Distinct requirements for chromatin assembly in transcriptional repression by thyroid hormone receptor and histone deacetylase

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Histone deacetylase and chromatin assembly contribute to the control of transcription of the *Xenopus* TR β A gene promoter by the heterodimer of *Xenopus* thyroid hormone receptor and 9-*cis* retinoic acid receptor (TR-RXR). Addition of the histone deacetylase inhibitor Trichostatin A (TSA) relieves repression of transcription due to chromatin assembly following microinjection of templates into *Xenopus* oocyte nuclei, and eliminates regulation of transcription by TR-RXR. Expression of *Xenopus* RPD3p, the catalytic subunit of histone deacetylase, represses the TR β A promoter, but only after efficient assembly of the template into nucleosomes. In contrast, the unliganded TR-RXR represses templates only partially assembled into nucleosomes; addition of TSA also relieves this transcriptional repression. This result indicates the distinct requirements for chromatin assembly in mediating transcriptional repression by the deacetylase alone, compared with those needed in the presence of unliganded TR-RXR. In addition, whereas hormone-bound TR-RXR targets chromatin disruption as assayed through changes in minichromosome topology and loss of a regular nucleosomal ladder on micrococcal nuclease digestion, addition of TSA relieves transcriptional repression but does not disrupt chromatin. Thus, TR-RXR can facilitate transcriptional repression in the absence of hormone through mechanisms in addition to recruitment of deacetylase, and disrupts chromatin structure through mechanisms in addition to the inhibition or release of deacetylase.

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Introduction

Recent evidence suggests that the thyroid hormone receptor regulates transcription through interaction with a variety of macromolecular complexes. These include heterodimeric partners (Glass *et al.*, 1989; Leblanc and Stunnenberg, 1995; Li *et al.*, 1997), the basal transcrip-

tional machinery (Baniahmad *et al.*, 1993; Fondell *et al.*, 1993), transcriptional intermediary factors (TIFs)/coactivators (Le Douarin *et al.*, 1995; Fondell *et al.*, 1996; Kamei *et al.*, 1996; Voegel *et al.*, 1996) and corepressors (Chen and Evans, 1995; Horlein *et al.*, 1995; Chen *et al.*, 1996). Transcriptional coactivators include p300 and associated proteins such as PCAF which possess histone acetyltransferase activity (Onate *et al.*, 1995; Chakravarti *et al.*, 1996; Kamei *et al.*, 1996; Ogryzko *et al.*, 1996; X.J. Yang *et al.*, 1996). Transcriptional corepressors include NCoR (Horlein *et al.*, 1995) and SMRT (Chen and Evans, 1995) and associated proteins such as SIN3 and histone deacetylase (Alland *et al.*, 1997; Heinzel *et al.*, 1997; Nagy *et al.*, 1997). The observation that transcriptional activation is associated with recruitment of histone acetyltransferase and repression with recruitment of histone deacetylase leads to a simple model where the modification of chromatin structure targeted by the thyroid hormone receptor might contribute to transcriptional control (Wolffe, 1997).

Nucleosome assembly has a role in both the silencing and activation of transcription mediated by the heterodimer of the thyroid hormone receptor and 9-*cis* retinoic acid receptor (TR-RXR) (Wong *et al.*, 1995, 1997a,b). The AF2/ τ 4 activation domain of TR-RXR (Baretino *et al.*, 1994; Baniahmad *et al.*, 1995) is required both for extensive chromatin disruption and for transcriptional activation (Wong *et al.*, 1997a). However, chromatin disruption can be distinguished as an independently regulated event, because it can be targeted by chromatin-bound TR-RXR on addition of hormone, without a requirement for subsequent transcriptional activation (Wong *et al.*, 1997a). Therefore chromatin disruption may be necessary to prepare the chromatin template for transcription by relieving repressive histone–DNA interactions, before the receptor can influence the activity of the general transcriptional machinery (Brownell *et al.*, 1996; Wolffe and Pruss, 1996a).

The molecular mechanisms by which TR-RXR might influence the organization of promoters within chromatin to facilitate transcriptional repression have not been defined. The targeted deacetylation of core histones represents one candidate mechanism for chromatin modification because histone deacetylase is present within the corepressor complex recruited by the unliganded receptor (Alland *et al.*, 1997; Heinzel *et al.*, 1997; Nagy *et al.*, 1997). However, it is possible that components of the corepressor complex such as NCoR, SMRT or SIN3 might exert repressive activities on components of the basal machinery or organize repressive chromatin structures directly without the requirement for histone deacetylase (for example, Le Douarin *et al.*, 1995). Potential mechanisms for the relief of transcriptional repression on addition of hormone would either be to release the histone deacetylase from the complex or to recruit histone acetyl-

transferase activities present in the transcriptional coactivators (Kamei *et al.*, 1996; Ogryzko *et al.*, 1996; X.J. Yang *et al.*, 1996). Other molecular machines capable of disrupting chromatin might also have a role in facilitating the transcription of nucleosomal templates (Yoshinaga *et al.*, 1992; Chiba *et al.*, 1994).

An important indicator of the molecular mechanisms involved in the disruption of chromatin is the change in DNA topology within the minichromosomal template on the addition of hormone to chromatin-bound TR-RXR. Hyperacetylation of the core histones alone has only minor consequences for the topological constraint of DNA within nucleosomes (Norton *et al.*, 1989; Bauer *et al.*, 1994). The TR-RXR-mediated targeted disruption of chromatin (Wong *et al.*, 1997a) leads to a much more severe loss of topological constraint than would be anticipated from histone acetylation alone (Norton *et al.*, 1989; Lutter *et al.*, 1992). This would indicate that histone acetylation might not be involved in the disruption process or might just be one component of the overall chromatin rearrangement (Wong *et al.*, 1997a). Nevertheless, histone acetylation does facilitate transcription factor access to nucleosomal DNA (Lee *et al.*, 1993; Vetesse-Dadey *et al.*, 1996), and does facilitate the transcription of chromatin templates (Ura *et al.*, 1997). In *Xenopus*, inhibition of histone deacetylase using the specific inhibitor Trichostatin A (TSA; Yoshida *et al.*, 1990, 1995) induces hyperacetylation of the core histones and specific gene activation (Almouzni *et al.*, 1994). Therefore the *Xenopus* oocyte system in which substantial changes in chromatin structure and gene activity mediated by TR/RXR can be obtained on minichromosomal templates, provides a useful system to explore the role of histone deacetylase and chromatin assembly in TR/RXR function.

In this work we demonstrate that histone deacetylase represses transcription of the *Xenopus* TR β A gene dependent on the assembly of a nucleosomal template and that this repression can be overcome either by hormone-bound TR-RXR or by inhibition of the histone deacetylase using TSA. However, there are distinct requirements for chromatin assembly in the establishment of transcriptional repression by the histone deacetylase alone compared with those needed in the presence of unliganded thyroid hormone receptor. The receptor can direct the repression of transcription under conditions where low levels of chromatin assembly have occurred on the template, while the deacetylase alone requires much higher levels of chromatin assembly to efficiently repress transcription. There are also remarkable differences in the disruption of repressive chromatin structures following the addition of hormone-bound TR-RXR compared with the addition of TSA. Whereas hormone-bound TR-RXR activates transcription with extensive chromatin disruption as assayed by DNA topological change and micrococcal nuclease digestion, addition of TSA leads to the same level of transcription without extensive chromatin disruption. Our results strengthen the link between the requirement for deacetylase activity on chromatin templates and transcriptional repression; however, they also indicate that alternate and/or auxiliary mechanisms operate to facilitate repression. In addition, since hormone-bound TR-RXR can induce changes in regulatory chromatin structures that far exceed those necessary for transcriptional activation

through the inhibition of deacetylase alone in a minichromosome, we suggest that the receptor has additional functions relevant to chromatin and chromosome structure beyond the control of histone acetylation.

Results

Trichostatin A relieves transcriptional repression due to replication-coupled chromatin assembly and eliminates transcriptional regulation by TR-RXR

Microinjection of single-stranded DNA templates into the nuclei of *Xenopus* oocytes leads to complementary strand synthesis and the rapid assembly of newly synthesized DNA into nucleosomes within 2 h (Almouzni and Wolffe, 1993). Comparable events can be recapitulated *in vitro* (Almouzni *et al.*, 1990a,b, 1991). This replication-coupled chromatin assembly leads to the repression of transcription *in vivo* and *in vitro* (Almouzni *et al.*, 1990b, 1991; Almouzni and Wolffe, 1993; Landsberger and Wolffe, 1995; Landsberger *et al.*, 1995). The histone H4 incorporated into newly synthesized chromatin is deacetylated (Ruiz-Carillo *et al.*, 1975; Shimamura and Worcel, 1989; Dimitrov *et al.*, 1993, 1994; Sobel *et al.*, 1995). Histone H4 is progressively deacetylated as chromatin matures; this deacetylation is inhibited in the presence of either sodium butyrate or TSA (Dimitrov *et al.*, 1993; Ura *et al.*, 1997). Under these conditions the transcriptional activity of class III genes is sustained even though they are efficiently assembled into chromatin (Ura *et al.*, 1997). Microinjection of single-stranded TR β A promoter templates into the nuclei of *Xenopus* oocytes (Figure 1A) also leads to a substantial repression of transcription during replication-coupled chromatin assembly (Figure 1B, lanes 1–3, Wong *et al.*, 1995). However in the presence of TSA transcriptional repression is relieved (Figure 1B, lanes 5–7).

Microinjection of double-stranded templates into the nuclei of *Xenopus* oocytes also leads to chromatin assembly; however, the rate of nucleosome formation is slower than observed on replicating templates (Almouzni and Wolffe, 1993). Under these circumstances double-stranded templates show a high level of basal transcription (Bienz, 1984; Almouzni and Wolffe, 1993; Landsberger and Wolffe, 1995, 1997; Wong *et al.*, 1995, 1997a,b). This high level of basal transcription occurs in spite of the eventual assembly of nucleosomes (see Figures 6–10 later). Expression of TR-RXR in oocytes following the microinjection of mRNA encoding both components of the heterodimer, represses the transcription of double-stranded DNA containing the TR β A promoter (Figure 1C and D, compare lanes 1 and 2). The subsequent addition of TSA relieves transcriptional repression dependent on unliganded TR-RXR (Figure 1D, compare lanes 2–5). These results suggest that deacetylase activity is important for transcriptional repression mediated by replication-coupled chromatin assembly and by the unliganded TR-RXR.

The relief of transcriptional repression established on the double-stranded TR β A promoter in the presence of unliganded receptor through the late addition of TSA (Figure 1C) indicates that simply inhibiting deacetylase is sufficient to activate transcription from a chromatin

Fig. 1. TSA treatment relieves the transcriptional repression established by unliganded TR/RXR as well as that established by replication-coupled chromatin assembly pathway. **(A)** Groups of oocytes were injected with ssDNA of pTR β A for 4 h to allow completion of chromatin assembly via the replicative pathway before being treated with TSA (TSA, 5 ng/ml) or maintained without TSA (Control) for periods of time as indicated. **(B)** Transcription was analyzed by primer extension. The position of the TR β A transcripts (Expt.) and of the endogenous histone H4 mRNA which serves as an isolation and leading control (Internal Control) are indicated. **(C)** Groups of oocytes were injected with TR/RXR and dsDNA of pTR β A and were incubated overnight to establish repression by unliganded TR/RXR. The oocytes were then treated with TSA (5 ng/ml) for periods of time as indicated. **(D)** Transcription was analyzed by primer extension.

template. Since no thyroid hormone is added in this experimental protocol, the positive recruitment of coactivators appears unnecessary to relieve transcriptional repression. Endogenous 'non-targeted' activities must be able to reverse the transcriptionally repressed state once deacetylase activity is inhibited by TSA.

In earlier work we examined the efficiency of transcriptional repression directed by TR-RXR when present either during replication-coupled chromatin assembly or during chromatin assembly on duplex DNA (Wong *et al.*, 1995). In order to quantitate the influence of TSA on transcriptional regulation mediated by chromatin assembly on replicative or double-stranded templates in the presence of TR-RXR with or without ligand, we assayed transcription under all of these circumstances using the strategy shown (Figure 2A). Oocytes were either injected with mRNAs encoding TR-RXR or were maintained as uninjected controls. After 14 h, double-stranded or single-stranded TR β A template DNA was injected. Oocytes were then maintained for a further 14 h either without further additions, or in the presence of TSA or thyroid hormone. At the end of the experiment mRNA was isolated and TR β A transcripts quantitated relative to an internal control of endogenous H4 mRNA (Figure 2B). The quantity of TR β A mRNA synthesized following the injection of double-stranded TR β A templates in the absence of any subsequent additions is arbitrarily expressed as 1 (Figure 2B, lane 1). We find that the presence of TSA eliminates practically

all transcriptional repression mediated by TR-RXR (Figure 2B, compare lanes 3 and 4, and lanes 8 and 9). The repressive effects of replication-coupled chromatin assembly are also eliminated (Figure 2B, compare lanes 6 and 7, and lanes 8 and 9). The final levels of transcription in the presence of TSA are very similar to those in the presence of ligand-bound TR-RXR (Figure 2B, compare lanes 4 and 5, and lanes 9 and 10). Therefore, relative to the repressed state in the presence of unliganded TR-RXR, either the addition of TSA or thyroid hormone provides comparable transcriptional activation. The range of transcriptional regulation achieved is ~8-fold for injections of double-stranded DNA and 30-fold for injections of single-stranded DNA.

TSA inhibits histone deacetylase specifically (Yoshida *et al.*, 1990, 1995). Since addition of TSA leads to the same functional consequences as hormone-bound TR-RXR, it is possible that the addition of thyroid hormone in the presence of chromatin-bound TR-RXR initiates events leading to the inhibition or removal of deacetylase activity in the vicinity of the TR β A promoter or the recruitment or activation of acetyltransferase activity at the promoter (Chen and Evans, 1995; Horlein *et al.*, 1995; Heinzl *et al.*, 1997; Nagy *et al.*, 1997). A prediction from this model would be that the addition of TSA would replace the necessity for the hormone-bound TR-RXR at the TR β A promoter, but that the basal transcriptional machinery and their binding sites in the TR β A promoter

Fig. 2. The histone deacetylase inhibitor TSA blocks the transcriptional regulation by TR/RXR. **(A)** Groups of oocytes were first injected with (+) or without (-) TR/RXR mRNAs and then injected with dsDNA or ssDNA of pTR β A as indicated. The oocytes were treated with (+) or without (-) TSA (5 ng/ml) or T3 (50 nM) overnight. **(B)** RNA was then prepared from the injected oocytes and the transcription from TR β A promoter was analyzed by primer extension (Expt.). The internal control represents the primer extension product derived from endogenous storage pool of histone H4 mRNAs which serves as an isolation and loading control. Levels of transcription from pTR β A were quantitated by phosphorimaging and normalized against the internal control. The level of transcription from control dsDNA of pTR β A was designated as 1 (lane 1) and the other lanes were compared with it.

would still be required to direct transcription. This would happen only after chromatin had been made accessible. We tested this prediction making use of single-stranded DNA templates containing mutations known to impair transcription of the TR β A promoter (Wong *et al.*, 1995). We microinjected single-stranded templates into *Xenopus* oocyte nuclei and incubated the oocytes for 4 h to allow completion of complementary strand synthesis and chromatin assembly. We then incubated these templates with TSA and assayed transcription 14 h later. We find that TSA addition to the repressed chromatin template replaces the need for hormone-bound TR-RXR to be present in chromatin in order for transcription to be activated (data not shown). The basal regulatory elements between -154 and the initiator element continue to be required for transcriptional activation (data not shown). Our results suggest that either TSA or hormone-bound TR-RXR can render chromatin accessible to the basal machinery in a manner that is sufficient to transcribe the TR β A promoter.

Expression of the catalytic subunit of histone deacetylase represses transcription of chromatin templates

Our evidence for a role of histone deacetylase in transcriptional regulation to this point relies only on experiments using the inhibitor TSA. It was essential to find an alternate approach to test for a role of the deacetylase in transcriptional regulation and potentially confirm that the deacetylase was the target of TSA. We cloned and characterized the *Xenopus* homolog of the yeast histone deacetylase catalytic subunit RPD3p (Vidal and Gaber, 1991; Parthun *et al.*, 1996; Rundlett *et al.*, 1996; Taunton *et al.*, 1996). The *Xenopus* RPDp homolog (xRPD3) is very similar to the human RPD3 sequence (89% identical; Taunton *et al.*, 1996) and yeast sequence (60% identical; Vidal and Gaber, 1991) (Figure 3).

Our next experiments wished to explore the functional consequences of expressing xRPD3 for TR β A promoter activity (Figure 4A). We synthesized a synthetic mRNA *in vitro* encoding xRPD3 and microinjected the mRNA into *Xenopus* oocyte cytoplasm (Figure 4B). The xRPD3 protein is robustly expressed as assayed by the radiolabeling of newly synthesized proteins with [³⁵S]methionine (Figure 4B, lanes 1–4). Translation of xRPD3 is not affected by the presence of TSA (Figure 4B, lanes 5 and 6). The xRPD3 protein is present in both the cytoplasm and the nucleus (Figure 4B, lanes 11 and 12). Note that measurements of the aqueous volume of *Xenopus* oocyte nuclei and cytoplasm reveal that each nucleus has one-tenth the volume of the cytoplasm (Dingwall and Allan, 1984). Thus, the amount of xRPD3 protein visible in lane 12 should be increased 10-fold to provide an estimate of relative concentration in the oocyte nucleus compared with the cytoplasm (Figure 4B, lane 11).

We microinjected oocytes first with varying amounts of mRNA encoding xRPD3 in the linear range for protein synthesis (Figure 4B, lanes 1–3), and allowed xRPD3 protein to accumulate for 14 h. We next microinjected oocyte nuclei with double-stranded TR β A templates. Following a further 14 h incubation we assayed transcription (Figure 4C). The double-stranded TR β A template in oocytes was actively transcribed (Figure 4C, lane 1). The injection of increasing amounts of xRPD3 mRNA leads to a strong repression of TR β A promoter activity (Figure 4C, lanes 2–4). We conclude that increasing the amount of xRPD3 in the *Xenopus* oocyte represses transcription from the TR β A promoter. This effect occurs in the absence of any targeting of the deacetylase by chromatin-bound TR-RXR because the receptor is not expressed in this experiment. Our next experiments examined whether xRPD3p exerted a dominant irreversible repressive effect on TR β A promoter activity, or whether the presence of hormone-bound TR-RXR could overcome this transcriptional repression.

Our experimental strategy (Figure 4A) was to express xRPD3, then to microinject double-stranded TR β A templates and to wait 14 h to obtain maximal repression of transcription, and then to microinject mRNAs encoding TR-RXR. The oocytes were incubated in thyroid hormone for a further 14 h and transcription from the TR β A promoter was assayed. Hormone-bound TR-RXR counteracts the repressive effects of xRPD3 on transcription and activates transcription (Figure 4D, compare lanes 2 and 3

Fig. 4. Expression of *Xenopus* histone deacetylase RPD3 (xRPD3) in oocytes represses transcription from TRβA promoter, and liganded TR/RXR overcomes the repression by xRPD3. (A) Scheme of experiment. (B) Expression of xRPD3 and its localization in oocytes. Groups of oocytes were co-injected with different amounts of *in vitro*-transcribed xRPD3 mRNA as indicated and [³⁵S]methionine in the absence or presence of Trichostatin A (TSA) (5 ng/ml). After a 14 h incubation, protein extracts were made from total, cytoplasmic as well as nuclear fractions as indicated and an amount of extract equivalent to one oocyte was separated by 10% SDS-PAGE. The [³⁵S]methionine-labeled xRPD3 was visualized by autoradiography (arrowhead). (C) Expression of the histone deacetylase xRPD3 represses transcription from the TRβA promoter. Groups of oocytes were injected with dsDNA of pTRβA and an increasing amount of xRPD3 mRNA (lanes 1–4, 0, 0.5, 1 and 2 ng respectively). The oocytes were incubated overnight and the levels of transcription were analyzed by primer extension. (D) Liganded TR/RXR can relieve the repression established in the presence of xRPD3. Groups of oocytes were injected with dsDNA of pTRβA, and with or without increasing amounts of xRPD3 mRNA as indicated (0.5 ng, lanes 2 and 4, and 1 ng lanes 3 and 5). 14 h later the oocytes were injected with or without TR/RXR mRNAs and treated with (+) or without (–) T₃ for 14 h, before the levels of transcription were analyzed by primer extension. (E) TSA can relieve repression by xRPD3. Groups of oocytes were injected with double-stranded pTRβA templates with or without prior injection of increasing amounts of xRPD3 mRNA (0.5 ng, lanes 3 and 5; 1 ng, lanes 2 and 4). The oocytes were then incubated 14 h in the presence (+) or absence (–) of TSA (5 ng/ml).

that regulation of histone deacetylase activity has a key role in transcriptional regulation mediated by the thyroid hormone receptor on the TRβA promoter.

The coactivators p300 and PCAF can acetylate substrates other than the histones, including components of the basal transcriptional machinery (Imhof *et al.*, 1997). We have also explored whether recombinant TR, RXR or TR-RXR might be efficient substrates for p300 and PCAF (Figure 5). These proteins were expressed in *Escherichia coli*, partially fractionated (Figure 5, lanes 4–6) and shown to reconstitute specific DNA binding for TRE containing DNA fragments and nucleosomes (Ranjan *et al.*, 1994; D.Guschin, Q.Li and A.P.Wolffe, in preparation). Neither PCAF (Figure 5B, lanes 4–6), nor p300 (Figure 5C, lanes 4–6), nor *Xenopus* cytoplasmic histone acetyltransferase (xHAT1, Figure 5D, lanes 4–6) acetylate the TR, RXR or TR-RXR proteins efficiently. Controls show that all of these acetyltransferases will acetylate the core histones with variable specificity (Figure 5E and F) and that PCAF and p300 will acetylate TFIIF and TFIIEβ (Figure 5B and C, lanes 2 and 3) (Imhof *et al.*, 1997). Although the TR-RXR complex is not a substrate for these acetyltransferases, TFIIF and TFIIEβ are targets for deacetylase activity (A.Imhof, D.Patterson and A.P.Wolffe, in preparation). It is therefore important to test whether a chromatin template is required for the transcriptional repression mediated by the deacetylase xRPD3. Our approach to this

problem is to make use of the relatively slow kinetics of chromatin assembly on double-stranded DNA templates (Almouzni and Wolffe, 1993; Kass *et al.*, 1997). The experimental strategy is shown in Figure 6A. We synthesize elevated amounts of xRPD3 following the microinjection of mRNA into the oocyte, and then microinject double-stranded TRβA templates and monitor both transcription and chromatin assembly over a 10 h time course. The assembly of a transcription complex is time-dependent (Birkenmeier *et al.*, 1978) and transcripts from the TRβA promoter begin to accumulate in detectable amounts within 1.5 h after injection (Figure 6B, lanes 1–4). Substantially more TRβA transcripts accumulate by 3 h (Figure 6B, compare lanes 5 and 6 with lanes 3 and 4). Remarkably, the prior expression of xRPD3 (Figure 6B, R lanes) has no effect on transcript accumulation relative to controls (C lanes) during the first 3 h following injection of the double-stranded TRβA template. The expression of the xRPD3 protein does not appear to influence the assembly and function of the transcription complex at these early times. At subsequent times, the control oocytes continue to drive the accumulation of TRβA transcripts through 8 h post injection (Figure 6B, compare lanes 5, 7, 9 and 11). In contrast, the oocytes in which xRPD3 has been expressed cease to accumulate TRβA transcripts (Figure 6B, compare lanes 6, 8, 10 and 12). These results are quantitated in Figure 6C. The template becomes

Fig. 5. *In vitro* histone acetyltransferase assays using TR and RXR, the basal transcription factors TFIIB, TFIIE β and TFIIF and core histones.

(A) Coomassie blue staining of polypeptides used in the *in vitro* histone acetyltransferase assays is shown. Molecular weight standards are indicated on the left and the identity of the polypeptides on the right-hand side of the panel. *In vitro* acetylation assays were carried out with 100 ng of PCAF [(B) and (F), lane 1], p300/CBP [(C) and (F), lane 2] or *Xenopus* HAT1 (xHAT1) which was partially purified from *Xenopus* oocytes [A.Imhof and A.P.Wolffe, unpublished results; (D) and (F), lane 3], 1 μ g of substrate, 40 pmol of [3 H]acetyl CoA (5.8 Ci/mmol, Amersham Life Science), 10 mM sodium butyrate in the HAT buffer (50 mM Tris-Cl, pH 8.0, 10% glycerol, 1 mM DTT, 1 mM PMSF) at 37°C for 30 min. Reactions were stopped (see Materials and methods) and analyzed on a 12% (B, C, D) or 18% (E, F) SDS PAA gel. Gels were stained with Coomassie blue [(A) and (E) are stained gels of autoradiographs (B) and (F) respectively], destained, treated with Amplify (Amersham Life Science) for 15 min and exposed for 2–5 days.

repressed only between 3–4.5 h after injection into *Xenopus* oocytes enriched in the xRPD3 deacetylase.

In previous work we have demonstrated that it takes more than 3 h to assemble a physiological density of nucleosomes (one per 180–190 bp) on double-stranded DNA microinjected into oocytes (Almouzni and Wolffe, 1993; Kass *et al.*, 1997). Agarose gel electrophoresis (in the presence of the drug chloroquine) of DNA extracted from minichromosomes at various times following microinjection into oocytes containing xRPD3 reveals that a physiological nucleosome density is not established until 3–4.5 h after injection (Figure 6D, data not shown; see also Figure 10 and Clark and Wolffe, 1991). Immediately following the microinjection of double-stranded DNA into the oocyte it is relaxed by endogenous topoisomerase activities and then progressively supercoiled following the assembly of nucleosomes. In Figure 6D, lane 1 there are <15 negative superhelical turns shortly after injection (<15 min) (data not shown). In Figure 6D at 3 h (lane 3) there are an average of 18 negative superhelical turns (arrowhead between lanes 3 and 4, density one nucleosome per 230 bp), and at 4.5 h after injection (lane 4) there are 23 turns on average (arrowhead between lanes 4 and 5, density one nucleosome per 180 bp). Thus, the capacity of xRPD3 to repress transcription correlates with the time taken to assemble a physiologically spaced nucleosomal array (see also Kass *et al.*, 1997). The kinetics of chromatin assembly are unaffected by the presence or absence of xRPD3 (see also Figure 7B).

Taken together, our results using oocytes in which we have expressed the catalytic subunit of the deacetylase xRPD3 are consistent with a repressive role for histone deacetylase in transcription. There is a requirement both for catalytic activity and for the presence of templates organized into physiologically spaced nucleosomal arrays to mediate transcriptional repression (see also Figures 9 and 10).

The unliganded TR-RXR can interfere with transcription and the addition of thyroid hormone can activate transcription before the complete assembly of a template into nucleosomes

A major difference between the transcriptional repression directed by xRPD3 and that directed by the unliganded TR-RXR, is that the TR-RXR has a mechanism to target repression to a particular promoter, since it can bind to a TRE even in the presence of chromatin (Wong *et al.*, 1995, 1997b). In contrast, no such targeting mechanism exists for xRPD3 in the absence of TR-RXR. It is possible that the requirement for a high nucleosomal density reflects the lack of a targeting function for the xRPD3 deacetylase, and that unliganded TR-RXR might be able to repress transcription at much lower nucleosomal densities. It is also possible that TR-RXR might be able to repress transcription through mechanisms independent of histone association with the template (Baniahmad *et al.*, 1993; Fondell *et al.*, 1993). In a parallel experiment (using the same batch of oocytes as used in Figure 6) we examined

Fig. 6. *Xenopus* histone deacetylase (xRPD3) requires a chromatin template to direct transcriptional repression. (A) Scheme of the experiment. Groups of oocytes were microinjected with (R) or without (C) xRPD3 mRNA (1 ng) and incubated 14 h to allow expression of xRPD3p. After this time, double-stranded TRβA template was injected. At various times as indicated, half of the oocytes were extracted to assess mRNA synthesis and half to assess DNA topology. (B) Transcriptional repression on the TRβA promoter takes more than 3 h to be established. Transcripts were analyzed by primer extension from oocytes injected with xRPD3 (R) and control oocytes (C). Time after injection of the TRβA template is indicated in hours. TRβA transcripts (Expt.) and the endogenous H4 mRNA (Internal Control) are indicated. (C) Quantitation of the data shown in (B). (D) The chromatin assembly onto double-stranded DNA pTRβA template is relatively slow. The DNA samples prepared from half of the oocytes were analyzed for chromatin assembly using 1% agarose gel with chloroquine (90 μg/ml) (see Materials and methods). After electrophoresis, DNAs were blotted to a nylon membrane and hybridized with random primer labeled TRβA promoter fragment (from -255 to -147). Nicked (form II) and linear DNA (form III) and supercoiled topoisomers (Topoisomers) are indicated. The arrowheads indicate the centers of distribution of the topoisomers present in the adjacent left hand lane.

the efficiency of transcriptional repression directed by the unliganded TR-RXR as chromatin assembly proceeds (Figure 7A). We find that at the earliest time point of 1.5 h, the unliganded TR-RXR is able to repress transcription from the TRβA promoter injected as double-stranded DNA (Figure 7B, lanes 2 and 9 and Figure 7D). Measurements of nucleosome density at this early time point indicate that there are <15 constrained negative superhelical turns per minichromosome, or one nucleosome per 300 bp on average, a density far below the physiological density of 180 bp per nucleosome (Figure 7C, lanes 1 and 8; note that use of other chloroquine concentrations reveals that these topoisomers are yet to reach the bottom of the gel whereas the other topoisomers are moving towards to

Fig. 7. Kinetics of transcriptional repression on the TRβA promoter by unliganded TR/RXR. (A) Scheme of experiment. (B) Transcriptional repression by unliganded TR/RXR is rapidly established. Groups of oocytes were injected with or without TR/RXR and incubated 14 h to allow the synthesis of TR/RXR. The oocytes were then injected with double-stranded pTRβA templates and collected at the indicated time points after injection of dsDNA. Half of the oocytes at each time point were used for RNA purification for assay of transcription and the other half were used for DNA purification for chromatin assembly assay in (C). (C) The chromatin assembly onto the double-stranded pTRβA template is relatively slow. The DNA samples prepared from half of the oocytes were analyzed for chromatin assembly using 1% agarose gel with chloroquine (90 μg/ml) (see Materials and methods). After electrophoresis, DNAs were blotted to a nylon membrane and hybridized with a random primer-labeled TRβA promoter fragment (from -255 to -147). Nicked (form II) and linear DNA (form III) and supercoiled topoisomers (Topoisomers) are indicated. The arrowheads indicate the centers of distribution of the topoisomers present in the adjacent left hand lane. (D) Quantitation of the levels of transcription in (B). Levels of transcription from pTRβA at each time point were quantitated by phosphorimaging and normalized against the internal control (histone H4).

top, data not shown). The presence of TR-RXR (or of xRPD3, Figure 6) does not influence the kinetics of nucleosome assembly (Figure 7C, compare lanes 1–7 with

Fig. 8. Kinetics of transcriptional repression by unliganded TR/RXR and relief of transcriptional repression on addition of TSA. Groups of oocytes were injected with or without TR/RXR and incubated 14 h to allow the synthesis of TR/RXR. The oocytes were then injected with double-stranded pTR β A templates in the absence or presence of TSA (5 ng/ml), and collected at the indicated time points after injection of dsDNA. TR β A transcripts (Expt.) and the endogenous H4 mRNA (Internal control) are indicated.

8–14). Superhelical density increases through 4.5 h after injection of the double-stranded template (Figure 7C). These results demonstrate that the unliganded TR-RXR can function to repress transcription at relatively low nucleosome densities. This is consistent with the capacity of the unliganded receptor to repress transiently transfected templates in chicken and mammalian cells (Damm *et al.*, 1987, 1989; Sap *et al.*, 1989; Zenke *et al.*, 1990; Baniahmad *et al.*, 1992). As discussed above, our results do not exclude a role for nucleosomes in transcriptional regulation, since the TR-RXR might recruit nucleosomes and direct the organization of chromatin in a process analogous to transcriptional silencers in yeast (for example, see Discussion; Edmondson *et al.*, 1996). However, it is probable that multiple repression mechanisms are targeted by the unliganded TR-RXR in addition to histone deacetylation (Chen and Evans, 1995; Horlein *et al.*, 1995; Alland *et al.*, 1997).

Since the presence of the unliganded TR-RXR can direct transcriptional repression at low densities of nucleosomes (Figure 7) we next explored whether the addition of TSA to inhibit deacetylase would also relieve transcriptional repression under these conditions. In the experiment shown in Figure 4, we show that the addition of TSA can relieve transcriptional repression at 14 h after injection of double-stranded templates when chromatin assembly is complete (Figure 7). We examined the effect of TSA on the transcription of double-stranded DNA templates that are incompletely assembled into chromatin shortly after microinjection into control oocytes (Figures 8, lanes 1–10) or experimental oocytes in which the TR-RXR protein had been previously expressed (Figure 8, lanes 11–20). We find that the presence of TSA provides a modest stimulation of transcription in the absence of TR-RXR (Figure 8, compare lanes 1–5 with lanes 6–10). From 2 to 5 h after injection the transcriptional stimulation is ~2-fold. We suggest that the acetylation of the basal transcriptional machinery might account for this modest stimulatory effect. Future experiments will explore this possibility. In the presence of unliganded TR-RXR we see substantial repression of transcription even at the earliest times (2 h) after injection of the template (Figure 8, compare lanes 1–3 with lanes 11–13). The addition of

Fig. 9. Comparison of chromatin disruption induced by liganded TR/RXR and TSA. Lanes 1–4: TSA has minimal effect on chromatin structure. Oocytes were injected with ssDNA or dsDNA of pTR β A and treated with (+) or without (–) 5 ng/ml of TSA overnight as indicated. The injected DNAs were recovered from the oocytes and the DNA topology was analyzed using a 1% agarose gel with 90 μ g/ml of chloroquine. After blotting to a nylon membrane, the DNAs were probed with a random primer-labeled TR β A promoter fragment (from –255 to –147). Lanes 5–8: liganded TR/RXR induces extensive chromatin disruption. Oocytes were injected with ssDNA or dsDNA templates containing the TR β A promoter and TR/RXR mRNAs and treated with (+) or without (–) T₃ (50 nM) overnight as indicated. Lanes 9–18: overexpression of xRPD3 in oocytes has little effect on chromatin structure. Oocytes were injected with ssDNA (lanes 9–10) or dsDNA (lanes 11–18) of pTR β A and injected with (+) or without (–) xRPD3 mRNA in the presence or absence of TSA (5 ng/ml) as indicated. Nicked (form II) and linear (form III) and supercoiled topoisomers (Topoisomers) are indicated.

TSA relieves this transcriptional repression (Figure 8, compare lanes 6–10 with lanes 16–20). Thus, deacetylase activity is important for transcriptional repression by the unliganded TR-RXR even at the earliest times that transcription can be detected and when chromatin assembly is far from complete. We suggest that deacetylation of the basal transcriptional machinery might contribute to the establishment of the repressed state under these conditions. Alternatively, the unliganded receptor must actively assemble a local chromatin structure sensitive to deacetylase. Future experiments will explore this issue.

Chromatin structural transitions associated with gene activation dependent on TSA or ligand-bound TR-RXR

Our results indicate that the addition of either TSA or ligand-bound thyroid hormone receptor to oocytes during replication-coupled chromatin assembly will relieve chromatin-mediated transcriptional repression (Figures 1 and 2). Each nucleosome constrains one negative superhelical turn (Germond *et al.*, 1975; Simpson *et al.*, 1985); therefore changes in the number of intact nucleosomes in a minichromosome can be assayed by changes in DNA topology. The ligand-bound TR-RXR is known to target a dramatic remodeling of chromatin (Wong *et al.*, 1997a,b) involving the loss of topological constraint equivalent to three nucleosomes per chromatin-bound receptor and the disruption of a regular nucleosomal array as assayed by micrococcal nuclease cleavage (Figure 9, compare lanes 5 and 6, and Figure 10A, compare lanes 1–4 with lanes 9–12). However, in remarkable contrast, the presence of TSA activates transcription without any significant topological change (Figure 9, lanes 1 and 2), and with only a modest increase in accessibility of the template to micrococcal nuclease in the vicinity of the TR β A promoter (Figure 10A, compare lanes 1–4 with lanes 5–8). Our experiments also demonstrate that no observable change

Fig. 10. TSA and liganded TR/RXR both induce the formation of chromatin structure with increased sensitivity to a nuclease probe (MNase). (**A**, **B**) Oocytes were injected with ssDNA or dsDNA of pTR β A with or without TR/RXR and treated with or without T₃ or TSA as indicated. After overnight incubation, the oocytes were proceeded for MNase digestion as in Materials and methods. After MNase digestion, DNA was recovered from each sample, resolved by a 1.5% agarose gel, blotted to a nylon membrane, and probed with random primer-labeled pTR β A promoter fragment (from -255 to -147). Each group of oocytes was proceeded for MNase digestion and analyzed as in (**A**). (**C**) xRPD3 and unliganded TR/RXR has no detectable effect on chromatin structure. Oocytes were injected with dsDNA of pTR β A and with or without TR/RXR or xRPD3 mRNA as indicated.

in topology occurs on templates assembled into chromatin on double-stranded DNA microinjected into oocyte nuclei using the protocol shown in Figure 2A dependent on the presence or absence of TSA (Figure 9, lanes 3 and 4), whereas large changes occur dependent on the presence of hormone-bound TR-RXR (Figure 9, lanes 7 and 8). TSA treatment leads to a slight increase in micrococcal nuclease accessibility in the vicinity of the TR β A promoter (Figure 10B, compare lanes 1–4 with lanes 5–8). In contrast, the presence of ligand-bound TR-RXR leads to a substantial increase in accessibility as seen most clearly by the large increase in the amount of subnucleosomal material (Figure 10B, compare lanes 1–4 with lanes 9–12). An important point is that the failure to see topological change in response to TSA is not due to a failure to examine the structure of transcriptionally responsive templates. The oocyte nucleus has remarkably high transcriptional activity (La Marca *et al.*, 1973; Gurdon and Melton, 1981). Transcription efficiencies in the presence of TSA are comparable with those of templates completely occupied with TR-RXR in the presence of thyroid hormone (Figure 2) as shown both by *in vivo* footprinting (Wong *et al.*, 1995) and by the changes in topology of the vast majority of templates microinjected into the oocyte nucleus (Figure 9, lanes 5–8).

We examined the consequences for chromatin organization of expressing xRPD3 in *Xenopus* oocytes in the presence or absence of TSA. These conditions lead to major changes in transcriptional activity of the TR β A promoter (Figures 4 and 6). However, there is no significant change either in minichromosome topology (Figure 9, lanes 11–18) or in micrococcal nuclease cleavage (Figure 10C, compare lanes 1 and 2 with lanes 5 and 6). The TR β A promoter is organized into nucleosomes in control oocytes, and in the presence of either unliganded TR-RXR or xRPD3 (Figure 10A, lanes 1–6). These results again confirm published work demonstrating that it is possible to have major changes in transcription of a chromatin template without large changes in overall DNA topology or chromatin organization as assayed by micrococcal nuclease (Pederson and Morse, 1990; Drabik *et al.*, 1997).

Discussion

The major conclusions from this work are that: (i) histone deacetylase activity is important for transcriptional regulation by TR-RXR (Figures 1–4, 6 and 8); (ii) that the unliganded TR-RXR can repress transcription without complete assembly of the template DNA into a physiologically spaced nucleosomal array, whereas repression mediated by the catalytic subunit of histone deacetylase, xRPD3 alone requires such an array (Figures 6 and 7); and (iii) that a significant range (from >8- to 30-fold) of transcriptional activity can be achieved without major changes in DNA topology or chromatin organization (Figures 2, 4, 9 and 10). Our results indicate that an existing model for transcriptional regulation by the TR-RXR in which reversible recruitment of corepressors containing histone deacetylase activity represses transcription in the absence of hormone (Chen and Evans, 1995; Horlein *et al.*, 1995; Alland *et al.*, 1997; Heinzl *et al.*, 1997; Nagy *et al.*, 1997), and where coactivators

possessing histone acetyltransferase activity are recruited in the presence of hormone (Kamei *et al.*, 1996; Ogryzko *et al.*, 1996; Yang, W.M. *et al.*, 1996) presents only one component of a complex regulatory process.

Our results suggest the existence of additional functions for TR-RXR, some anticipated and some not. The simple model of targeted histone modification as a major source of transcriptional control cannot easily explain the rapid repression of transcription mediated by the unliganded TR-RXR where the template is incompletely assembled into chromatin. Likewise the inhibition of deacetylase activity using TSA relieves transcriptional repression under conditions of incomplete chromatin assembly (Figure 8). A second complication is that while the liganded TR-RXR has the capacity to target an extensive disruption of histone–DNA interactions (Figures 9 and 10; Wong *et al.*, 1995, 1997a,b), this disruptive activity is not essential to sustain high levels of transcription from the TR β A promoter in the minichromosome. We suggest that the TR-RXR is manipulating chromatin structure to provide structural changes that might allow for a more permanent change in gene expression.

A role for histone deacetylase in transcriptional regulation

Saccharomyces cerevisiae RPD3 is a gene required for both the maximal repression and activation of several inducible genes (Vidal and Gaber, 1991; Rundlett *et al.*, 1996). These include genes such as *PHO5* in which chromatin structure has a well-established functional role (Almer and Horz, 1986; Almer *et al.*, 1986; Straka and Horz, 1991; Fascher *et al.*, 1993; Svaren *et al.*, 1994). RPD3p functions on the same pathway as RPD1p (SIN3) (Vidal *et al.*, 1991). These two proteins interact and can be targeted by DNA-binding transcription factors (Kadosh and Struhl, 1997; see also W.M. Yang *et al.*, 1996). The identification of RPD3p as a histone acetyltransferase (Taunton *et al.*, 1996) suggested a pathway by which transcriptional repression might be achieved through the modification of chromatin structure. It should be noted that the histones are bona fide substrates for the RPD3p deacetylase activity since this protein functions in the modification of chromatin structure through the deacetylation of H4 at the replication fork. The biology of this process is well defined in *S. cerevisiae* (Parthun *et al.*, 1996; Rundlett *et al.*, 1996; Verreault *et al.*, 1996). The yeast model was extended to metazoans by the observation that the mammalian SIN3 homolog interacted with both the corepressors NCoR and SMRT (Alland *et al.*, 1997; Heinzel *et al.*, 1997; Nagy *et al.*, 1997) as well as the mammalian histone deacetylase homologs (Alland *et al.*, 1997; Hassig *et al.*, 1997; Heinzel *et al.*, 1997; Nagy *et al.*, 1997). Since the association of NCoR and SMRT together with SIN3 and histone deacetylase with the unliganded TR-RXR occurs in the absence of hormone (Chen and Evans, 1995; Horlein *et al.*, 1995), histone deacetylase will be recruited to the TR-RXR in the absence of hormone. The release of NCoR and SMRT from TR-RXR in the presence of hormone is also likely to release histone deacetylase. In addition, the recruitment of coactivators having histone acetyltransferase activity in the presence of thyroid hormone (Kamei *et al.*, 1996; Ogryzko *et al.*, 1996; X.J. Yang *et al.*, 1996) might be

expected in this model to counteract any residual deacetylase activity.

Our experimental results support an active role for deacetylase and acetyltransferase activities in the transcriptional regulation of the TR β A promoter. In a physiological context, the TR-RXR will have to function within a nucleosomal array and the TR β A promoter will have to be transcribed after it is assembled into chromatin. Replication-coupled chromatin assembly will normally repress TR β A transcription (Figures 1 and 2). Deacetylase activity is essential for mediating this repression (Figures 1 and 2). Expression of the catalytic subunit of histone deacetylase xRPD3 in oocytes drives the transcriptional repression of chromatin templates—but only at a physiological nucleosome density (Figures 4 and 6). Inhibition of deacetylase activity with TSA prevents this repression of transcription by xRPD3 (Figure 4). This result indicates that the deacetylase requires a chromatin substrate to direct transcriptional repression in this non-targeted process. All of these transitions in transcriptional activity occur without major changes in DNA topology, consistent with the maintenance of DNA within a largely intact nucleosomal environment (Figures 9 and 10). Variations in histone acetylation will influence the integrity of histone–DNA interactions without significant changes in DNA topology (Norton *et al.*, 1989; Lutter *et al.*, 1992; Bauer *et al.*, 1994). TSA has been shown to increase the acetylation of core histones in chromatin in *Xenopus* (Almouzni *et al.*, 1994). Therefore, although other targets for acetyltransferase and deacetylase exist (Imhof *et al.*, 1997), the hyperacetylation of the histones is one proven consequence of inhibiting deacetylase. Histone hyperacetylation is causal for promoting transacting factor access to nucleosomal DNA (Lee *et al.*, 1993; Vetesse-Dadey *et al.*, 1996) and transcription (Ura *et al.*, 1997). Other work *in vivo* has documented that robust transcription of a chromatin template can occur without significant chromatin disruption as assayed by topological change (Pederson and Morse, 1990; Drabik *et al.*, 1997). Our results are consistent with these observations. There is excellent precedent for chromatin remodeling and histone hyperacetylation having a role in transcriptional regulation of hormone-inducible promoters. The mouse mammary tumor virus (MMTV) long terminal repeat (LTR) is organized into a positioned array of nucleosomes *in vivo* (Richard-Foy and Hager, 1987). Nucleosome positioning modulates the accessibility of regulatory proteins to the MMTV LTR (Pina *et al.*, 1990). The addition of hormone induces binding of glucocorticoid receptor and other transcription factors to a rearranged nucleosome on the MMTV LTR (Truss *et al.*, 1995). This chromatin remodeling can be reproduced by a moderate increase in histone acetylation levels (Bartsch *et al.*, 1996), suggesting that targeted histone acetylation might have a regulatory function on the MMTV LTR. Nevertheless, further exploration of the regulatory process on the TR β A gene in the presence of unliganded TR-RXR as discussed below, indicates that chromatin modification is only one component of the repression and activation pathway.

The role of TR-RXR in transcriptional regulation and chromatin disruption

The TR-RXR has the capacity to bind to DNA in chromatin (Wong *et al.*, 1995, 1997a,b). Unliganded TR-RXR can

repress transcription from pre-assembled chromatin templates, as well as during chromatin assembly (Wong *et al.*, 1995). We find that unliganded TR-RXR can repress transcription at a time following microinjection of double-stranded templates well before chromatin assembly is complete (Figure 7). This result differs from the requirement for chromatin assembly in order to obtain transcriptional repression by histone deacetylase (Figure 6). However, it is also important to note that even when unliganded TR-RXR represses transcription during chromatin assembly, inhibition of histone deacetylase blocks that repression (Figures 1 and 2). The addition of TSA to inhibit deacetylase activity will also relieve transcriptional repression when templates are only partially assembled into nucleosomes (Figure 8). Therefore, deacetylase activity is essential for maintaining transcriptional repression and the simple inhibition of deacetylase activity appears sufficient for maximal relief of transcriptional repression (Figures 1B, 2 and 8). Thus, the positive targeted recruitment of coactivators with histone acetyltransferase activity may be unnecessary in this system.

Possible explanations for the repression of transcription by unliganded TR-RXR occurring under conditions of incomplete chromatin assembly, yet still requiring histone deacetylase include: (i) The active recruitment of histones to the transcriptional corepressor complex so that they are used as part of the regulatory nucleoprotein architecture to repress transcription after deacetylation (Roth *et al.*, 1990, 1992; Cooper *et al.*, 1994; Alevizopoulos *et al.*, 1995; Hecht *et al.*, 1995; Edmondson *et al.*, 1996; Wolffe and Pruss, 1996b). (ii) Deacetylase may work on basal transcription factors and not histones (Imhof *et al.*, 1997). In this model, acetylated components of the basal transcriptional machinery (Figure 5) would have enhanced functional properties. We do not detect efficient acetylation of TR-RXR in our *in vitro* assays (Figure 5). At this time there exists no direct evidence for an enhancement of transcription factor function following acetylation (Imhof *et al.*, 1997). (iii) The initial process of establishing transcriptional repression might involve interactions between TR/RXR and/or the corepressor complex with the basal transcriptional machinery that might interfere with transcription (Baniahmad *et al.*, 1993; Fondell *et al.*, 1993). This state of repression would be subsequently stabilized by other events requiring deacetylase activity, potentially including nucleosome assembly. (iv) Any combination of the above.

An unexpected finding is that the chromatin disruption process directed by hormone-bound TR-RXR (Figures 9 and 10; Wong *et al.*, 1995, 1997a,b) appears unnecessary for transcriptional activation (Figures 1, 2, 9 and 10). The lack of change in chromatin on transcriptional activation by TSA is surprising because chromatin disruption as assayed by topological change and changes in micrococcal nuclease cleavage has generally been interpreted as essential for transcriptional activation (Hirschhorn *et al.*, 1992; Imbalzano *et al.*, 1994; Tsukiyama *et al.*, 1994; Varga-Weisz *et al.*, 1995; Wong *et al.*, 1997a). However, it is a fact that transcriptional activation can occur with minimal changes to DNA topology (Pederson and Morse, 1990; Drabik *et al.*, 1997; Figure 9, lanes 1–4). This would be anticipated if histone acetylation was the only alteration to chromatin structure necessary for transcriptional activation

(Norton *et al.*, 1989; Lutter *et al.*, 1992; Bauer *et al.*, 1994). It is possible that the pathways directing chromatin disruption in the oocyte nucleus in response to hormone-bound TR-RXR do not involve histone acetylation, and that alternative means of disrupting histone–DNA contacts are employed leading to similar transcriptional consequences. Candidate mechanisms include recruitment of molecular machines such as SWI/SNF (Côté *et al.*, 1994; Imbalzano *et al.*, 1994; Wang *et al.*, 1996), NURF (Tsukiyama *et al.*, 1994) or RNA polymerase itself (Wilson *et al.*, 1996; Gaudreau *et al.*, 1997).

Chromatin disruption directed by TR-RXR might serve other purposes aside from the activation of transcription on minichromosomes. It might be necessary to destabilize higher-order chromatin structures (Hansen *et al.*, 1991; Schwarz and Hansen, 1994). Such substantial alterations in chromatin structure, including loss of DNA wrapping in the nucleosome, might serve as an epigenetic mark for the propagation of gene activity states through replication and chromosomal duplication (Weintraub, 1985), or for the recruitment of the promoter to a nuclear site competent for transcription in a true chromosomal environment (Cook, 1994). Future studies will explore these possibilities.

Materials and methods

Plasmid constructs

pSP64(polyA)-xTR β A and -xRXR α constructs have been described before (Wong *et al.*, 1995). The pTR β A construct which contains 1.6 kb of TR β A promoter and 0.3 kb CAT gene sequence in pBluescript II KS(+) has been described before (Wong *et al.*, 1995). TRp(*Pst*I) construct was generated from pTR β A by deleting all *Pst*I fragments from the TR β A promoter. TRpm2 and TRpm3 plasmids were all constructed by PCR based on the TRp(*Pst*I) construct. 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 a SP6 Message Machine Kit (Ambion) as described by 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/ μ l) or double-stranded DNA (100 ng/ μ l) into the nuclei of the oocytes. The TR mRNAs were usually mixed with an equal concentration of RXR α 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. 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 thyroid hormone (T_3) as indicated.

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 μ l 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 μ l of RNeasyTM reagent together with 50 μ l of chloroform was 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 μ l) 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 ready for primer extension analysis using primer I (ATCCTTATAACGGTG-AGTAGTGATGTCATCAG) 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 was immediately added to the remaining half of the samples. The samples were treated with RNase A (100 µg/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 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). In brief, 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 8% glycerol]. The extract was 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 3M 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 sample was loaded onto a 1–1.2% agarose gel in 1× TPE (40 mM Tris, 30 mM NaH₂PO₄, 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. Different concentrations of chloroquine can be used to resolve minichromosome with different densities of nucleosomes. 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 TRβA promoter.

In vitro acetyltransferase assays

We purified TR and RXR as previously described (Ranjan *et al.*, 1994). The *Xenopus* TRβ and RXRα were cloned into pET15b (Novagen) as a *Nde*I–*Bam*HI fragment, respectively. The resulting pET15b–TRβ and pET15b–RXRα were transformed into *E. coli* BL21 (DE3). A single colony was inoculated in LB medium with ampicillin (200 µg/ml). The culture was grown at 30°C with vigorous shaking. Induction was carried out by the addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 1 mM when cell density (*A*₆₀₀) reached 0.6 and continued for 2 h. Cells were harvested by centrifugation. Purification was achieved by using nickel column from Novagen as recommended by manufacturer. After this single step purification, TRβ and RXRα were ~70% pure based on Coomassie blue-stained SDS-gel (not shown) and used for gel mobility shift assays and acetyltransferase assays.

Core histones were purified as previously described (Wolffe and Hayes, 1993). Recombinant general transcription factors and histone acetyltransferases were expressed and purified as described (Ge *et al.*, 1996; Mizzen *et al.*, 1996; Ogryzko *et al.*, 1996; Yang, X.-J. *et al.*, 1996). Concentrations of each factor were determined by both the Bradford method and SDS–PAGE. *In vitro* acetylation assays (20 nl) were carried out with 100 ng of acetyltransferase, 1 ng of substrate, 43 pmol of [³H]acetyl CoA (5.8 Ci/mmol, Amersham Life Science), 10 mM sodium butyrate in the HAT buffer (50 mM Tris–Cl, pH 8.0, 10% glycerol, 1 mM DTT, 1 mM PMSF) at 37°C for 30 min (Brownell *et al.*, 1996).

Reactions were stopped by adding 10 nl of 3× Laemmli buffer and analyzed on an 18% SDS PAA gel. Gels were stained with Coomassie blue, destained, treated with Amplify (Amersham Life Science) for 15 min and exposed for 2–5 days.

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