [Fig. 2A]. Injection of mRNA encoding TRp or RXRa leads to the appearance of the protein as detected by immunoblotting [Fig. 2A]. Approximately equivalent amounts of TRp and RXRa accumulate in the nucleus in the presence or absence of T3 [Fig. 2A]. As a result of the difference in aqueous volume between the oocyte nucleus and cytoplasm, the accumulation of more receptor in the nuclear fraction compared with the cytoplasmic fraction reflects at least a 10-fold preferential localization of TRp and RXRa in the oocyte nucleus (Dingwall and Allan 1984).

We investigated whether the independent expression of either TRp or RXRa would lead to the appearance of TRE binding activity, or whether both proteins would have to be coexpressed. We find that expression of both TRp and RXRa are required to generate a specific complex with the TRE from the TRpA gene [Fig. 2B, lane 5]. No binding is detected in extracts prepared from uninjected oocytes [Fig. 2B, lane 2]. Competition experiments with wild-type and mutant TREs demonstrated the specific nature of the TRp/RXRα complex [data not shown; see Ranjan et al. 1994]. This binding occurs in the absence or presence of T3 [data not shown].
Figure 2. A T₃-responsive oocyte transcription system can be established by introducing exogenous TRp and RXRα into oocytes. (A) Expression of TRβ and RXRα in oocytes and T₃-independent localization in the nuclei upon microinjection of corresponding mRNA. The mRNA encoding TRβ and mRNA encoding RXRα were injected into oocytes. The oocytes were then incubated in the presence (+) or absence (−) of T₃ as indicated. The proteins from total oocytes or individually dissected nuclei and cytoplasm were isolated by centrifuging the homogenate to remove insoluble materials and analyzed on a 10% SDS gel. On this gel, the total oocyte proteins (lanes 1,4,7,8,11,14) or cytoplasmic proteins (lanes 3,6,10,13) equivalent to one-third of an oocyte were loaded, whereas proteins equivalent to one nuclei were loaded in lanes 2,5,9,12. The total protein from uninjectected oocytes were used as control (lanes 1,8). The expression of TRβ or RXRα was detected by immunoblotting using antibody against the recombinant Xenopus TRβ or RXRα. Note that no endogenous TRβ or RXRα can be detected by this analysis, consistent with lack of T₃ response of uninjection oocytes [Fig. 1]. (B) DNA binding by heterodimers of TRβ and RXRα produced in oocytes. Extract was prepared from the oocytes injected with TRβ (lane 3) or RXRα (lane 4) mRNA individually or both mRNAs together (lane 5) (2.7 ng/oocyte) and used for mobility retardation assays with a labeled TRE oligonucleotide [probe alone, lane 1]. Note that no binding was observed by TRβ or RXRα alone or when the extract from uninjected oocytes was used (lane 2). (C) T₃-dependent transcriptional regulation of TRβA promoter by TRβ/RXRα in oocytes is dependent on TRE. Oocytes were either injected with (+) or without (−) an equal amount of TRβ and RXRα mRNA (0.27 ng/oocyte) as indicated. After 6 hr, ssDNA template with wild-type TRE (lanes 1–4) or mutant TRE (mTRE) (lanes 5–8) or a S’ truncated promoter (−252 to +316) containing the mutated TRE [mTRE(d)] (lanes 9–12) (see Materials and methods) was injected into the nuclei of the oocytes and incubated in the presence (+) or absence (−) of T₃ as indicated. Transcripts from the TRβA promoter were analyzed by primer extension using CAT primer, which also detected an endogenous oocyte mRNA indicated as internal control.

We conclude that expression of exogenous TRβ and RXRα leads to the assembly of a heterodimeric TRβ/RXRα protein that accumulates in the nucleus, recognizes TRE elements, represses transcription of the TRβA promoter in the absence of T₃, and activates transcription in the presence of T₃.

A role for chromatin in the transcriptional regulation of the TRβA promoter

We have established that repressive effects as a result of chromatin assembly influence basal Xenopus TRβA promoter activity [Fig. 1]. We have also described repressive effects on transcription as a result of the association of unliganded TRβ/RXRα with a TRE in the TRβA promoter and the activation of transcription by TRβ/RXRα in the presence of thyroid hormone [Fig. 2]. We then wished to examine how these phenomena might com-
bine to reconstitute regulation of TRβA promoter activity.

We determined the transcriptional activity of the TRβA promoter activity under a variety of conditions: in the absence or presence of TRβ/RXRα, in the absence or presence of T₃, and when chromatin assembly occurred on replicating or nonreplicating templates (Fig. 3). Most importantly, we were able to examine the effect of adding template and, thus, directing the assembly of a minichromosome before or after TRβ/RXRα was synthesized. This was accomplished either by injecting a mixture of mRNAs encoding TRβ/RXRα 6 hr before the DNA templates (Fig. 3A, lanes 5–8) or, alternatively, by allowing chromatin assembly to occur on the TRβA promoter following injection of the DNA templates before expressing TRβ/RXR through the injection of mRNA (Fig. 3A, lanes 9–12). In all cases, transcription efficiencies were again normalized against an endogenous mRNA present in the oocyte (Fig. 3A, internal control).

In agreement with earlier work (Fig. 1; Almouzni and Wolffe 1993), we find that the injected ssDNA template is less active than double-stranded templates containing the TRβA promoter (Fig. 3A, cf. lanes 1 and 2 with 3 and 4). When TRβ/RXR is expressed before chromatin assembly, TRβA promoter transcription is repressed from both the injected ssDNA and dsDNA templates in the absence of T₃, compared with transcription when TRβ/

**Figure 3.** Replication-coupled chromatin assembly in the presence of unliganded TR/RXR heterodimer is required for efficient repression of transcription from the TRβA promoter. [A] Groups of 20 oocytes were injected without (−) or with (+) TRβ/RXRα mRNAs (0.9 ng/oocyte), either 6 hr before (early injection mRNAs) or 6 hr after (early injection DNA) the injection of either ssDNA or dsDNA of the pTRβA. The oocytes were then treated for 16 hr with (+) or without (−) T₃ as indicated. The mRNA transcribed from the TRβA promoter was analyzed as described in Fig. 2C. The mRNA level in each case was quantitated by densitometer and normalized against the internal control. Fold transcriptional activation by T₃ was quantitated by comparing the mRNA level in the presence of T₃ with that in the absence of T₃. [B] In vivo nuclear run-on assay for TRβA promoter activity. Oocytes were injected with mRNAs and TRβA promoter DNA as in A. After overnight incubation, [³²P]CTP was injected into the nuclei of the oocyte and the oocytes were incubated for another hour to label the newly transcribed RNA. The RNA was isolated and hybridized to a slot–blot containing a 300-bp CAT DNA fragment (+316 to ~+600, Fig. 1A). [C] Equal amounts of TRβ were present in oocytes with early mRNA injection in the presence or absence of T₃ or late mRNA injection.

**Table:**

| Early Injection | No mRNAs | DNA + ssDNA | DNA + dsDNA |
|-----------------|----------|-------------|-------------|
| TRβ-RXRα mRNAs |          |             |             |
| pTRβA           | +        | +           | +           |
| T₃ (50 nM)      | -        | +           | +           |
| ssDNA           | -        | +           | +           |
| dsDNA           | +        | -           | -           |

**Activation by T₃**

- Early mRNAs
- Late mRNAs

2X 1X 280X 17X 28X 6X
assembly is further emphasized by the results obtained if chromatin assembly is allowed to occur before the synthesis of TRβ/RXR and the addition of T3 [Fig. 3A, lanes 9–12]. Using this protocol, both injected ssDNA and dsDNA templates show similar reduced levels of transcription in the absence of T3 [Fig. 3A, cf. lanes 9 and 11]. Thus, transcriptional silencing on the ssDNA template is much more effective if chromatin is assembled after TRβ/RXR is expressed [Fig. 3A, cf. lanes 5 and 9]. We suggest that the association of TRβ/RXR with the TRβA promoter during replication-coupled chromatin assembly is necessary to direct truly efficient transcription silencing of the TRβA promoter. This could be by either a direct or indirect effect. A direct effect might involve contacts with some component of the transcriptional preinitiation complex that prevents complete assembly of a competent complex in the face of ongoing chromatin assembly. An indirect effect would involve some role for the TRβ/RXR protein in establishing a repressive chromatin structure that might influence the function or assembly of a preinitiation complex, for example, the role of the Saccharomyces cerevisiae α2/MCM1 protein in repressing transcription [Roth et al. 1990; Cooper et al. 1994].

A complication to the simple interpretation of the TRβA mRNA accumulation results shown in Figure 3A is that during the ordered addition experiments in which template is injected first [Fig. 3A, lanes 9–12], 6 hr elapse before TRβ and RXR mRNAs are injected. This period of time might allow significant quantities of TRβA mRNA to accumulate before the unliganded TRβ/RXRα heterodimer could establish silencing. We therefore made use of an independent run-on methodology to directly determine transcription rates from the ssDNA templates assembled into chromatin in the presence of unliganded TRβ/RXRα [Fig. 3A, lanes 5–8] or those that are assembled into chromatin before TRβ/RXRα accumulates [Fig. 3A, lanes 9–12]. We find that transcription from the ssDNA template assembled into chromatin in the absence of unliganded receptor is more active [~fivefold] than the same template to which unliganded receptor is present during chromatin assembly [Fig. 3B, cf. lanes 7 and 8]. Moreover, there is a good correlation between the accumulation of TRβA mRNA [Fig. 3A] and the transcriptional efficiency of the TRβA promoter [Fig. 3B]. Independent measurement of TRβA mRNA stability indicated that the mRNA was relatively unstable having a half-life of <3 hr [data not shown; see also Almouzni and Wolffe 1995]. This result is consistent with the correspondence between transcription rate and mRNA accumulation [Fig. 3]. As an additional control, immunoblotting [Fig. 3C] indicated that equal amounts of TRβ were present in oocytes at the conclusion of the mRNA accumulation or transcription assays independent of late or early injection of the template DNA.

The most effective way of approaching the full physiological regulation of the TRβA promoter (~280-fold) is to have unliganded TRβ/RXR protein available to bind and act on the TRβA promoter during replication-coupled chromatin assembly. This is, of course, the state of affairs that most closely resembles that occurring within the living cell.

The TRβ/RXR heterodimer is constitutively bound to chromatin within chromatin

Our results on the transcriptional activation and repression of the TRβA promoter are dependent on the presence of an intact TRE in the promoter [Fig. 2]. This implies that the TRβ/RXR heterodimer is constitutively bound to the TRE both in the absence or presence of thyroid hormone. Our next experiments tested this prediction. Prior expression of TRβ/RXR [following injection of mRNAs encoding the proteins] before assembly of the TRβA promoter into chromatin in the oocyte nucleus leads to footprinting of the TRE between +264 and +280 [relative to the start of transcription at +1] in the absence or presence of T3 [Fig. 4A, lanes 7–12]. There are no significant differences between footprinting of the TRE in the absence or presence of T3. Thus, we conclude that the switch from active repression to transcriptional activation occurs with the same TRβ/RXR complex bound to the TRβA promoter within chromatin.

The transcription results presented earlier on templates assembled into chromatin before synthesis of TRβ/RXR [Fig. 3A, lanes 9–12] lead to the suggestion that although the unliganded TRβ is less effective in repressing transcription on a preexisting chromatin template, the protein can still bind to the TRE in a chromatin context. We explored this possibility by comparing footprinting of the TRE of the TRβA promoter when chromatin was assembled before synthesis of TRβ/RXR and vice versa [Fig. 4B]. We find that the TRE is efficiently protected from DNase I digestion under both sets of circumstances [Fig. 4B, lanes 4–9]. We suggest that the TRβ/RXR complex can bind to the TRE within chromatin independently of the prior association of the template with histones. This observation is consistent with earlier biochemical studies indicating the stable association of TR with chromatin independent of the presence of ligand [Perlman et al. 1982].

Thyroid hormone-dependent chromatin disruption concomitant with transcriptional activation

We then examined whether TRβ/RXR association disrupted the canonical nucleosomal array within which the TRE was assembled. We therefore assembled minichromosomal templates following injection of ssDNA and examined the micrococcal nuclease digestion patterns over the promoter including the TRE at +264 [Fig. 5A] or over a downstream transcribed region [Fig. 5B] in the presence or absence of TRβ/RXR with or without T3. We find that the TRE remains within a canonical nucleosomal array independent of the presence or absence of T3 or in the presence of unliganded receptor [Fig. 5A, lanes 1–12]. This result implies that unliganded TRβ/RXR binds to DNA within chromatin but does not disrupt nucleosomes around the binding site. The tran-
The TRβ/RXRα heterodimer binds to nucleosomal DNA in vitro

Our hypothesis from the experiments shown in Figures 4, 5, and 6 is that the unliganded TRβ/RXRα is able to associate with DNA within a chromatin environment. We then attempted to test this hypothesis by reconstituting the association of TRβ/RXRα with a nucleosome in a purified system. Our first analysis examined the capacity of TR/RXR to bind to a reconstituted nucleosome in a gel retardation assay [Fig. 7]. We assembled a histone octamer onto a 160-bp DNA fragment of the TRβA promoter including the TRE +264. We then mixed this nucleosome with an increasing mass of an oocyte extract in which TRβ/RXRα had or had not been expressed. We find a clear dependence on TRβ/RXRα expression for binding to naked DNA [Fig. 7, lanes 5–8]. Mutation of the TRE at +264 prevents TRβ/RXRα association with naked DNA [Fig. 7, lanes 1–4]. With the reconstituted nucleosome, a clear supershifted complex containing TRβ/RXRα, the histones, and DNA is resolved above the complex of TRβ/RXRα with naked DNA [Fig. 7, lanes 13–16]. No binding is detected to nucleosome if the TRE is mutated [Fig. 7, lanes 9–12]. This result demonstrates the assembly of a tertiary complex of the TRβA promoter, the histone octamer, and the TRβ/RXRα heterodimer.

The TRβ/RXRα heterodimer can bind to the TRE in a T3-independent manner and even when the TRE was preassembled into chromatin in vivo. (A) TRβ/RXRα heterodimer binds to the TRE independently of T3. Groups of 30 oocytes were injected with (+, lanes 7–12) or without (−, lanes 1–6) TRβ/RXRα mRNAs (2.7 ng/oocyte) 6 hr before single-stranded pTRβA was injected and treated with (+, lanes 4–6, 10–12) or without (−, lanes 1–3, 7–9) T3 as indicated. After overnight incubation, the oocytes were collected and processed for PCR-mediated DNase I footprinting analysis with increasing amounts of DNase I (400 U/ml, 600 U/ml, and 800 U/ml, respectively). [Lanes A, C, G, T] Sequencing ladders of pTRβA using the same end-labeled primer used for the PCR-mediated DNase I footprinting. The diagram at right of B illustrates the position of the TRE in pTRβA promoter. Note that the protection of the region further upstream (top of the gel) is also dependent on the presence of the TRβ/RXRα. The nature of this protection is unknown. (B) TRβ/RXRα heterodimer binds to the TRE preassembled into chromatin in vivo. Groups of 30 oocytes were injected with mRNAs (lanes 4–6) or single-stranded pTRβA first (lanes 7–9) followed by the injection of single-stranded pTRβA or mRNAs, respectively, 6 hr later. Control indicates no mRNA injection (lanes 1–3). DNase I footprinting was processed as in A. Note that the region upstream of the TRE was also protected in a TRβ/RXRα heterodimer-dependent manner under both injection conditions.

The described region [Fig. 5B] also appears within a canonical nucleosomal array independent of the presence or absence of T3 or in the presence of unliganded receptor [Fig. 5B, lanes 1–12]. However, the addition of both receptor and T3 results in the disruption of canonical nucleosomal arrays including the TRE +264 and the downstream transcribed region [Fig. 5A, B, lanes 13–16]. We conclude that the presence of liganded receptor and the concomitant increase in transcriptional activity lead to the disruption of chromatin structure. We then examined whether transcription itself was necessary for the disruption of chromatin.

We established conditions under which the transcriptional activation of the TRβA promoter by TRβ/RXRα in the presence of T3 could be inhibited by the injection of α-amanitin [Fig. 6A]. We assembled ssDNA into minichromosomes in the presence of unliganded TRβ/RXRα and examined the micrococcal nuclease accessibility of the chromatin including the TRE at +264 [Fig. 6B]. In the absence of T3, the TRE is assembled into a canonical nucleosomal array [Fig. 6B, lanes 1–3]. In the presence of T3, this nucleosomal array is disrupted [Fig. 6B, lanes 4–6]. This nucleosomal disruption is directed by the ligand-bound receptor even when transcription is inhibited by α-amanitin [Fig. 6B, lanes 7–12]. Thus, transcription is not required for chromatin disruption at this inducible promoter.
We wished to understand why the TRβ/RXR complex could bind to nucleosomal DNA. DNase I footprinting of the mixture of histone complexes with the TRβA promoter, followed by isolation of the tertiary complex, deproteinization, and resolution on a denaturing gel [Hayes and Wolfe 1992, Lee et al. 1993], reveals that DNA is rotationally positioned on the surface of the histone octamer [Fig. 8A, lanes 3,4]. This rotational positioning is such that the TRE can be recognized by the TRβ/RXR heterodimer [Fig. 8A, lanes 5,6]. The cleavage pattern of the tertiary complex of the TRβA promoter fragment with the histone octamer and TRβ/RXR reveals that the specific association of the TRβ/RXR occurs with the rotationally positioned TRE [Fig. 8B]. The DNase I cleavage pattern flanking the receptor binding site shows some alterations; however, a 10- to 11-bp cleavage periodicity is also apparent indicating continued histone association. We conclude that the TRβ/RXR recognizes the TRE incorporated into a nucleosome. This recognition provides an explanation for the constitutive binding of this protein within chromatin [Fig. 4] and for the lack of disruption of nucleosomal arrays following TRβ/RXR association in the absence of hormone [Figs. 5 and 6]. An important issue that we have not yet resolved is how the TRβ/RXR heterodimer disrupts chromatin structure. Although our gel retardation experiments suggest that a fraction of the receptor coexists with histones on DNA, we are yet to examine their stoichiometry and the exact nature of histone–DNA interactions in the presence or absence of thyroid hormone. Our current hypothesis is that other nucleosome disrupting activities will have to be recruited by the TR to effect chromatin reorganization [Figs. 5 and 6]. It is always possible that experiments involving in vitro chromatin assembly do not recapitulate the conditions operative in vivo.

Discussion

The major conclusions from these experiments are that the unliganded TRβ/RXR heterodimer can make use of replication-coupled chromatin assembly to direct the repression of the TRβA promoter [Fig. 3], that the TRβ/RXR heterodimer can constitutively bind to DNA within chromatin [Figs. 4, 7, and 8], and that addition of

![Figure 5](https://www.cshlp.org/2703/3/6-1.f5a.png)
ligand induces the disruption of chromatin structure [Figs. 5 and 6]. These results demonstrate both the adaptation of TRβ/RXR to function in a chromatin environment and the exploitation of chromatin assembly and disassembly pathways to effect gene regulation.

**Transcriptional repression by TRβ/RXR**

Roeder and colleagues have demonstrated that unliganded TR bound to a TRE will inhibit assembly of a functional preinitiation complex (Fondell et al. 1993). In their in vitro study, the role of chromatin assembly was not explicitly examined; however, overall repression by TR was greater in a nuclear extract. These investigators suggested that unliganded TR has direct interactions with the basal transcriptional machinery including TFIIB and that TFIIB might be frozen in a nonfunctional conformation.

There are several similarities and differences between our work and that of Fondell et al. (1993). First of all, in our experiments unliganded TR/RXR can repress even a previously active promoter in vivo [Fig. 3], in contrast to their in vitro result in which preexisting transcription complexes are refractory to TR repression in vitro. In addition, unlike their work, we require both TRβ and RXR to be expressed to have binding to the TRE of the TRβA promoter [Fig. 2]. Moreover, only unliganded TRβ/RXR represses transcription at the range of mRNAs injected such that the repression is dependent on the TRE [Fig. 2]. Synthesis of TR alone at comparable levels has an effect that is an order of magnitude lower [data not shown]. Thus, we require the RXR component to exert transcriptional silencing, presumably because the RXR component of the TR/RXR heterodimer influences the association of TR with the TRE (Perlmann et al. 1993).

In agreement with Fondell et al. (1993), we find that unliganded TR/RXR heterodimer can repress transcription on templates incompletely assembled into chromatin [Figs. 1D and 3A, cf. lanes 3 and 7; Fig. 3B, cf. lanes 2 and 4]. However, we also find a much more complete repression of transcription under conditions of efficient replication-coupled chromatin assembly [Figs. 1D and 3A, cf. lanes 1 and 5; Fig. 3B, cf. lanes 4 and 8]. One aspect of transcriptional silencing is that replication-coupled chromatin assembly is more efficient in repressing basal transcription [Figs. 1 and 2; Almouzni and Wolffe 1993]. A second aspect may be that the unliganded TR/RXR complex may alter the competition between preinitiation complex assembly and nucleosome assembly more toward the assembly of repressive chromatin. This might be accomplished actively either by preventing stable association of general transcription factors like TFIIB (Baniahmad et al. 1993; Fondell et al. 1993) with the preinitiation complex or the TRβ/RXR heterodimer might destabilize the association of TBP/TFIID allowing key regulatory elements to become histone associated (Workman and Roeder 1987; Workman et al. 1988). Alternatively, the TRβ/RXR could be involved in actively directing the assembly of a repressive
TR/RXR and histones regulate TRβA promoter activity

| Free Probe          | Reconstituted Nucleosome |
|---------------------|--------------------------|
| Control Extract (μl) | mTRE  TRE                |
| TR-RXR (μl)         | 1  2 - 1  2             |
|                     | 1  2 - 1  2             |

Figure 7. TR/RXR heterodimer binds to the TRE but not the mutated TRE reconstituted into nucleosome in vitro. A 160-bp end-labeled DNA fragment from TRβA promoter (from +163 to +322) containing either the wild-type TRE (TRE) or the mutated TRE (mTRE) was generated by PCR amplification with one of the two primers end-labeled with 32P (position +322), purified, and reconstituted into nucleosome in vitro with histone octamers purified from chicken erythrocytes. The reconstituted nucleosome was then incubated with extract from oocytes with or without (control) overproduction of TRp/RXRα (lanes 9-16). For a comparison, the binding experiment was also conducted with the end-labeled naked DNA (lanes 1-8). In a 10-

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Figure 8. DNase I footprinting reveals that TR/RXR heterodimer bound to a rotationally positioned nucleosome in vitro. 

To observe the footprint of TR/RXR on the reconstituted nucleosome, scale-up binding reactions of that shown in Fig. 7 (lane 16) were digested with two different concentrations of DNase I resolving the complexes on a 4% polyacrylamide gel. The complexes correspond to nucleosome (lanes 3,4) and TR/RXR-nucleosome (lanes 5,6) were cut out from the gel, electroeluted, deproteinized, and then analyzed with a 6% sequencing gel. As controls, free probes incubated either with 2 μl of control oocyte extract (lane 1) or with 2 μl of oocyte extract with TR/RXR (lane 2) in 25 μl of binding reaction were digested with DNase I and analyzed on the same gel. [B] PhosphorImager scanning of lanes 4 and 6, demonstrating strong protection of the TRE region by TR/RXR in the nucleosome.

Constitutive binding of TR/RXR in chromatin

A feature of TR/RXR function is that the protein is constitutively bound to chromatinized DNA in the absence or presence of thyroid hormone (Fig. 4). Although another member of the steroid hormone receptor superfamily, the glucocorticoid receptor (GR), can also bind to a glucocorticoid response element (GRE) in a nucleosome [Perlmann and Wrangle 1988; Pina et al. 1990a,b; Archer et al. 1991; Li and Wrangle 1995], it does so only in the presence of its cognate hormone. In the absence of the hormone, GR remains in the cytoplasm. On the other hand, TR is constitutively nuclear. The progesterone receptor also binds to chromatin constitutively, but it disrupts chromatin in the presence or absence of hormone [Mymryk and Archer 1995]. In contrast, TR/RXR can associate with a preassembled chromatin template without disrupting the nucleosomal array (Figs. 4, 5, and 6). The addition of hormone is necessary to disrupt chromatin (Figs. 5 and 6).

In vitro reconstruction demonstrates that the region of the TRβA promoter including the TRE contains intrinsic structural features that direct the rotational positioning of DNA on the surface of the histone octamer (Fig. 8). The positioning of DNA within the nucleosome containing the TRE is such that the TR/RXR complex can stably bind to this element despite the presence of histone-DNA contacts [Figs. 7 and 8]. Precedent for the rotational positioning of hormone recognition elements on the surface of a translationally positioned histone octamer includes the GREs within nucleosome B of the mouse mammary tumor virus long terminal repeat (MMTV LTR) [Perlmann and Wrangle 1988; Pina et al. 1990a,b; Archer et al. 1991]. This nucleosome lies within a phased array of at least five nucleosomes [Richard-Foy and Hager 1987]. Association of the GR with this nucleosomal array leads to the local disruption of chromatin structure [Zaret and Yamamoto 1984; Lee and Archer 1994], whether the GR receptor is continually present within chromatin during transcriptional activation and subsequent repression is still under investigation [Lee et al. 1986; Archer et al. 1992]. Transcription is not required for chromatin disruption [Fig. 6; Fascher et al. 1993]; neither is replication [Archer et al. 1989; Schmid et al. 1992]. In our experiments [Figs. 5 and 6], chromatin disruption occurs after the minichromosome is assembled and DNA synthesis is complete, thus, replication is unlikely to contribute to chromatin disruption. Xenopus oocytes do not replicate dsDNA [Almouzni and Wolffe 1993]. Several investigators have shown that molecular machines including the metazoan homologs of the yeast SWI/SNF complex can disrupt chromatin structure [Côté et al. 1994; Imbalzano et al. 1994]. Other proteins engaged in ATP-dependent processes have been shown to reconfigure nucleosomal complexes [Pazin et al. 1994; Tsukiyama et al. 1994; Varga-Weisz et al. 1995]. It has been clearly demonstrated that yeast SWI/SNF will facilitate the activation of transcription by glucocorticoid receptor in yeast [Yoshinaga et al. 1992]. This activation might be related to the disruption of chromatin structure mediated by SWI/SNF. Potentially comparable events might lead to chromatin disruption and transcriptional activation by the liganded TRβ/RXRx heterodimer.
and Archer 1994; Truss et al. 1995). Our studies suggest that the TRβ/RXRα remains bound to DNA even following chromatin disruption [Figs. 4, 5, and 6]. Moreover, we can clearly separate the stable association of the TRβ/RXR receptor with chromatin from the disruption process itself.

Future studies will investigate whether the nucleosome incorporating the TRE is part of a precise nucleoprotein architecture in vivo as has been proposed for the MMTV promoter and the S. cerevisiae PHOS promoter (Richard-Foy and Hager 1987; Almouzni et al. 1986). In both of these examples, positioned nucleosomes are essential components of a regulatory nucleoprotein structure within which transcription can be reversibly activated and repressed (Hager et al. 1993; Svanen and Horz 1993). The TRβα promoter should provide a useful example for the further analysis of the mechanisms of transcriptional silencing and activation within a chromatin environment.

Materials and methods

Plasmid constructs

The pTRβα construct was generated by cloning a 1.9-kb EcoRI fragment containing 1.6 kb of TRβα promoter sequence and ~0.3 kb of CAT gene sequence from plasmid pCAT-WT (Ranjan et al. 1994) into pBluescript II KS(−) (Stratagene, CA). The dsDNA of pTRβα was prepared using a Qiagen kit as described by the manufacturer. The ssDNA of pTRβα was prepared from phagemids induced with VCS M13 as described (Sambrook et al. 1989). The TRE mutant pTRβα (TRβAm) was generated by PCR as described (Ranjan et al. 1994). To remove the imperfect TREs located upstream of the transcriptional start site, the TRE mutant TRβα promoter was digested with PstI that excises the proximal TRβα promoter fragment, which was then cloned into pBluescript II KS(−) to generate pTRβAm[Δ]. To produce Xenopus RXRα mRNA for microinjection, the cdNA encoding Xenopus RXRα [the kind gift of Dr. E. De Robertis (Blumberg et al. 1992)] was cloned into pSP64poly(A) vector. Briefly, cDNAs encoding Xenopus full-length RXRα was amplified by PCR with Vent polymerase (New England Biolab) and then cloned into pSP64poly(A) to generate pSP64(A)-xRXRα. The pSP64(A)-xTRβα, which contains cDNA encoding full-length TRβα receptor, was a kind gift from Drs. A. Karamori and D. Brown at Carnegie Institute of Washington (Washington, D.C.).

Microinjection of Xenopus oocytes

The microinjection of Xenopus stage VI oocytes and the microinjection procedure were essentially as described (Almouzni and Wolffe 1993). Briefly, ~20 injected oocytes were collected for each sample, rinsed with 400 μl of MBSH buffer, and then homogenized in 300 μl of 0.25 m Tris (pH 8.0). Half of the sample was used to isolate RNA using RNAzol TM reagent, and the remaining half was used for DNA recovery. To isolate RNA, 500 μl of RNAzol TM reagent was added to the sample, vortexed, and then incubated on ice for 15 min before centrifugation. The clean supernatant was transferred to a new tube and extracted once with equal volumes of phenol/chloroform. The RNA was then precipitated with 0.7 volumes of isopropanol, rinsed with 70% ethanol, and dissolved in DEPC-treated water. For primer extension analysis, RNA from one or two oocyte equivalents was annealed with either the end-labeled CAT primer [5’-GGTGGTATATCCAGTGATTATTATTCTCCAT-3’] or primer I [5’-ATCCTTATAAGCGGTGACTAGTGACTGTCAT-3’] in 10 μl of 0.4 M KCl at 65°C for 10 min, 55°C for 25 min, and 42°C for 5 min. Thirty microliters of reverse transcription mixture (67 mM Tris-HCl at pH 8.3, 8 mM MgCl2, 5 mM DTT, 1 mM dNTP mix, 1 unit of RNasin, and 10 units of Superscript IITM) was then added. The reaction was incubated at 42°C for 1 hr and then stopped by ethanol precipitation. The products were displayed on a 6% sequencing gel and visualized by autoradiography.

For DNA recovery, the remaining half of the sample was mixed with an equal volume of 20 mM EDTA, 0.2% SDS and then treated with protease K [100 μg/ml] at 55°C for 2–3 hr.
After two phenol/chloroform extractions, the sample was precipitated with isopropanol and then resuspended in 100 µl of TE buffer. DNA was then treated with RNase A (100 µg/ml) at 37°C for 1 hr followed by phenol/chloroform extraction and ethanol precipitation. DNA was quantitatively analyzed either by Southern blot or slot-blot as described (Sambrook et al. 1989). Blots were probed with ssDNA of pTRpA labeled with a random prime labeling kit (Amersham) as described by the manufacturer.

Nuclear run-on assay

The injection of mRNA and DNA was as described above. Groups of oocytes were injected into the nucleus with [α-32P]CTP (5 µCi/µl) in a volume of 10 nl/oocyte. The oocytes were cultured for 1 hr and then processed for RNA purification as described above. The purified RNA was then hybridized to 0.5 ng of CAT DNA (from +316 to +600) bound to a nitrocellulose strip in a final volume of 1 ml of 1× PBS plus 160 mM NaCl, 0.1% SDS at 65°C overnight. The strips were washed in 0.1× SSC, 0.1% SDS at 65°C and visualized with a Phosphor-Imager (Molecular Dynamics).

In vivo DNase I footprinting

For in vivo DNase I footprinting, oocytes were injected with either ssDNA alone or ssDNA plus mRNA as described above. After overnight incubation, 25–30 oocytes were collected from each sample and homogenized in 200 µl of buffer containing 20 mM Tris-HCl (pH 7.6), 70 mM KCl, 5 mM MgCl2, 1 mM DTT, and 5% glycerol. The extract was aliquoted into three tubes with 100 µl each and treated with 30, 60, and 90 units of DNase I, respectively, at room temperature for 5 min. The reaction was stopped with an equal volume of stop solution (0.2% SDS, 20 mM EDTA). DNA was purified as described above. To further remove the contaminating RNA, DNA sample was adjusted to 100 mM NaOH in a final volume of 100 µl and incubated at 95°C for 10 min. After adjusting the reactions to a neutral pH with 10 µl of 1 M HCl, 12 µl of 3 M NaOAc (pH 5.2) was added followed by ethanol precipitation. This DNA was used for PCR-mediated primer extension (25 cycles, 94°C for 1 min, 55°C for 1 min, 72°C for 2 min) using the end-labeled CAT primer with Taq polymerase (Perkin-Elmer).

Gel retardation assay and DNase I footprinting analysis of the binding of TR/RXR heterodimer to the TRE reconstituted into nucleosome in vitro

To examine the in vitro reconstitution of the TRE of the TRβA promoter into a nucleosome, a 159-bp DNA fragment [from +322 to +163] was PCR amplified with the end-labeled primer II (5'-AGACAGGAGATCTACA-3') and cold primer III (5'-AAGCTGCTGTGTACTATA-3'). The PCR product was then gel-purified. The reconstitution was accomplished with the octamer exchange method as described [Hayes and Wolffe 1992] using the histone octamers purified from chicken erythrocytes. The gel retardation assay was performed essentially as described [Ranjan et al. 1994] except the oocyte-produced TR/RXR heterodimer was used. Briefly, the oocytes were injected with TR/RXR mRNAs (2.7 ng/oocyte each). After overnight incubation, the injected and un.injected oocytes were rinsed with TE buffer once and then homogenized in 20 µl/oocyte of buffer E (10 mM Tris-HCl at pH 7.6, 60 mM KCl, 0.1 mM EDTA, 5 mM DTT, 1 mM PMSF, and 8% glycerol). The extracts used for binding assays were obtained by microcentrifuging the crude extracts at top speed at 4°C for 20 min to remove yolk proteins and lipids. To analyze TR/RXR–nucleosome ternary complexes by DNase I footprinting, two 10-fold scale-up reactions (in 150 µl with 20 µl of the TR/RXR oocyte extract) were digested with 3 units and 6 units of DNase I, respectively, at room temperature for 1.5 min. DNase I digestion was stopped by the addition of 3 µl of 0.5 M EDTA. The mixtures were then loaded on a 4% native polyacrylamide gel containing 3% glycerol in 0.5× TBE prerun at room temperature for 1 hr (150 V) and run for two more hours. After autoradiography, the complexes corresponding to nucleosome alone and TR/RXR–nucleosome were excised from the gel. DNA was electroeluted from the gel slices, deproteinized, and ethanol-precipitated. As controls, binding reactions were done in a 25 µl volume with free probe alone or free probe plus 2 µl of TR/RXR oocyte extract. The reactions were digested with 0.1, 0.2, or 0.3 unit of DNase I for 40 sec at room temperature. DNase I digestions were stopped by the addition of 75 µl of 0.4 M NaOAc [pH 5.2], 0.2% SDS. The DNA in the reactions was recovered by ethanol precipitation. The products were displayed on a 6% sequencing gel and visualized with Phosphor-Imager (Molecular Dynamics).

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In vivo assays of nucleosomal arrays over the TRβA promoter and transcribed regions by micrococcal nuclease (MNase) digestion and Southern hybridization

Oocytes were injected as described above. After overnight incubation, groups of 25–30 oocytes were collected and homogenized in 360 µl of buffer with 20 mM Tris-HCl (pH 7.6), 70 mM KCl, 3 mM CaCl2, 1 mM DTT, 5% glycerol. The extract was aliquoted into either four fractions (60 µl each) and digested with 7.5 units, 2.5 units, 0.83 unit, 0.24 unit of MNase, respectively, [Pharmacia Biotech] [Fig. 6] of three fractions (90 µl each) and digested with 7.5 units, 2.5 units, and 0.83 unit of MNase [Fig. 7], respectively, at room temperature for 25 min. DNA was purified as described above except without NaOH treatment. DNA was resolved on 1.5% agarose gels run at 60 V for 6 hr in 1× TBE buffer, blotted to Nytran Plus membranes (Schleicher & Schuell), and probed with either a random primer-labeled DNA fragment from TRβA promoter [from +314 to +218] or a 270-bp fragment of the CAT gene (+318 to +588).
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