Determinants of chromatin disruption and transcriptional regulation instigated by the thyroid hormone receptor: hormone-regulated chromatin disruption is not sufficient for transcriptional activation

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Chromatin disruption and transcriptional activation are both thyroid hormone-dependent processes regulated by the heterodimer of thyroid hormone receptor and 9-*cis* retinoic acid receptor (TR–RXR). In the absence of hormone, TR–RXR binds to nucleosomal DNA, locally disrupts histone–DNA contacts and generates a DNase I-hypersensitive site. Chromatin-bound unliganded TR–RXR silences transcription of the *Xenopus* TR β A gene within a canonical nucleosomal array. On addition of hormone, the receptor directs the extensive further disruption of chromatin structure over several hundred base pairs of DNA and activates transcription. We define a domain of the TR protein necessary for directing this extensive hormone-dependent chromatin disruption. Particular TR–RXR heterodimers containing mutations in this domain are able to bind both hormone and their thyroid hormone receptor recognition element (TRE) within chromatin, yet are unable to direct the extensive hormone-dependent disruption of chromatin or to activate transcription. We distinguish the hormone-dependent disruption of chromatin and transcriptional activation as independently regulated events through the mutagenesis of basal promoter elements and by altering the position and number of TREs within the TR β A promoter. Chromatin disruption alone on a minichromosome is shown to be insufficient for transcriptional activation of the TR β A gene.

Keywords: chromatin/disruption/thyroid hormone receptor/transcriptional activation

Introduction

The thyroid hormone receptor (TR) regulates gene activity through alternately silencing or activating transcription dependent on the absence or presence of thyroid hormone (T₃) (Evans, 1988; Damm *et al.*, 1989; Glass *et al.*, 1989; Graupner *et al.*, 1989; Sap *et al.*, 1989; Baniahmad *et al.*, 1990, 1992; Brent *et al.*, 1993). Silencing of transcription by TR is dependent on the action of structurally related co-repressor molecules N-CoR (Horlein *et al.*, 1995) and SMRT (Chen and Evans, 1995). These co-repressors bind to the receptor in the absence of T₃ and are released in

the presence of T₃. The exact molecular mechanism by which they inhibit transcription is unknown. In addition, the TR itself makes contact with components of the basal transcriptional machinery including TFIIB (Baniahmad *et al.*, 1993; Fondell *et al.*, 1993; Hadzic *et al.*, 1995). Several structurally independent domains of the TR are required for silencing, these include regions that do not interact with TFIIB (Baniahmad *et al.*, 1993), indicative of multiple silencing mechanisms (Baniahmad *et al.*, 1992, 1995a; Damm and Evans, 1993; Lee and Mahdavi, 1993; Qi *et al.*, 1995; Uppaluri and Towle, 1995; Wagner *et al.*, 1995). Transcriptional activation by the TR also involves multiple structurally independent domains. A ligand-dependent transactivation domain (AF2) is located at the carboxy-terminus of the receptor (Zenke *et al.*, 1990; Baretino *et al.*, 1994). This domain is required for the release of a putative co-repressor necessary for transcriptional silencing (Casanova *et al.*, 1994; Baniahmad *et al.*, 1995a). A second transactivation domain includes part of the hinge region (D region) that links the DNA-binding and ligand-binding domains of the receptor (Lee and Mahdavi, 1993; Uppaluri and Towle, 1995). The mechanism of transcriptional activation by the TR has not yet been defined, although potential co-activators have been isolated (Halamchmi *et al.*, 1994; Baniahmad *et al.*, 1995b; Le Douarin *et al.*, 1995; Lee *et al.*, 1995a,b; Onate *et al.*, 1995; Chakravarti *et al.*, 1996).

Nucleosome assembly and disruption have an essential regulatory role in the transcription of many inducible genes in yeast and metazoans (Zaret and Yamamoto, 1984; Almer *et al.*, 1986; Straka and Horz, 1991; Archer *et al.*, 1992; Lee and Archer, 1994; Truss *et al.*, 1995). In yeast, co-repressors organize repressive chromatin structures (Roth *et al.*, 1990, 1992; Vidal and Gaber, 1991; Cooper *et al.*, 1994; Wolffe, 1995, 1996), whereas co-activators modify histones and disrupt chromatin structure (Hirschhorn *et al.*, 1992; Kruger *et al.*, 1995; Brownell *et al.*, 1996; Wolffe and Pruss, 1996). The TR retains the capacity to bind to DNA packaged into nucleosomes and silences transcription effectively in the context of a positioned nucleosomal array (Wong *et al.*, 1995). This canonical nucleosomal array is disrupted on the addition of T₃ and transcription is activated (Wong *et al.*, 1995).

In this work, we make use of mutagenesis of the *Xenopus* thyroid hormone β receptor to determine the role of the AF2 regulatory domain in hormone binding, association with nucleosomal DNA, transcriptional silencing, transcriptional activation and hormone-dependent disruption of nucleosomal arrays. We localize a ligand-dependent chromatin disruption function to the AF2 domain containing the C-terminal nine amino acids of the receptor (Baretino *et al.*, 1994). We then establish that chromatin disruption is a distinct hormone-regulated event

targeted by the TR bound to its recognition element in chromatin. Using this information, we demonstrate that chromatin disruption alone is insufficient for transcriptional activation of the TR β A gene.

Results

Transcriptional regulation and chromatin disruption by the *Xenopus* thyroid hormone receptor

Microinjection of *Xenopus* oocyte nuclei with single-stranded DNA leads to the assembly of chromatin (Almouzni and Wolffe, 1993). Nucleosome assembly coupled to complementary strand synthesis using single-stranded DNA as a template occurs with rapid kinetics and represses basal transcription of the TR β A gene promoter (Wong *et al.*, 1995). *Xenopus* oocytes are deficient in TR (Eliceiri and Brown, 1994; Wong and Shi, 1995), thus the capacity of the receptor to regulate transcription from the TR β A gene promoter can be followed from the microinjection of mRNA encoding TR β and/or the 9-*cis* retinoic acid α receptor (RXR α) which encode the two subunits of the heterodimeric receptor. Increasing masses of a mixture of TR β and RXR α mRNAs lead to the synthesis of more receptors able specifically to bind a thyroid response element (TRE) within the TR β A gene (Wong *et al.*, 1995). Basal transcription is very low from templates in which chromatin assembly is coupled to replication (Figure 1A, lane 1). However, expression of increasing amounts of TR in the presence of T₃ prevents transcriptional repression caused by chromatin (Figure 1A, lanes 2–6). To examine the consequences of transcriptional activation for chromatin disruption on the *Xenopus* TR β A promoter, we established both conditions for robust ligand-inducible transcription in the presence of TR–RXR (Figure 1B, lanes 4 and 5) and conditions where transcription was inhibited in the presence of α -amanitin (Figure 1B, compare lanes 5 and 6). Quantitation of the small amount of transcription from the TR β A promoter in the absence of exogenous TR–RXR (Figure 1B, lane 1) reveals no significant activation on addition of T₃ (Figure 1B, lane 2) relative to the internal control. Note that the internal control was not affected by α -amanitin since it derives from endogenous mRNA already synthesized in the oocyte. We next made use of a supercoiling assay for circular plasmid DNA molecules to determine the extent of chromatin disruption. This assay is based on the fact that each nucleosome constrains a single negative superhelical turn (Germond *et al.*, 1975; Simpson *et al.*, 1985). Disruption of nucleosomal architecture can lead to changes in the topology of DNA in the nucleosome even if histones are not displaced from DNA (Norton *et al.*, 1989; Bauer *et al.*, 1994). Addition of ligand to chromatin-bound TR induces a change in the topology of DNA in the minichromosome containing the TR β A promoter that is equivalent to the loss of six nucleosomes (Figure 1C, compare lanes 4 and 5), i.e. the loss of at least 20% of the nucleosomes of the entire minichromosome (25 nucleosomes are estimated to be assembled on a plasmid of 4.5 kb). This topological change is independent of transcriptional activation (Figure 1C, compare lanes 5 and 6). These results indicate that following the addition of T₃ the TR is inducing a major

transition in chromatin structure. We next wished to examine the extent of the disruption process.

The *Xenopus* TR β A promoter contains a TRE at 264 bp downstream from the start site of transcription (Shi *et al.*, 1992; Ranjan *et al.*, 1994; Wong *et al.*, 1995). In our experiments, we utilize a construct containing 1336 bp upstream and 316 bp downstream of the transcription start site of the TR β A gene fused to a segment of the bacterial chloramphenicol acetyltransferase (CAT) gene (Figure 2A). We probed for disruption of a canonical nucleosomal array using a probe containing the TRE (Figure 2B, probe II), a probe upstream of the TRE (Figure 2B, probe I), probes including the CAT gene (Figure 2C, probe III) and downstream of the CAT gene (Figure 2C, probe IV) and a vector probe of pBluescript II sequences (Figure 2D). We find that some disruption of a canonical nucleosomal array is obtained over all DNA sequences in the vicinity of the transcription unit, as determined by a more diffuse micrococcal nuclease (MNase) cleavage pattern dependent on the presence of TR and T₃ (Figure 2B and C, compare lanes 1–4 and 9–12 with lanes 5–8 and 13–16). A more regular pattern of MNase is found on the pBluescript vector sequences in the presence or absence of ligand-bound TR (Figure 2D). It should be noted that probes I, II, III and IV are close to each other and that the disruption of nucleosomal organization in either one of the adjacent DNA segments might contribute to the appearance of disruption over both sequences. This is particularly important for longer DNA fragments such as those found in tri- and tetra-nucleosomes. These experiments make use of α -amanitin to inhibit transcriptional activation (Figure 1B), so that any disruption of chromatin is a primary effect of transcriptional activators and not a secondary effect of transcription itself. We conclude that extensive alterations in chromatin organization occur on the TR β A promoter in the presence of TR–RXR and T₃. Future experiments will be necessary to determine the degree to which these transitions in nucleosomal structure are precisely targeted.

The role of TR activation domain mutants in hormone binding, association with nucleosomal DNA, transcriptional silencing, transcriptional activation and hormone-dependent chromatin disruption

We next examined the role of the hinge and C-terminal transactivation domains of TR in the various biological functions of the TR–RXR protein (Zenke *et al.*, 1990; Baretino *et al.*, 1994; Casanova *et al.*, 1994; Uppaluri and Towle, 1995). We introduced six independent point mutations in the hinge and C-terminal domains and created one deletion mutation of the C-terminal nine amino acids of the *Xenopus* TR β A protein (Figure 3A). These mutations were selected initially because they had been shown earlier to influence T₃-induced transcriptional activation by mammalian/avian TRs, but had been found not to influence the T₃ or DNA binding affinity (Baretino *et al.*, 1994; Baniahmad *et al.*, 1995a). Microinjection of mRNA encoding these different proteins into oocyte cytoplasm led to equivalent synthesis of the corresponding TRs as detected by immunoblotting (Figure 3B). In the presence of the heterodimeric partner RXR α , equivalent

Fig. 1. The transcriptional activation by liganded TR-RXR from the repressive chromatin assembled by the replication-coupled pathway is accompanied by extensive chromatin disruption. **(A)** Liganded TR-RXR activates transcription from the repressive chromatin template assembled via the replication-coupled chromatin assembly pathway. The scheme shown at the top of the panel illustrates the features of chromatin assembly from injected single-stranded DNA (ssDNA). Groups of 20 oocytes were first injected with an increasing amount of TR-RXR mRNAs (1.2, 3.7, 11.1, 33.3 and 100 ng/ml, lanes 2–6) and then injected with ssDNA of pTR β A (100 ng/ml, 23 nl/oocyte). The oocytes were incubated at 18°C overnight with T₃. The transcription was analyzed by primer extension. To make sure that injection of ssDNA is uniform for each group of samples, DNAs were recovered from each group and analyzed by slot-blot hybridization with a probe from the TR β A promoter (from +218 to +314). **(B)** The transcriptional activation by liganded TR-RXR can be inhibited by α -amanitin. Groups of 20 oocytes were injected with ssDNA (100 ng/ml, 23 nl/oocyte) and TR-RXR mRNAs (100 ng/ml, 27 nl/oocyte) and treated with or without hormone as indicated. α -Amanitin was co-injected with ssDNA at a concentration of 10 mg/ml. The transcription was analyzed by primer extension using CAT primer, and the internal control is the primer extension product from an unknown endogenous mRNA as described (Wong *et al.*, 1995). **(C)** The DNA topology assay indicates that liganded TR-RXR also induces extensive chromatin disruption and that this chromatin disruption is not the by-product of processive transcription. The injections were the same as in (B). The DNA was purified from each group and the topological status of the DNA was analyzed using chloroquine agarose gel as described in Materials and methods. The top band in each lane represents the nicked form of plasmid.

DNA-binding activity was recovered (Figure 3C, and data not shown).

We next examined the capacity of these same mutations to regulate transcription in a chromatin environment. Using a template that is assembled into a repressive chromatin structure during replication, we find that deletion of the C-terminal nine amino acids of *Xenopus* TR β prevents the activation of transcription in chromatin (Figure 4A, TRm1, lanes 5 and 6). Likewise, the point mutants TRm4 and TRm6 fail to activate transcription within chromatin (Figure 4A, lanes 7–10). The other mutant TRs all retain the capacity to fully regulate transcription. Transcriptional

activation by wild-type TR β has been correlated with chromatin disruption on the *Xenopus* TR β A promoter used in these experiments (Wong *et al.*, 1995). We wished to determine which domains of *Xenopus* TR were required for the hormone-dependent disruption of chromatin. We find that every TR mutant that activates transcription within chromatin also directs the disruption of the canonical nucleosomal array including the TR β A promoter (Figure 4B, lanes 9–16). In contrast, TR mutants that do not activate transcription within chromatin do not disrupt the canonical nucleosomal array (Figure 4B, lanes 3–8). Analysis of the nucleosome disruption process using the

Fig. 2. Liganded TR-RXR instigates extensive chromatin disruption. (A) The diagram shows the structure of the construct and the locations of the probes used for hybridization. Probe I is a 108 bp *PstI*-*RsaI* fragment (from -255 to -147); probe II is a 96 bp fragment generated by PCR which covered the TRE site; probe III is a 268 bp CAT gene sequence from *Bgl*II (+306) to *Eco*RI (+584) and probe IV is a 270 bp fragment of pBluescript KS(+) from *Eco*RI (+584) to *Pvu*II (+854). (B and C) Hybridizations with probes from different regions of the constructs all show hormone-dependent chromatin disruption. The oocytes were injected with ssDNA (100 ng/ml, 23 nl/oocyte) and TR-RXR mRNAs (100 ng/ml each, 27 nl/oocyte) and treated with or without hormone in the presence of α -amanitin (as in Figure 1, lane 6) and processed for MNase assay. The amounts of MNase used are zero (lanes 1 and 5), 10 (lanes 2 and 6), 5 (lanes 3 and 7) and 2.5 U (lanes 4 and 8). The same filter was hybridized successively with random-primed labeled probes as indicated. (D) Hybridization with the entire vector DNA sequence (pBluescript II). Conditions were as described in (B) and (C).

topological assay (Figure 4C) reveals that for the TR mutants that fail to activate transcription, the topology of the template is retained (lanes 2–4), whereas those that do activate transcription lose topological constraint equivalent to six nucleosomes (Figure 4C, lanes 5–8). Thus the transactivation domain containing the C-terminal nine amino acids of *Xenopus* TR β is required for the activation of transcription within chromatin, and for the disruption of canonical nucleosomal arrays on the addition of T_3 . These results establish a strong link between the capacity of the TR to regulate transcription and to disrupt chromatin.

There are several potential explanations for the failure of the TR mutants (TRm1, TRm4 and TRm6) to activate transcription in a chromatin environment. The mutant receptors might not bind hormone, they might not bind to the TRE in a nucleosomal environment, they might be non-functional in their capacity to interact with the basal transcriptional machinery, with the various molecular machines that have been suggested to disrupt chromatin (Yoshinaga *et al.*, 1992; Kingston *et al.*, 1996; Wilson *et al.*, 1996) or with co-activators that modify histones (Ogryzko *et al.*, 1996). We began to discriminate between these possibilities by examining the capacity of the mutant receptors to direct transcriptional silencing from a double-stranded template microinjected into the oocyte nucleus. Double-stranded DNA is not rapidly assembled into chromatin (Almouzni and Wolffe, 1993), and in the absence of TR-RXR shows a high level of basal transcription

(Wong *et al.*, 1995). The capacity of the mutant receptors to silence this basal transcription reflects their interaction with both the TRE in the absence of rapid chromatin assembly and their interference with the function of the basal transcription machinery. Expression of all the mutant proteins in the absence of T_3 leads to the silencing of basal transcription from a microinjected double-stranded template (Figure 5A, compare lane 1 with lanes 3, 5, 7, 9, 11, 13, 15 and 17). However, in three cases (TRm1, TRm4 and TRm6, Figure 5A, lanes 5–10), silencing is not relieved by the addition of ligand. The other point mutations behave like the wild-type TR, fully regulating transcription (Figure 5A, lanes 11–18). These results are consistent with earlier work demonstrating that certain mutations in the C-terminal transactivation domain can still bind DNA and act as constitutive silencers, but that addition of hormone does not lead to significant relief of the silencing function (Baniahmad *et al.*, 1995a). We note that TR mutants that can relieve the silencing of transcription on addition of ligand (Figure 5A, lanes 11–18) can also activate transcription in a chromatin environment (Figure 4A, lanes 11–18). TR mutants (TRm1, TRm4 and TRm6) that silence transcription, but that cannot relieve this silencing on addition of ligand (Figure 5A, lanes 5–10), do not have the capacity to activate transcription within chromatin (Figure 4A, lanes 5–10). Therefore, the transactivation domain containing the C-terminal nine amino acids of *Xenopus* TR β is

A. TR Activation Domain Mutants **B. Expression** **C. DNA Binding**

Fig. 3. Characterization of TR mutants. (A) The diagram shows the structural and functional domains of TR. A/B, N-terminus variable domain; C, DNA-binding domain; D, hinge domain between the DNA-binding and hormone-binding domains; E, hormone-binding and dimerization domain; F, C-terminal hormone-dependent activation domain. The amino acid change in each TR mutant is indicated. TRm1 has the change of proline to a stop codon. (B) Western blotting analysis of TR mutants expressed in oocytes. mRNAs encoding mutant TRs were synthesized *in vitro* and injected into groups of oocytes (Materials and methods). After overnight incubation, the groups of oocytes were collected and homogenized, and half an oocyte equivalent was resolved with a 10% SDS-PAGE, blotted to a piece of nitrocellulose filter and visualized with antibody against recombinant TR using an ECL kit (KPL) (Materials and methods). (C) All TR mutants can form heterodimers with RXR and bind TRE as effectively as wild-type TR. For the gel retardation assay, 1 ml of TR or TR mutant oocyte extract (lanes 4–11) was mixed with 1 ml of RXR oocyte extract and incubated with an end-labeled double-stranded oligonucleotide containing the TRE from the TR β A promoter in 10 μ l of binding buffer as described (Wong and Shi, 1995). As controls, uninjected oocyte extract, TR or RXR oocyte extract alone also were used (lanes 1–3). Note that TR alone does not bind to the probe.

required both for the relief of silencing and for the activation of transcription within chromatin dependent on the addition of T₃.

We next examined the capacity of wild-type and mutant TR to associate with radiolabeled T₃ (Eliceiri and Brown, 1994). We find that TRm1 and TRm6 are substantially reduced in their capacity to bind hormone, whereas TRm4 binds hormone with an efficiency comparable with the wild-type TR (Figure 5B). Thus the failure of TRm1 and TRm6 to activate transcription in a chromatin environment (Figure 4) potentially can be accounted for by a failure to interact efficiently with hormone.

Our next experiments examined the capacity of the mutant and wild-type receptors to associate with nucleosomal DNA *in vivo*. Earlier work had established that TR–RXR would interact with chromatin in the presence or absence of hormone *in vivo* (Wong *et al.*, 1995). The association of wild-type TR–RXR with DNA in a nucleosome leads to a local disruption of histone–DNA contacts in the nucleosome (Wong *et al.*, 1995). This hormone-independent local disruption of chromatin can be detected as a DNase I-hypersensitive site (Figure 5C, arrowheads, compare lanes 1–7 with lanes 8–13). On addition of hormone, the DNase I hypersensitivity generated by the wild-type receptor becomes more pronounced

(Figure 5C, arrowheads, compare lanes 8–10 with 11–13). An additional region of DNase I sensitivity is revealed upstream of the TRE in the presence of hormone (lanes 11–13, indicated by an asterisk). This is consistent with the extensive hormone-dependent disruption of chromatin directed by the wild-type receptor (see Figure 4B). Some of the sites that are preferentially accessible to DNase I in the upstream region might reflect the association of other specific transcription factors (see Figure 6 later). Comparison of the wild-type and mutant receptors demonstrates that they can all associate with chromatin to generate a DNase I-hypersensitive site, albeit with varying efficiency. However, the TRm1 mutation in which the C-terminal nine amino acids of the receptor are deleted assembles a receptor that only weakly generates any DNase I hypersensitivity (Figure 5C, lanes 18–21). We suggest that the failure of the TRm1 mutant receptor to activate transcription in a chromatin environment might be due to a deficiency in the capacity of the receptor to interact with chromatin. Importantly, the TRm4 mutant receptor retains the capacity to bind both T₃ (Figure 5B) and nucleosomal DNA (Figure 5C) yet fails to activate transcription in a chromatin environment (Figure 4A) and to disrupt chromatin (Figure 4B and C). These results indicate that mutations in the C-terminal nine amino acids

Fig. 4. The transcriptional activation domain is required for both hormone-dependent transcriptional activation and chromatin disruption. (A) The transcriptional activation of the TRβA promoter by TR mutants. The groups of oocytes were injected with ssDNA of pTRβA (100 ng/ml, 23 nl/oocyte) and with mRNAs encoding RXR and TR or TR mutants (100 ng/ml each, 23 nl/oocyte) and treated with T₃ (50 nM) overnight. Half of the oocytes in each group were used for transcriptional analysis by primer extension and half for chromatin disruption analysis by MNase assay. Note that TRm1, TRm4 and TRm6 failed to activate transcription from the TRβA promoter (compare lanes 6, 8 and 10 with lane 4). The internal control represented the extension product of the endogenous oocyte H4 mRNA using the H4 primer. (B) The mutants impaired in transcriptional activation also failed to disrupt canonical chromatin structure as assayed by MNase. Partial MNase digestions of minichromosomes were carried out with 10 U of MNase in the even lanes and 5 U of MNase in the odd lanes at room temperature for 20 min as described in Materials and methods. The DNAs were purified, resolved with a 1.5% agarose gel, blotted to Nytran Plus membrane and probed with random primer-labeled DNA fragment including the TRE (from +218 to +314). Also indicated on the right are the positions of mono-, di-, tri-, tetra- and penta-nucleosomal DNAs. (C) The DNA topological assay again indicates that the mutants deficient in transcriptional activation are defective in hormone-dependent chromatin disruption. The injection of oocytes was as described above. After overnight treatment with hormone, the groups of oocytes were used for DNA purification and topology assay. The filter is probed with the random primer-labeled probe III (Figure 2A).

of TR can interfere with transcriptional activation and chromatin disruption in a highly selective manner. We next sought to establish whether chromatin disruption was a primary function of the *Xenopus* thyroid hormone receptor, or a secondary effect dependent on the association of other sequence-specific DNA-binding proteins with the TRβA promoter.

Chromatin disruption is targeted by the thyroid hormone receptor

The *Xenopus* TR binds to nucleosomal DNA *in vitro* and *in vivo*, generating local alterations in nucleosome structure detected as DNase I hypersensitivity but without extensive disruption of chromatin in the absence of ligand (Figure 5, Wong *et al.*, 1995). Addition of ligand leads to extensive

hormone-dependent chromatin disruption that is also dependent on a defined transactivation domain (Figures 3 and 4). We wished to determine if the association of other sequence-specific DNA-binding proteins necessary for transcription from the TRβA promoter was a prerequisite for chromatin disruption. We made use of 5' deletion mutants of the TRβA promoter and the internal location of the TRE to determine the role of the TRE and sequences 5' to the TRE in transcriptional activation and chromatin disruption (Figure 6A). Sequence-selective hormone-dependent initiation of transcription required sequences 5' to the TRE itself (Figure 6B, compare lanes 1 and 2 with lanes 5 and 6 and 9–12). Deletion of these sequences 5' to –258 significantly reduces both the efficiency of chromatin disruption (Figure 6C, compare lanes 1 and 2

Fig. 5. The transcriptional activation domain is required for relief of transcriptional repression by TR-RXR, but not for the binding of TR-RXR to chromatin. (A) The TR mutants defective in hormone-dependent transcriptional activation are constitutive transcriptional repressors. The oocyte injections and transcription analysis were the same as described in Figure 4 except that the dsDNA of pTRβA (100 ng/ml, 23 nl/oocyte) was used. (B) The TR mutant defective in hormone-dependent transcriptional activation can still retain high affinity for binding of hormone. The TR and TR mutants, overexpressed in oocytes as described in Figure 3, were used for filter binding assay using [¹²⁵I]T₃ as ligand. The result shown represents the mean value of three independent experiments. (C) The mutants defective in hormone-dependent transcription activation and chromatin disruption can bind to the TRE in chromatin as wild-type receptor and induce the formation of DNase I-hypersensitive sites. The groups of oocytes were injected with or without TR-RXR mRNAs (100 ng/ml each, 27 nl/oocyte) and ssDNA of pTRβA (100 ng/ml, 23 nl/oocyte) and treated with or without T₃ as indicated. After overnight incubation, the groups of oocytes were collected and used for the DNase I sensitivity assay. The DNase I concentrations were 80 (lanes 14, 18, 22 and 26), 40 (lanes 5, 8, 11, 15, 19, 23 and 27), 20 (lanes 6, 9, 12, 16, 20, 24 and 28) and 10 U (lanes 7, 10, 13, 17, 21, 25 and 29). The naked plasmid controls were treated with a 200-fold smaller amount of DNase I. Also indicated are the positions of *Eco*RI sites and the TRE. The probe used is probe III (Figure 2). The asterisk represents the position of the increase of DNase I cleavage in response to hormone-bound receptor 5' to the TRE (lanes 11-13).

with 5 and 6) and transcriptional activation (Figure 6B, compare lanes 1 and 2 with 7 and 8). A contributory factor to these changes is the elimination of a second TRE at -785 relative to the start site of transcription (+1) (Machuca *et al.*, 1995; Wong *et al.*, 1995). The binding of the TR-RXR to this site might contribute some specificity to the DNase I cleavage sites seen in Figure 5C upstream from the start site of transcription. Further deletion of the region from -258 to +264 has no major effect on the efficiency of chromatin disruption (Figure 6C, compare lanes 7 and 8 with lanes 5 and 6 and 9-12), whereas these sequences are essential for transcription (Figure 6B, compare lanes 7 and 8 with lanes 5 and 6 and 9-12). Thus the efficiency of chromatin disruption

appears independent of high levels of specific transcriptional activation. Removal of the TRE, together with sequences 5' including the transcription start site, leads to the loss of both sequence-selective hormone-dependent transcriptional activation and chromatin disruption (Figure 6B and C, lanes 3 and 4). Thus the TRE alone is sufficient to confer hormone-dependent chromatin disruption in the presence of TR.

Our results further suggest that chromatin disruption *per se* is not sufficient for transcriptional activation and that while the TR alone is sufficient to target the chromatin disruption process (Figure 6C, lanes 5 and 6), it is not sufficient to target the accurate initiation of transcription by RNA polymerase II (Figure 6B, lanes 5 and 6).

Fig. 6. TRE is essential and sufficient for instigating chromatin disruption but not transcription activation by liganded TR-RXR. (A) The diagrams illustrate the differences among the different constructs. (B) The TRE, initiator and an upstream element are all required for the transcriptional activation of TR β A promoter by liganded TR-RXR. The groups of oocytes were injected with or without TR-RXR mRNAs (50 ng/ml each, 27 nl/oocyte) and ssDNA constructs (100 ng/ml, 23 nl/oocyte) as indicated. After incubation with hormone overnight, the groups of oocytes were collected for RNA purification. The transcription from the constructs was then analyzed by primer extension. (C) The TRE alone is sufficient to target chromatin disruption by liganded TR-RXR. Injection and treatment of the oocytes were performed as above. DNAs were purified from each group of injected oocytes and analyzed for topology changes using chloroquine agarose gel. DNAs were then transferred to the Nytran Plus membrane and probed with random primer-labeled probe III (Figure 2).

Chromatin disruption is not sufficient for transcriptional activation

Our experiments demonstrate that the TR-RXR in the presence of T₃ can direct chromatin disruption indicated by the loss of approximately three negative superhelical turns from a single TRE (Figure 6C, lanes 5 and 6). In contrast, the wild-type promoter containing two TREs (Ranjan *et al.*, 1994; Machuca *et al.*, 1995; Wong *et al.*, 1995) directs the disruption of approximately six nucleosomes (Figure 1C, lanes 5 and 6; Figure 6C, lanes 1 and 2). We next examined whether chromatin disruption directed by hormone-bound receptor would depend simply on the number of TREs or whether a spatial separation of

these regulatory elements was also required. We also explored the role of the TRE as an enhancer of transcription by examining whether transcriptional stimulation was independent of the distance of the TRE from the promoter and how this might influence both chromatin disruption and transcriptional activation.

The constructs employed in these experiments use a single TRE 1132 bp downstream of the start site of transcription (Figure 7A) or four clustered TREs at this position separated individually by 26 bp. Control gel-shift experiments indicate that this spacing is sufficient for four TR-RXR complexes to bind (data not shown). We also generated templates with two, three and four TREs separ-

Fig. 7. TREs at separate locations but not the multiple TREs clustered at a single location increase the chromatin disruption instigated by liganded TR-RXR. (A) The diagram illustrates the number of TREs and their relative positions in each construct. (B) The increasing number of dispersed TREs leads to increasing levels of chromatin disruption in the presence of TR-RXR and hormone. The groups of oocytes were injected with TR-RXR mRNAs (50 ng/ml each, 27 nl/oocyte) and the ssDNA constructs (100 ng/ml, 23 nl/oocytes) and then treated with or without hormone overnight as indicated. The oocytes were then collected for purification of DNA for supercoiling assay. After transfer to Nytran Plus membrane, DNAs were probed with random primer-labeled probe III (Figure 2). Also shown on the right are the scans of the corresponding supercoiling assay data by phosphorimaging (Molecular Dynamics). (C) The increasing levels of chromatin disruption do not correlate with increasing levels of transcriptional activation. The groups of oocytes were injected without (lanes 1, 4, 7, 10 and 13) or with TR-RXR mRNAs of 10 ng/ml (lanes 2, 5, 8, 11 and 14) or 100 ng/ml (lanes 3, 6, 9, 12 and 15) and the ssDNA constructs as indicated. Each group of oocytes was treated with hormone overnight and then collected for purification of RNA for primer extension analysis.

ated by distances of 548, 274 and 578 bp, as indicated (Figure 7A). We find that one or four clustered TREs at the same site 1132 bp downstream of the transcription start site give very similar extents of hormone-dependent chromatin disruption (Figure 7B, lanes 1–4). Densitometric scanning suggests a disruption, in both instances corres-

ponding to the loss of three negative superhelical turns of topological constraint. In contrast, the separation of TREs over hundreds of base pairs leads to a progressive increase in hormone-dependent chromatin disruption (Figure 7B, lanes 5–10). This suggests that the chromatin disruption is centered on each TRE and is transmissible *in cis* for a limited but undefined range. Surprisingly, although both a single and four clustered TREs 1132 bp downstream of the transcription start site disrupt chromatin to equivalent extents (Figure 7B, compare lanes 1 and 2 with 3 and 4), there are very different consequences for transcriptional activation. A single TRE cannot activate transcription at this position (Figure 7C, lanes 1–3), whereas four clustered TREs activate transcription (Figure 7C, lanes 4–6). Placement of multiple dispersed TREs closer to the start site of transcription activates transcription as efficiently as the four clustered TREs at 1132 bp downstream (Figure 7C, compare lanes 6, 9, 12 and 15). However, increasing levels of chromatin disruption do not correlate with progressive increases in transcriptional efficiency (Figure 7C). We suggest that not only is chromatin disruption regulated independently by the TRE but that a localized region of chromatin disruption itself at this distal site is insufficient for transcriptional activation (Figure 7B, lanes 1–4; Figure 7C, lanes 1–6).

We next examined the distance-dependent effects of single or multiple TREs from the start site of transcription on both chromatin disruption and transcriptional activation (Figure 8A). Single or multiple clustered TREs are able to direct hormone-dependent chromatin disruption to equivalent extents, independently of their position relative to the transcription start site (Figure 8B). In contrast, a single TRE is able to direct transcriptional activation 264 and 584 bp downstream from the start site, but not 1132 bp downstream (Figure 8C, compare lanes 7, 8, 11 and 12 with 15 and 16). These results again suggest that hormone-dependent chromatin disruption does not require transcriptional activation and that hormone-dependent chromatin disruption alone at a distal site is insufficient for transcription.

Discussion

The major conclusions from this work are: (i) that the TR bound to its recognition site within chromatin is sufficient to instigate the disruption of nucleosomal arrays dependent on both a defined transactivation domain and the addition of T₃ (Figures 1–6); and (ii) that hormone-dependent chromatin disruption itself on a mini-chromosome is insufficient for transcriptional activation (Figures 6–8). Our results indicate that although chromatin disruption is linked tightly to transcriptional activation (Figure 4), it is possible to separate these two processes *in vivo* (Figures 6–8). Although chromatin disruption is an independent hormone-regulated function of the thyroid hormone receptor, additional activities of the receptor are required for transcriptional activation.

Silencing, relief of silencing, transcriptional activation in chromatin and chromatin disruption by the thyroid hormone receptor

Minichromosomes assembled on microinjected double-stranded DNA have high levels of basal transcription that

Fig. 8. Chromatin disruption is not sufficient for transcriptional activation. (A) The diagram indicates the position and number of TREs in each construct. In TRpm(P) and all of its derivative constructs, the TRE was mutated and no longer can bind to TR-RXR (Wong *et al.*, 1995). (B) The supercoiling assay indicates that a single TRE or multiple TREs clustered at a single site at different locations mediate the chromatin disruption induced by liganded TR-RXR to similar extents. The groups of oocytes were injected with TR-RXR mRNAs (100 ng/ml each, 27 nl/oocyte) and the ssDNA constructs (100 ng/ml, 23 nl/oocytes) and then treated with or without hormone overnight as indicated. The oocytes were then collected for purification of DNA for the supercoiling assay. After transfer to Nytran Plus membrane, DNAs were probed with random primer-labeled probe III (Figure 2). (C) Four TREs, but not one TRE >1.1 kb downstream of the start site, are able to activate the transcription of TRβA promoter by liganded TR-RXR. The groups of oocytes were injected without (lanes 1–6) or with TR-RXR mRNAs of 10 ng/ml (lanes 7, 9, 11, 13, 15 and 17) or 100 ng/ml (lanes 8, 10, 12, 14, 16 and 18) and the ssDNA constructs as indicated. Each group of oocytes was treated with hormone overnight and then collected for purification of RNA for primer extension analysis.

are silenced efficiently upon the expression of the receptor in the absence of T₃ (Figure 5A). All of the mutant TR proteins used in our experiments silence basal transcription. Some of these mutant TRs will not relieve silencing on addition of T₃ (Figure 5A, lanes 3–11). The nature of these mutations confirms earlier work (Baniahmad *et al.*,

1995a), which demonstrated that deletion of the conserved nine amino acids PLFLEVFED significantly reduces the relief of silencing (TRm1), as does point mutation of this sequence at the glutamic acid residue at position five (TRm4). The point mutation (TRm6) which alters the first proline to serine adds another TR variant deficient in relief of silencing (Figure 5A). Importantly, these mutant forms of the TR do not activate transcription within a repressive chromatin environment assembled on a replicating template (Figure 4, lanes 5–11). For two of these mutants, control experiments indicate that either a deficiency in hormone binding (TRm1 and TRm6) or a deficiency in binding to nucleosomal DNA (TRm1) might interfere with hormone-induced functions of the receptor in chromatin. This failure to bind nucleosomal DNA might account for the relative inefficiency of TRm1 in silencing transcription in chromatin (Figure 4A, compare lanes 1 and 5). Importantly, one mutant, TRm4, retains the capacity to bind both hormone (Figure 5B) and nucleosomal DNA (Figure 5C). However, TRm4 remains deficient in disrupting chromatin and in activating transcription (Figure 4). Thus the C-terminal transactivation domain previously defined (Zenke *et al.*, 1990; Baretino *et al.*, 1994; Baniahmad *et al.*, 1995a) has an essential novel role both in transcriptional activation within a chromatin environment and in targeting chromatin disruption.

The rat TR has been crystallized recently and its structure in the presence of ligand resolved (Wagner *et al.*, 1995). The C-terminal activation domain forms an amphipathic helix, with the hydrophobic face actually constituting part of the hormone-binding cavity. The potential structural role for the ligand provides an attractive explanation for the allosteric transition in the TR that enables the protein to activate transcription (Wagner *et al.*, 1995). Whatever contacts that are made by this domain with other proteins, such as co-repressors or co-activators, must control transcription in a chromatin environment. In contrast to the clear role of the C-terminal domain of the TR in gene regulation, the role of the hinge (D) region in silencing or activation (Lee and Mahdavi, 1993; Uppaluri and Towle, 1995) is not revealed in our assay system. We use microinjection of *Xenopus* oocytes as an assay system (Almouzni and Wolffe, 1993); the earlier work indicative of a regulatory role for the D region made use of transfected mammalian cells (Lee and Mahdavi, 1993) or yeast (Uppaluri and Towle, 1995). It is possible that the *Xenopus* oocyte lacks appropriate co-activators or co-repressors present in these other cells, or that chromatin assembly in our system introduces constraints not apparent in the earlier assays.

We conclude that microinjection of the *Xenopus* TRβA promoter into *Xenopus* oocyte nuclei allows confirmation of earlier functions attributed to the C-terminal transactivation domain of the receptor (Baretino *et al.*, 1994; Baniahmad *et al.*, 1995a; Wagner *et al.*, 1995) together with the attribution of a new function—the hormone-dependent disruption of chromatin.

Chromatin disruption instigated by the thyroid hormone receptor

Chromatin disruption is tightly correlated with the inducible transcription of many genes (Burch and Weintraub, 1983; Zaret and Yamamoto, 1984; Elgin,

1988; Gross and Garrard, 1988). Genetic experiments in *Saccharomyces cerevisiae* indicate an essential role for histone–DNA contacts in repressing basal transcription and demonstrate that relief of this repression is a major component of transcriptional activation (Han and Grunstein, 1988; Han *et al.*, 1988; Kruger *et al.*, 1995). For example, disruption of histone–DNA contacts through reduction in histone gene copy number activates *PHO5* transcription independently of an inductive signal (Han *et al.*, 1988). During normal induction of *PHO5*, the action of a transcriptional activator PHO4p on an organized chromatin structure (Straka and Horz, 1991; Fascher *et al.*, 1993; Svaren *et al.*, 1994) is required to disrupt nucleosomal arrays (Almer and Horz, 1986; Almer *et al.*, 1986). Recent evidence suggests that a co-repressor RPD3p that probably functions as a histone deacetylase also contributes to *PHO5* gene regulation (Vidal and Gaber, 1991; Taunton *et al.*, 1996; Wolffe, 1996). In metazoans, experiments using the mouse mammary tumor virus long terminal repeat paradigm have shown that the ligand-bound glucocorticoid receptor disrupts chromatin (Zaret and Yamamoto, 1984). In this case, the chromatin consists of a phased nucleosomal array (Richard-Foy and Hager, 1987). Positioned nucleosomes facilitate the association of ligand-bound glucocorticoid receptor (Perlmann and Wrangé, 1988; Pina *et al.*, 1990). Once bound to chromatin, the glucocorticoid receptor initiates a process leading to chromatin disruption and the assembly of a functional pre-initiation complex (Archer *et al.*, 1989, 1992; Lee and Archer, 1994). Thus, for these inducible systems, chromatin disruption is linked intimately to transcriptional activation. TR differs from these systems in that it binds to a phased nucleosomal array *in vivo* in the absence of ligand, but fails to activate transcription (Wong *et al.*, 1995). We find that the association of unliganded receptor with nucleosomal DNA is sufficient to generate a DNase I-hypersensitive site (Figure 5C) indicative of local disruption of histone–DNA contacts; however, chromatin structure, as indicated by a defined nucleosomal array or topological constraint, is not disrupted under these conditions (Figures 1C and 2B and C). Only on the addition of ligand does a major topological change (Figure 1C) occur, coupled to an extensive loss of definition in MNase cleavage (Figure 2). These results indicate that chromatin remodeling at the TR β A promoter is a two-step phenomenon. The first step represents the generation of a DNase I-hypersensitive site dependent on unliganded receptor, but hormone-dependent chromatin disruption involves the disruption of nucleosomes flanking the TRE over several hundred base pairs (Figure 2). The more extensive disruption can also be detected by DNase I (Figure 4C, lanes 11–13, indicated by the asterisk).

We have shown that the TR alone and its recognition element are sufficient to initiate a process of chromatin disruption over an extended segment of DNA sequence (Figures 2, 4, 6 and 7). The disruption of chromatin from the wild-type promoter containing two TREs is equivalent to the loss of the topological constraint found in six nucleosomes (Figures 2 and 4). Transcription is not required for chromatin disruption (Figure 2), and additional proximal promoter elements are necessary to facilitate hormone-dependent activation of transcription by the TR (Figure 6). The hormone-dependent chromatin disruption

obtained in our experiments does not depend on DNA binding of the transactivator alone, but is also dependent on a ligand-binding transactivation domain (Figure 4). Thus we can dissect the process of TR β A gene activation into three steps: binding of the receptor to chromatin in the absence of ligand (Figure 5C), disruption of chromatin on addition of ligand (Figures 2 and 4B and C), and transcriptional activation (Figures 1, 4, 5, 7 and 8).

Chromatin disruption is insufficient for transcriptional activation

Our results with single versus clustered multiple TREs indicate that chromatin disruption is insufficient for complete transcriptional activation. A single TRE at a distance of 1132 bp from the transcription start site disrupts chromatin to the same extent as four clustered TREs at the same position (Figures 7 and 8). Nevertheless, the single TRE does not activate transcription efficiently, whereas the four TREs can. There are two possible explanations for these results: (i) that chromatin disruption is an epiphenomenon that is not required for transcriptional activation, or (ii) that chromatin disruption might be permissive for transcriptional activation from the TR β A promoter but that additional interactions between the TR–RXR and the transcriptional machinery are necessary to activate transcription. We favor the latter possibility.

Chromatin disruption leads to major topological changes in minichromosomes (Figures 1, 4, 6, 7 and 8), consistent with a loss of wrapping of DNA around the histones (Germond *et al.*, 1975; Bauer *et al.*, 1994). Although histone acetylation is associated with targeted co-activators (Brownell *et al.*, 1996; Wolffe and Pruss, 1996), including the p300 protein involved in nuclear receptor signaling (Chakravarti *et al.*, 1996; Ogryzko *et al.*, 1996), it is difficult to account for the large topological change observed in our experiments through acetylation alone (Norton *et al.*, 1989; Bauer *et al.*, 1994). Moreover, histone acetylation alone is insufficient to relieve transcriptional repression without the removal of histone H1 (Ura *et al.*, 1997). The removal of histone H1 from chromatin is associated with gene activation (Bresnick *et al.*, 1992). Histone H1 deficiency increases nucleosome mobility (Pennings *et al.*, 1994; Ura *et al.*, 1995) and thereby facilitates transcription (Ura *et al.*, 1995; Varga-Weisz *et al.*, 1995). However, the topological change following from the removal of histone H1 is unlikely to be large (Hamiche *et al.*, 1996). The most likely explanation for the observed topological change is an unfolding of the nucleosome, reflecting a disruption of core histone interactions with DNA and with each other. Such changes might reflect the loss of DNA wrapping observed when mononucleosomes are exposed to large excesses of the SWI–SNF general activator complex (Côté *et al.*, 1994; Imbalzano *et al.*, 1994). These changes clearly can disperse over an extensive region of chromatin (Figure 2) and need not be localized to the complete disruption of nucleosomes immediately adjacent to the TRE. Future experiments will attempt to define the nature of this chromatin reorganization and its enzymatic basis.

Materials and methods

Plasmid constructs

pSP64(polyA)-xTR β A and -xRXR α constructs have been described before (Wong *et al.*, 1995). All TR β A mutants were generated using a

Table I. List of primers used for generating TR mutants^a

TRβ1	CAACGGTCGACATGGAAGGTATATACCC
TRβ2	CATTTGGATCCATGTCTATTTAGAACG
TRm1	CTTCGGATCCTAGGGAACAGTTCAGTGG
TRm2	GTGCGGATCCTAGTCCGCAAAACACTTCCAAGAA
TRm4	GTGCGGATCCTAGTCCCTCAAACACTTTCA
TRm6	GTGCGGATCCTAGTCCCTCAAACACTTCCAAGAACAGTGAG
TRm5	GTGCGGATCCTAGTCCCTCAGGCACTTCCAA
TRm7	GCAAAAAGAAAGCTCACAAGAAAACAGAG
TRm8	CTCTGTTTCTTCTGTGAGCTTCTTTTTC
TRm9	CTCATAGAAGAAAACACGAAAAAGACGGAAA
TRm10	TTTCCGTCTTTTTCGTGTTTCTTCTATGAG

^aThe mutated residues are underlined.

PCR method based on pSP64(polyA)-xTRβA. In brief, TRβm1–TRβm6 were constructed by replacing the *Afl*III–*Bam*HI fragment of the pSP64(polyA)-xTRβA with the *Afl*III–*Bam*HI fragments of the PCR products generated with a TRβ1 primer and a corresponding mutant primer, as listed in Table I. To make TRβm7 and TRβm9, pSP64(polyA)-xTRβA was first amplified with a pair of primers, TRβ1 and TRβm7, and another pair of primers, TRβm8 and TRβ2, respectively. Two PCR products were gel purified, mixed and then PCR amplified with TRβ1 and TRβ2 primers. The full-length PCR product was gel purified and digested with *Sa*II and *Afl*III. The resulting *Sa*II–*Afl*III fragment was used to replace the corresponding fragment in the wild-type pSP64-(polyA)-xTRβA construct to generate pSP64(polyA)-TRβm7. The pSP64(polyA)-TRβm9 was generated in the same way as pSP64(polyA)-TRβm7 except that TRβm9 and TRβm10 primers were used. The PCR fragment in each construct was verified by DNA sequencing.

The pTRβA construct which contains 1.6 kb of TRβA promoter and 0.3 kb of CAT gene sequence in pBluescript II KS(+) has been described before (Wong *et al.*, 1995). The TRp(PstI) construct was generated from pTRβA by deleting all *Pst*I fragments from the TRβA promoter. TRpm2, TRpm3, TRp4(–TRE) and TRp5(+TRE) plasmids were all constructed by PCR based on the TRp(PstI) construct. TRpm1 plasmid containing a 5 bp deletion (GGGGG), which included the transcriptional start site (underlined) in the initiation region, was generated by site-directed mutagenesis based on the TRp(PstI) using an Amersham kit as described by the manufacturer. The TRpm(P) construct has been described before (Wong *et al.*, 1995). The plasmids TRpm(P)–1.TRE and TRpm(P)–4.TRE were generated respectively by inserting one and four TREs (5' AGCTTGCAAGGTCATTTTCAGGACAGCA 3') into the *Hind*III site of the TRpm(P) construct. To generate the plasmids TRpm(PE)–1.TRE and TRpm(PE)–4.TRE, a 548 bp *Eco*RI fragment from the *Xenopus* RXRγ gene was cloned into the *Eco*RI site of the plasmids TRpm(P)–1.TRE and TRpm(P)–4.TRE and the clones with the insert in the same orientation were selected. This 548 bp *Eco*RI fragment contains no TRE site based on the sequence search. To generate the plasmid TRpm(PE)–TRE(E), the plasmid TRpm(PE)–1.TRE was partially digested with *Eco*RI and then ligated with the TRE duplex with *Eco*RI sites at both ends. The clone with a single TRE (5' AATTCAGGTCATCCTAGGTCAG 3') inserted into the *Eco*RI site between the CAT fragment and the inserted 548 bp fragment was selected. The plasmid TRpm(PE)–TRE(E)(B) was a derivative of TRpm(PE)–TRE(E) with a single TRE (5' GATCGAGGTCATCCTAGG TCAC 3') inserted into the *Bgl*II site. The plasmid TRpm(PE)–TRE(E)(B)(B) was then generated by inserting a single TRE (5' GATCGAGGTCATCCTAGGTCAG 3') into the *Bam*HI site of the plasmid TRpm(PE)–TRE(E)(B). The single-stranded DNAs were prepared from phagemids induced with helper phage VCS M13 as described (Sambrook *et al.*, 1989).

In vitro mRNA synthesis and microinjection of *Xenopus* oocytes

All the constructs for *in vitro* transcription were linearized with *Eco*RI, deproteinized with phenol/chloroform and ethanol precipitated. The *in vitro* mRNA synthesis was carried out with an SP6 Message Machine Kit (Ambion) as described by the manufacturer. The preparation and microinjection of *Xenopus* stage VI oocytes were essentially as described (Almouzni and Wolffe, 1993). For transcription and chromatin disruption analysis, a group of ~20 oocytes were injected for each sample to minimize the variations of injection, and the mRNAs were usually injected 2–3 h before injection of DNA. The DNA was injected (23 nl/oocyte) either as single- (100 ng/μl) or double-stranded DNA (100 ng/μl) into the nuclei of the oocytes. The TR mRNAs were usually

mixed with an equal concentration of RXRα mRNA to give a final concentration of 100 ng/ml for each and injected into the cytoplasm of the oocyte at a volume of 27.6 nl per oocyte, except in Figure 1 in which a series of 3-fold dilutions (from 100 ng/μl up to 81-fold dilution) of TR–RXR were used to examine the dosage effect of TR–RXR. The injected oocytes were incubated at 18°C overnight in MBSh buffer (Peng, 1991) with 50 U/ml of ampicillin and streptomycin in the presence (+) or absence (–) of 50 nM of T3 as indicated.

Gel retardation assay and Western blotting analysis

The gel retardation assay and Western blotting analysis of TR–RXR expressed in oocytes injected with TR or TR–RXR mRNAs have been described elsewhere (Wong and Shi, 1995).

Ligand-binding assay

The assay for the binding of T₃ to the receptor and the receptor mutants utilizing [¹²⁵I]T₃ was performed essentially as described (Eliceiri and Brown, 1994), except that the *Xenopus* oocyte-expressed receptors were used.

Transcription analysis and DNA analysis

Preparation of RNA from injected oocytes and transcription analysis by primer extension were performed as described (Wong *et al.*, 1995). In brief, 20 healthy oocytes for each sample were collected after overnight incubation, rinsed once with MBSh buffer and homogenized in 200 μl of 0.1 M Tris (pH 8.0) and 20 mM EDTA (pH 8.0). The homogenates were then divided into two halves, one for RNA purification and another for DNA purification. For RNA purification, 500 μl of RNazol™ reagent together with 50 μl of chloroform were added to each sample. The samples were vortexed vigorously and incubated on ice for 15 min before centrifugation at top speed in a bench top centrifuge. The clean supernatants (350 μl) were transferred to new tubes and RNAs were precipitated with 0.7 volume of isopropanol. After rinsing with 70% ethanol, RNAs were resuspended in DEPC-treated water and prepared for primer extension analysis using the primer I (ATCCTTATAAA–CGGTGAGTAGTGATGTCATCAG) as described (Wong *et al.*, 1995). The internal control is the primer extension product of the endogenous histone H4 mRNA using primer H4 (GGCTTGGTGATGCCCTGG–ATGTTATCC).

To recover DNA for either quantifying the amount of injected DNA in different samples or examining the supercoiling status of the DNA, 200 μl of 1% SDS and 50 mM EDTA were immediately added to the remaining half of the samples. The samples were treated with RNase A (100 mg/ml) at 37°C for 2 h, followed by treatment with proteinase K (200 mg/ml) for 2–3 h at 55°C. After addition of 30 μl of 3 M NaOAc, pH 5.4, the samples were phenol/chloroform extracted twice, precipitated with 0.7 volume of isopropanol and rinsed with cold 70% ethanol. DNAs were resuspended in 50 μl of TE buffer and used either for quantitative analysis by slot hybridization as described (Wong *et al.*, 1995) or supercoiling assay as described below.

Micrococcal nuclease assay and supercoiling assay of chromatin disruption

The MNase assay of chromatin structure was performed as described previously (Wong *et al.*, 1995). Briefly, groups of 20–25 injected oocytes were collected after overnight incubation and homogenized in 300 μl of MNase buffer [10 mM HEPES, pH 8.0, 50 mM KCl, 5 mM MgCl₂, 3 mM CaCl₂, 1 mM dithiothreitol (DTT), 0.1% NP-40 and 5% glycerol]. The extract was either divided into four fractions (60 μl each) and digested with 10, 5, 2.5 and 1.25 U/ml of MNase (Worthington) respectively at room temperature for 20 min. MNase digestions were stopped by addition of 200 μl of 20 mM EGTA, 1% SDS. The reactions were treated with RNase A (100 μg/ml) at 37°C for 2 h, followed by treatment with proteinase K (200 μg/ml) for 2–3 h at 55°C. After addition of 30 μl of 3 M NaOAc, pH 5.4, the reactions were phenol/chloroform extracted twice, precipitated with 0.7 volume of isopropanol and rinsed with cold 70% ethanol. DNAs were resolved by 1.5% agarose gel, blotted to nylon membrane and probed with the indicated labeled DNA fragments.

The supercoiling assay using chloroquine agarose gel was performed essentially as described (Clark and Wolffe, 1991). To analyze DNA topology, 10 μl of each DNA samples were loaded onto a 1–1.2% agarose gel in 1× TPE (40 mM Tris, 30 mM NaH₂PO₄ and 10 mM EDTA) containing 90 μg/ml of freshly prepared chloroquine in both gel and running buffer. Under these gel conditions, the minichromosomes with nucleosome density from 180 to 270 bp/nucleosome can be resolved optimally into isomers with positive supercoiling due to the binding of

chloroquine to DNA, dependent on the differences in negative supercoiling which resulted from the difference in nucleosome density. The gels were run in the dark overnight at ~3.5 V/cm. The gels were then washed with distilled water several times to remove chloroquine and processed for DNA transfer to Nytran Plus membrane as described (Sambrook *et al.*, 1989). The DNA was probed with a random-primed labeled DNA fragment from +218 to +314 of the TR β A promoter.

DNase I-hypersensitive site analysis

Briefly, groups of 25–30 injected oocytes were collected after overnight incubation and homogenized in 400 μ l of MNase buffer (10 mM HEPES, pH 8.0, 50 mM KCl, 5 mM MgCl₂, 1 mM DTT, 0.1% NP-40 and 5% glycerol). The extract was divided into four fractions (100 μ l each) and digested with 80, 40, 20 and 10 U of DNase I (BRL) respectively at room temperature for 1 min. The reactions were stopped with addition of 200 μ l of 1% SDS and 20 mM EDTA. After the purification procedure as described for the MNase assay, each DNA sample was resuspended in 100 μ l of TE buffer and treated again with RNase A (100 μ g/ml) for 1 h, followed by phenol/chloroform extraction and isopropanol precipitation to further clean the DNA samples. The DNA samples were then resuspended in 50 μ l of 1 $\times$ EcoRI buffer (BRL) and digested with 15 U of EcoRI (BRL) for 2 h. The digested DNA samples were resolved by a 1.2% agarose gel, blotted to Nytran Plus membrane and probed with a random primer-labeled CAT fragment. The naked DNA controls were treated with 200-fold less DNase I than used for chromatin samples.

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