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Chromatin Disruption and Histone Acetylation in Regulation of the Human Immunodeficiency Virus Type 1 Long Terminal Repeat by Thyroid Hormone Receptor

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The human immunodeficiency virus type 1 (HIV-1) long terminal repeat (LTR) controls the expression of HIV-1 viral genes and thus viral propagation and pathology. Numerous host factors participate in the regulation of the LTR promoter, including thyroid hormone (T_3) receptor (TR). In vitro, TR can bind to the promoter region containing the NF-κB and Sp1 binding sites. Using the frog oocyte as a model system for chromatin assembly mimicking that in somatic cells, we demonstrated that TR alone and TR/RXR (9-cis retinoic acid receptor) can bind to the LTR in vivo independently of T_3. Consistent with their ability to bind the LTR, both TR and TR/RXR can regulate LTR activity in vivo. In addition, our analysis of the plasmid minichromosome shows that T_3-bound TR disrupts the normal nucleosomal array structure. Chromatin immunoprecipitation assays with anti-acetylated-histone antibodies revealed that unliganded TR and TR/RXR reduce the local histone acetylation levels at the HIV-1 LTR while T_3 treatment reverses this reduction. We further demonstrated that unliganded TR recruits corepressors and at least one histone deacetylase. These results suggest that chromatin remodeling, including histone acetylation and chromatin disruption, is important for T_3 regulation of the HIV-1 LTR in vivo.

Human immunodeficiency virus type 1 (HIV-1) is responsible for the development of AIDS and AIDS-related complex (ARC) (56). After acute infection, HIV-1 maintains latency for many years, but the mechanism of its reactivation to cause AIDS and ARC remains largely unclear. It has been suggested that viral reactivation involves complex interactions among chromatin-associated proviral DNA, cellular transcription factors, and viral proteins (11). A critical factor in viral reactivation is the transcription of the viral genome, which is directed by the long terminal repeat (LTR). Numerous studies have identified essential DNA elements within the LTR as well as many host and viral proteins for the transcriptional activation of the LTR in vitro and in tissue culture cells (35, 56). The DNA elements important for the LTR activity are located between −454 and +184, where +1 is the transcription start site. This region contains the TATA box and binding sites for host transcription factors Sp1 and NF-κB, etc. (Fig. 1).

The involvement of multiple host factors in HIV-1 transcription suggests that HIV-1 propagation and pathological progression of AIDS and ARC are likely to be influenced by physiological conditions that affect the availability and function of host factors. Studies on thyroid hormone (T_3) levels in AIDS and ARC patients suggest that T_3 may affect disease development (27, 51). The biological effects of T_3 are presumed to be mainly mediated by T_3 receptors (TRs), which are high-affinity T_3-binding nuclear proteins (7, 38, 44, 59). TRs belong to the superfamily of nuclear hormone receptors, with two subfamilies of TRs in vertebrates, TRα and TRβ (12, 28, 52, 70). Transcriptional activation by T_3 requires the binding of TRs, most likely as heterodimers with 9-*cis*-retinoic acid receptors (RXRs), to the T_3 response elements (TREs) in T_3 response genes. The binding of TREs by TR/RXR heterodimers is, however, independent of T_3 (36, 52, 65), implicating a role of unliganded TR in gene regulation. Indeed, various studies have revealed that unliganded TRs repress target transcription, while in the presence of T_3, they enhance the transcription of these same genes (13, 52, 60, 65).

Both transcriptional repression by unliganded TRs and activation by T_3-bound TRs are likely mediated by TR-interacting cofactors (2, 4, 20, 30, 39, 68, 69, 73). Many such factors have been isolated based on their ability to interact with TRs in the presence or absence of T_3. The corepressors bind preferentially or exclusively to unliganded TR, while the coactivators generally require T_3 for binding to TR. Recent studies have shown that corepressors, such as SMRT and N-CoR, form multimeric complexes containing histone deacetylases while many, but not all, coactivators, such as SRC-1 and CBP/p300, are histone acetyltransferases or acetylases (2, 3, 15, 16, 18, 21, 22, 26, 30, 32, 40, 43, 55, 68). These findings suggest that histone acetylation levels are important for gene regulation by TR, consistent with studies on *Xenopus* TR function in frog oocytes (62).

The role of T_3 in LTR regulation is supported by several reports demonstrating the binding of TRs to a region of the LTR that contains the binding sites for Sp1 and NF-κB in vitro (8, 17, 41, 67). Surprisingly, Rahman et al. (41) reported that TR alone activates the LTR while the addition of T_3 reverses this effect, which contrasts findings by us (17) and other groups (8, 67). One possible explanation may be the use of different model systems. In our study, we took advantage of the ability

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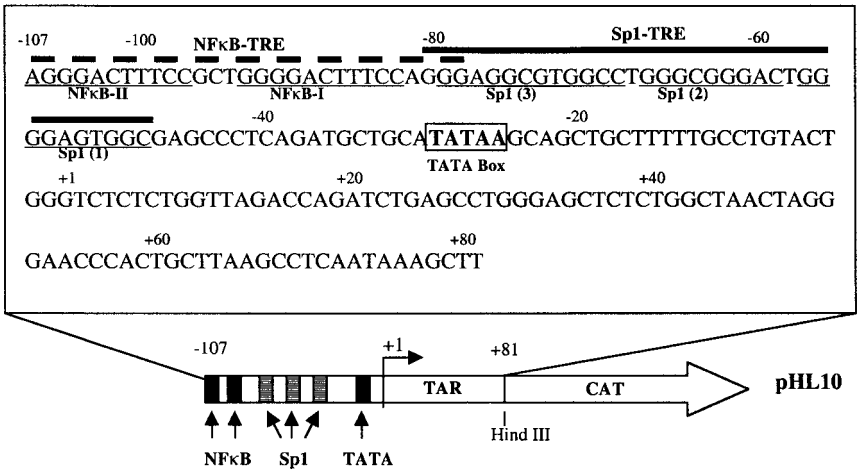

FIG. 1. Schematic representation of the LTR promoter plasmids. pHL10 contained the HIV-1 LTR promoter region from −107 to +81 in front of a 300-bp CAT reporter fragment in pBluescript KS(−) vector. The promoter fragment included two NF-κB sites, three Sp1 sites, and a TATA box followed by a regulatory TAR sequence. Two TREs are located in the regions containing the NF-κB and Sp1 binding sites (NF-κB TRE and Sp1 TRE). The Sp1 TRE is a stronger TRE than the NF-κB TRE (17).

of frog oocytes to replicate and chromatinize exogenous single-stranded DNA (ssDNA) in a process that mimics the process in normal somatic cells (1). We microinjected ssDNA containing the LTR promoter into *Xenopus* oocytes that had or had not been preinjected with the mRNAs encoding a TR and an RXR. Our studies revealed that TR/RXR represses the LTR in a chromatin context in the absence of T_3. The addition of T_3 reverses this repression and further activates the promoter to a level higher than that observed in the absence of T_3 and TR/RXR.

In the present study, we investigated how TR regulates the LTR in vivo. We demonstrate here that TR alone or as a heterodimer with RXR binds to the LTR in chromatin in vivo independently of T_3. In the absence of T_3, this binding leads to a local reduction of histone acetylation levels and repression of the LTR. T_3 treatment not only reverses the decrease in histone acetylation level but also activates the promoter. In addition, it causes drastic chromatin disruption. These results suggest that chromatin remodeling plays an important role in the regulation of LTR by TR.

MATERIALS AND METHODS

Plasmids. The plasmid pHIV-CAT was obtained from the NIH AIDS Research and Reference Program. This plasmid contains the full-length HIV-1 LTR and the chloramphenicol acetyltransferase (CAT) reporter. The HIV-1 LTR promoter plasmid pHL10 (Fig. 1) contains the core HIV-1 LTR fragment and the 5′ part of the CAT reporter. It was generated by PCR with forward primer F1 (5′-GGGGTACCACAAG GGACTTTCCCT-3′) and reverse primer B16 (5′-TGAGCATTCATCAAGCGGGC-3′) with pHIV-CAT as the PCR template. The amplified product was inserted into pCR2.1-TOPO with the TA cloning kit (Invitrogen, Carlsbad, Calif.). The resulting plasmid was then double digested with *Kpn*I and *Eco*RI, and the insert was purified and ligated into the *Kpn*I-*Eco*RI-digested plasmid p4/5A (17) to make pHL10. Single-stranded and double-stranded pHL10 were generated as described previously (29).

In vitro synthesis of capped mRNA. In vitro transcription was performed using the mMESSAGE mMACHINE SP6 kit (Ambion, Austin, Tex.) essentially as described by the manufacturer. The templates used for the reactions were pSP64(A)-TRβA (63), pSP64(A)-RXRα (63), and pSP64(A)-TRαΔDBD (37) for synthesis of mRNAs encoding *Xenopus* TRβ, RXRα, and TRα lacking the DNA binding domain, respectively. The capped mRNA was purified and resuspended in diethyl pyrocarbonate-treated water at a concentration of 50 ng/μl.

Microinjection of *Xenopus* oocytes. Microinjection experiments were performed as described previously (63). Briefly, plasmid pHL10 was injected at 23 nl/oocyte as either ssDNA (0.25 ng/oocyte) or double-stranded DNA (0.5 ng/oocyte) into the germinal vesicle (nucleus) of the oocytes. When indicated, in vitro-synthesized TRβA and/or RXRα mRNAs were injected (27 nl/oocyte) into the cytoplasm of oocytes 6 h before the DNA injection to ensure protein expression prior to chromatin assembly. The injected oocytes were incubated at 18°C in MBSH buffer (63) overnight in the presence or absence of 50 nM T_3 and/or 5 ng of trichostatin A (TSA), a specific inhibitor of histone deacetylases (71), per ml. For transcription analysis, a group of 20 oocytes was used for each sample to minimize the variation among oocytes and injections.

RNA isolation and transcription analysis. The transcription level from the LTR promoter was evaluated by primer extension assay as described previously (63). RNAzol B reagent (Tel-Test, Inc., Friendswood, Tex.) was used to isolate total RNA from the oocytes according to the manufacturer's instruction. RNA from the equivalent of two oocytes was annealed with an end-labeled CAT1 primer (5′-GGTGGTATATCCAGTGATTTTTTTCTCCAT-3′), which hybridized to the CAT region of the transcript from the LTR promoter, in 10 μl of 1× annealing buffer (50 mM Tris-HCl [pH 8.3], 50 mM KCl, 10 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM spermidine, and each deoxynucleoside triphosphate at 1 mM). The annealing reaction mixture was preheated to 65°C for 10 min and then annealed at 55°C for 30 min, followed by a 5-min incubation at 37°C. For reverse transcription, 10 μl of a mixture containing 1× annealing buffer, 5.6 mM sodium pyrophosphate, and 1 U of avian myeloblastosis virus reverse transcriptase (Promega, Madison, Wis.) was added to the annealing mixture followed by incubation of the final mixture at 42°C for 1 h. The products were analyzed directly on a 6% polyacrylamide sequencing gel. A histone H_4 antisense primer (5′-GAGGCCG GAGATGCGCTTGAC-3′) was included as an internal control to quantify the endogenous H_4 mRNA level. Each experiment was done at least twice with similar results.

Micrococcal nuclease (MNase) digestion assay. Single-stranded pHL10 DNA was microinjected into the germinal vesicle of oocytes (0.25 ng/oocyte). After overnight incubation, a group of 20 oocytes was homogenized with 100 μl of homogenization buffer: 10 mM Tris-HCl (pH 8.0), 50 mM NaCl, 1 mM EDTA, 5% glycerol, 5 mM $MgCl_2$, 3 mM $CaCl_2$, and 1 mM dithiothreitol. Lyophilized MNase (Worthington Biochemical Corp, Lakewood, N.J.) was dissolved in storage buffer (50 mM Tris-HCl [pH 8.0], 0.05 mM $CaCl_2$, and 20% glycerol) at 100 U/μl. Homogenized mixture equivalent to 10 oocytes was digested with MNase at room temperature for 20 min with enzyme at 0.16, 0.8, or 4 U/100-μl reaction mixture. The reaction was stopped with the addition of an equal volume of 2× TNESK buffer (20 mM Tris-HCl [pH 7.4], 200 mM NaCl, 2 mM EDTA, and 2% sodium dodecyl sulfate [SDS]) followed by RNase A treatment (10 mg/ml) and proteinase K digestion (15 mg/ml). The DNA was purified by phenol-chloroform extraction and isopropanol precipitation. The purified DNA was analyzed by electrophoresis on a 1.5% agarose gel and Southern hybridization with a ^{32}P-labeled LTR probe. Each experiment was done at least twice with similar results.

Plasmid DNA supercoiling assay. The supercoiling assay was based on the chloroquine agarose gel system described previously (5). To analyze the topology of injected DNA, DNA was purified from the oocytes and electrophoresed on a 1.2% agarose gel in 1× TPE (40 mM Tris, 30 mM NaH_2PO_4, 10 mM EDTA) containing 90 μg of chloroquine per ml in both the gel and running buffer. Gel electrophoresis was carried out at 3.5 V/cm in the dark for 6 h, and the gels were washed with water to remove chloroquine and then subjected to Southern analysis with a ^{32}P-labeled LTR promoter probe. Each experiment was done at least twice with similar results.

ChIP assay. *Xenopus* oocytes were microinjected with pHL10 and/or TR/RXR mRNA and incubated as described above. A group of 10 oocytes was then treated with a 2% formaldehyde solution (700 μl of 0.25 M Tris and 40 μl of 37% formaldehyde) for 30 min at room temperature. The oocytes were then sonicated on ice for 8 bursts of 10 s each in 400 μl of SDS lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris-HCl [pH 8.1]) containing 1 mM phenylmethylsulfonyl fluoride, 1 μg of aprotinin per ml, and 1 μg of pepstatin A per ml. The sonicated, lysed oocyte mixture was spun for 10 min at 15,000 rpm with a refrigerated Eppendorf microfuge at 4°C, and the supernatant was diluted 10-fold with dilution buffer (25 ml; 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl [pH 8.1], and 167 mM NaCl) containing protease inhibitor as described above. Immunoprecipitation was then performed with a chromatin immunoprecipitation (ChIP) assay kit essentially as described by the manufacturer with an antibody against one of the following proteins: acetylated H_4, H_3 acetylated at residue K9 (Upstate Biotechnology, Lake Placid, N.Y.), *Xenopus* TR and RXR (63), *Xenopus* N-CoR (22), *Xenopus* Rpd3 and Sin3 (58), and *Xenopus* SMRT (L. M. Sachs, P. L. Jones, and Y.-B. Shi, unpublished data). To analyze the DNA immunoprecipitated by the antibodies, PCR amplification was performed on the precipitated DNA with LTR primers (5′-TAC CAC AAG GGA CTT TCC GCT G-3′ and 5′-TGT TCT TTA CGA TGC CAT TGG G-3′) or control primers (5′-CGA TGC CTG TAG CAA TGC CAA C-3′ and 5′-AGC AA AAA CCA GCC AGC CG-3′), which amplified a fragment of the ampicillin resistance gene of the plasmid. For each sample, a 50-μl, 25-cycle PCR was carried out in the presence of 10 pmol of the primers. Each cycle consisted of 1 min at 94°C, 40 s at 50°C, and 1 min at 72°C. Each experiment was done at least twice with similar results.

RESULTS

Both TR and TR/RXR bind to the HIV-1 LTR in vivo and regulate its activity. TRs are known to be able to bind to TREs as monomers, homodimers, or heterodimers formed with RXRs. As the putative HIV-1 TREs diverge considerably from the consensus TRE, made up of two direct repeats of AGG TCA separated by 4 bp (Fig. 1), we investigated TR binding to the TREs in the presence or absence of RXR. We focused on the HIV-1 TRE located in the region containing the Sp1 binding sites, i.e., Sp1 TRE, as it is a stronger TRE for TR/RXR heterodimer than NF-κB TRE (Fig. 1) (17). For comparison, we used the TRE in the *Xenopus* TRβA gene (TRβA TRE), a strong TRE consisting of two near-perfect repeats of AGGTCA separated by 4 bp (42). In vitro binding studies using ^{32}P-labeled double-stranded TRE oligonucleotides and purified *Xenopus* TRβ and RXRα produced in *Escherichia coli* showed that TR/RXR heterodimer bound strongly to TRβA TRE but only weakly to Sp1 TRE (data not shown), in agreement with earlier observations (17, 42). Interestingly, TR alone appeared to have affinities comparable to those of TRβA TRE and Sp1 TRE and bind to Sp1 TRE with an affinity equal to or slightly higher than that of TR/RXR (data not shown).

Although TR and TR/RXR can bind to the HIV-1 TREs in vitro, it is unknown if TR binds to the TREs in vivo in the context of chromatin. The weak affinities of the TREs for TR or TR/RXR make it impossible to detect the binding in vivo with traditional methods such as in vivo DNase I footprinting. Thus, we investigated the binding of TR to the HIV-1 TREs in vivo by using the ChIP assay. We cross-linked the DNA and proteins in the plasmid minichromosome directly in the oocyte and sonicated the sample to shear the minichromosome to an average of about 0.5 kb (Fig. 2A). The sonicated minichromosome was immunoprecipitated with antibodies against TR (Fig. 2B) or RXR (Fig. 2C). Analysis of the immunoprecipitated DNA by qualitative PCR showed that the HIV-1 TRE region was bound by TR independently of RXR or T_3 (Fig. 2B). This indicates that TR binds to the LTR constitutively and is in agreement with the similar affinities for the TRE of TR and TR/RXR in vitro (see above). As a control, a fragment within the ampicillin resistance gene was shown to be not bound by TR under any conditions, as only a very weak signal was present independently of the presence or absence of TR in the oocytes (Fig. 2B).

The binding of RXR to the LTR, on the other hand, was dependent on the presence of TR but not T_3 (Fig. 2C). Thus, the LTR fragment was not immunoprecipitated by RXR antibodies when only TR or RXR was expressed in the oocytes. When TR was coexpressed with RXR, RXR was found to be associated with the LTR in the absence or presence of T_3 (Fig. 2C, lanes 2 and 3). These results indicate that both TR/RXR and TR bind to the LTR in vivo.

We then investigated whether TR alone could regulate the LTR similarly to TR/RXR. Oocytes were injected into the cytoplasm, with mRNAs encoding TR or TR and RXR followed by the nuclear injection of single-stranded pHL10. As shown in Fig. 3, TR alone functioned similarly to TR/RXR. In the absence of T_3, both TR and TR/RXR efficiently repressed the LTR. T_3 and the histone deacetylase inhibitor TSA (71) each relieved the repression and further activated the promoter, suggesting that both TR and TR/RXR employ histone deacetylase complexes to repress the LTR in the absence of T_3. The similar levels of regulation by TR and TR/RXR were also in agreement with the fact that TR and TR/RXR bound to Sp1 TRE with similar affinities (see above).

T_3-bound TR/RXR disrupts LTR chromatin independently of transcriptional activation. To investigate whether transcriptional regulation of the LTR by TR alters the chromatin configuration at the LTR promoter, we studied the structure of the LTR plasmid minichromosome in vivo by using an MNase digestion assay. Transcriptional activation of the LTR promoter by TR/RXR in the presence of T_3 led to the disruption of the ordered nucleosomal structure of the minichromosome as indicated by the loss of the nucleosomal ladder generated by the MNase digestion of the minichromosome (Fig. 4A, compare lanes 4 to 6 to lanes 1 to 3). Similar results were found when only TR was present (data not shown). To rule out the possibility that the disruption was due to the movement of the huge RNA polymerase complex along the chromatin, thus altering the structure of the chromatin, we treated the oocytes with the drug α-amanitin, to block transcription elongation from the LTR promoter (Fig. 4B), and analyzed the resulting chromatin structure. Again, we found that TR/RXR disrupted the chromatin in the presence of T_3 (Fig. 4A, lanes 7 to 9). Thus, chromatin disruption at the LTR by T_3-bound TR/RXR is independent of transcription process itself and is an intrinsic property of the liganded TR bound to the LTR.

To demonstrate that the disruption was due to direct binding of liganded TR to the LTR, we replaced TR with a mutant TR lacking the DNA binding domain (37). MNase digestion assay

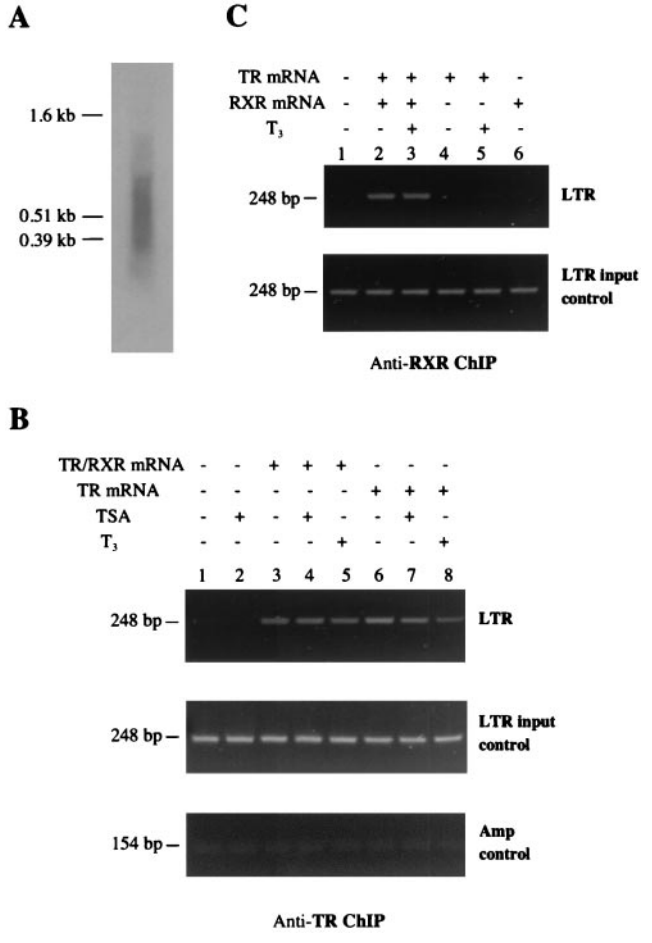

FIG. 2. A ChIP assay demonstrates the binding of TR or TR/RXR to the LTR promoter in vivo. (A) The LTR minichromosome was sheared to an average of 0.5 kb by sonication during the ChIP assay. Oocytes were injected with the LTR plasmid, treated with formaldehyde to cross-link the DNA and protein in the minichromosome, and sonicated. The cross-links were then reversed as in the ChIP assay. The DNA was purified and subjected to Southern blot hybridization with a random-primed, ^{32}P-labeled plasmid probe. DNA size markers are on the left. (B) ChIP assay shows that TR binds to LTR constitutively in vivo. The oocytes injected with the LTR plasmid and indicated mRNAs were treated with formaldehyde and sonicated after treatment with T_3 and/or TSA. The sonicated LTR minichromosome was immunoprecipitated with an anti-TRβ antibody (63), and the precipitated DNA was analyzed by qualitative PCR. Note that TR bound to the LTR in the presence or absence of RXR and independently of T_3 and/or TSA treatment. Little LTR DNA was immunoprecipitated by the anti-TR antibody when no TR mRNA was injected into the oocytes (controls, lanes 1 and 2), and equal amounts of DNA were present in all samples prior to immunoprecipitation (LTR input control). In addition, only a very weak signal independent of TR expression was detected for the ampicillin resistance gene (Amp control), indicating that the sonication sheared the minichromosome sufficiently to separate the LTR from the ampicillin resistance gene in the plasmid and that the antibody was specific in precipitating TR-bound DNA. (C) ChIP assay indicates that RXR binds to the LTR only as a TR/RXR heterodimer. The experiments were done as described for panel B except for the use of anti-RXR antibody (63). Note that the anti-RXR antibody precipitated the LTR only when TR was also present (lanes 2 and 3) but independently of T_3. Again, equal amounts of DNA were present prior to immunoprecipitation (LTR input control).

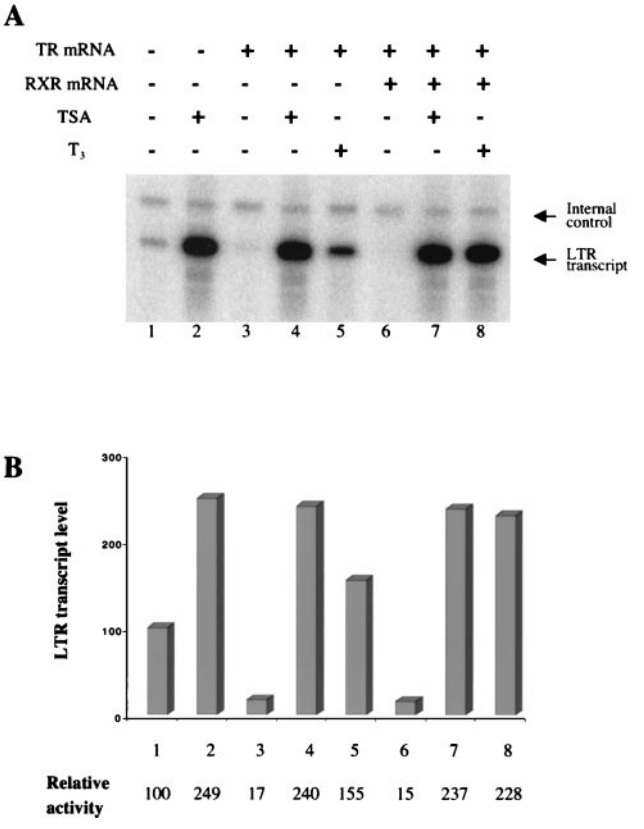

FIG. 3. Both TR and TR/RXR repress the LTR promoter in the absence of T_3 in a histone deacetylase-dependent manner. (A) Oocytes were injected with either TR or TR/RXR mRNAs followed by injection of the LTR plasmid. After overnight treatment, the promoter activity was analyzed by primer extension with a CAT1 primer (LTR transcript) and histone H_4 primer (internal control). (B) Quantification of the data in panel A by PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.). Note that unliganded TR and TR/RXR repressed the promoter similarly (about 6-fold, lanes 3 and 6). The addition of either T_3 or TSA reversed the inhibition and further activation, resulting in an overall change of 9- to 15-fold in promoter activity.

showed that the mutant TR had no detectible effect on the chromatin structure in the presence of T_3 (Fig. 4C, lanes 7 to 9), while under the same conditions, the wild-type TR disrupted the ordered nucleosomal array (lanes 4 to 6); thus, DNA binding by TR is essential for its ability to alter the LTR chromatin structure in the presence of T_3.

Based on the finding that like T_3, TSA could relieve the repression by unliganded TR/RXR and further activate the LTR (Fig. 3), we investigated whether TSA treatment could alter the chromatin structure similarly to T_3. MNase digestion of the LTR minichromosome revealed that again TR/RXR disrupted the chromatin structure in the presence of T_3 (Fig. 5A, lanes 13 to 15) but not in its absence (lanes 7 to 9). Interestingly, while TSA could relieve the repression by unliganded TR/RXR, it had little effect on chromatin structure either by itself (Fig. 5, lanes 4 to 6) or in the presence of TR/RXR (lanes 10 to 12).

To further investigate the chromatin disruption, we used a more sensitive method that measures the superhelical density of the plasmid DNA. This is based on the fact that closed circular plasmids with different superhelical densities migrate

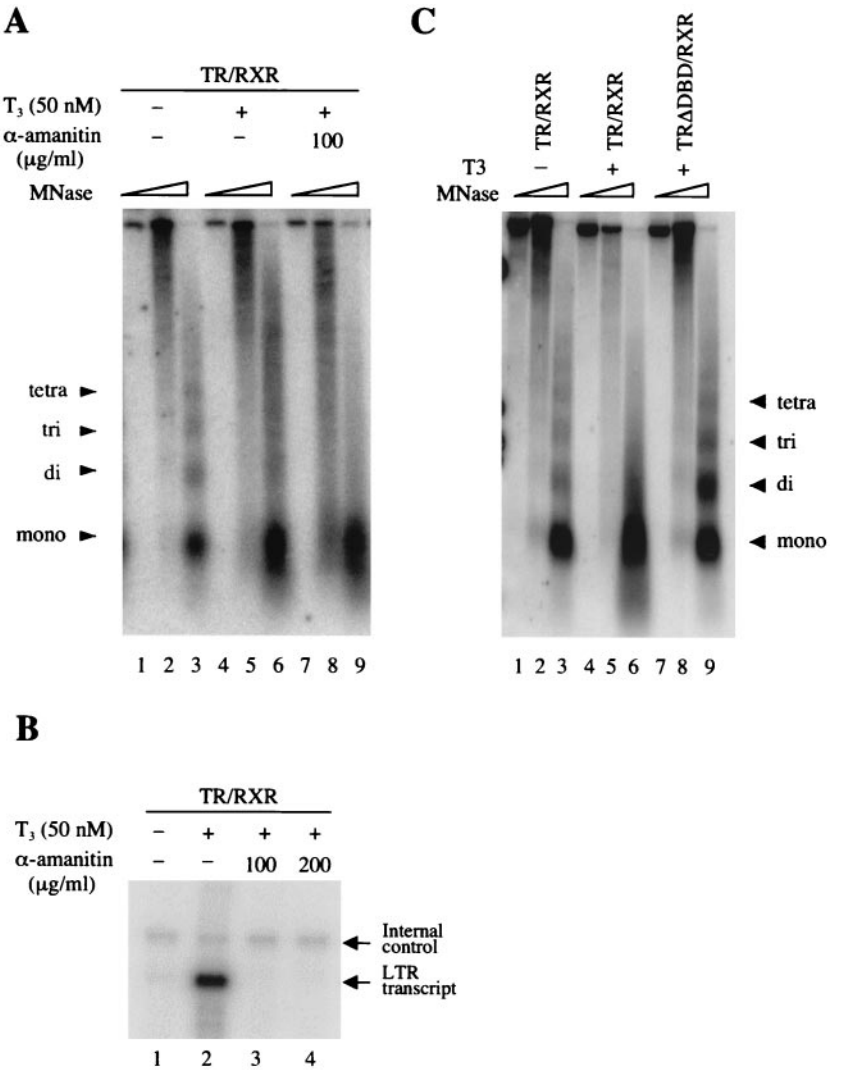

FIG. 4. Liganded TR disrupts chromatin at the LTR through direct binding to the LTR but independently of transcriptional elongation. (A) An MNase digestion assay reveals that liganded TR disrupts the ordered nucleosome array on the LTR plasmid. Oocytes were injected and treated as described for Fig. 3 except for the presence or absence of 100 μg of α-amanitin per ml. After overnight incubation, the oocytes were harvested for the MNase digestion assay with increasing amounts of MNase (0.16, 0.8, and 4 U). The digested DNA was purified and analyzed by Southern blot analysis with a labeled LTR probe. Note that in the absence of T_3 (lanes 1 to 3) or TR/RXR (not shown), an ordered nucleosomal array was present on the LTR plasmid, as indicated by the presence of the mono-, di-, and trinucleosome bands, etc. In the presence of T_3 and TR/RXR, this ordered structure was disrupted, as indicated by the presence of a smear instead of discrete oligonucleosomal bands (lanes 4 to 6). Blocking transcriptional elongation with α-amanitin had no effect on the T_3-induced chromatin disruption (lanes 7 to 9), suggesting that the disruption is an intrinsic property of liganded TR bound at the LTR. (B) Transcriptional activation of the LTR is inhibited by the elongation inhibitor α-amanitin. Oocytes were injected and treated as described above and were then processed for primer extension analysis. Note that TR/RXR activated the LTR in the presence of T_3, and α-amanitin blocked transcription from the LTR. (C) Chromatin disruption requires the DNA binding domain of TR. mRNA encoding TR or a mutant TR lacking the DNA binding domain (TRΔDBD) (37) was injected into oocytes with RXR mRNA followed by the LTR plasmid injection. After overnight incubation in the presence or absence of T_3, the plasmid minichromosome was isolated and analyzed as described for panel A. Note that disruption of the ordered nucleosomal array was observed with the liganded TR/RXR (compare lanes 4 to 6 to lanes 1 to 3) but not with the liganded TRΔDBD/RXR (lanes 7 to 9), indicating that the binding of TR to the LTR in the presence of T_3 is necessary for chromatin disruption.

differently on a native chloroquine-containing agarose gel. As shown in Fig. 5B, T_3 treatment of oocytes containing the HIV-1 plasmid pHL10 and TR/RXR altered the superhelical structure of the plasmid DNA. On the average, a loss of two to three negative superhelical turns was observed in the presence of T_3 and TR/RXR but not TR/RXR alone (Fig. 5B and data not shown). As each nucleosome generates one negative supercoil in the plasmid DNA after deproteination, these results suggest that the change caused by liganded TR/RXR is equivalent to the loss of two to three nucleosomes. When a similar analysis was done for the TSA-treated sample, we failed to detect a shift in the average superhelical turns of the minichromosome (Fig. 5B, lane 2). In addition, the mutant TR lacking the DNA binding domain also failed to alter the superhelical structure of the DNA in the presence of the ligand (data not shown), in agreement with the MNase digestion data above.

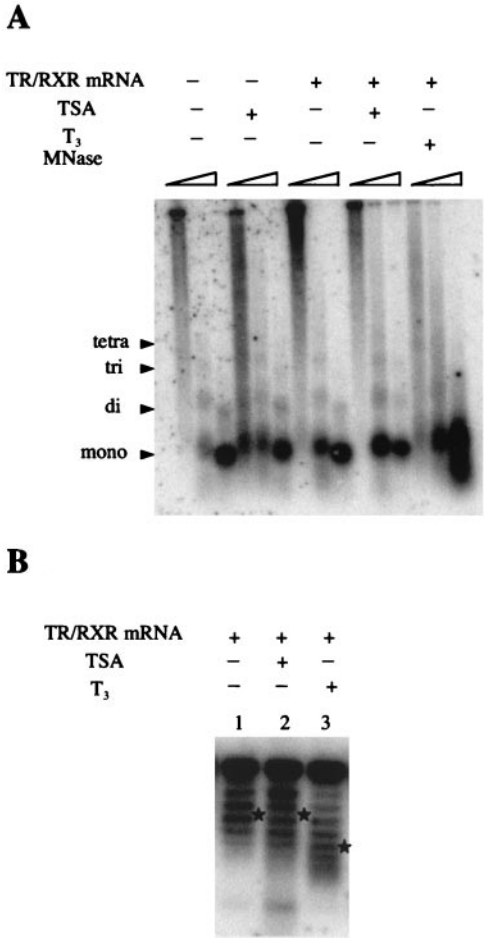

FIG. 5. Transcription activation of the HIV-1 LTR by T_3 but not TSA leads to chromatin disruption. (A) An MNase digestion assay shows that TSA treatment does not affect the nucleosomal array on the LTR plasmid. The oocytes were injected and treated as described for Fig. 4, except for TSA treatment where indicated. They were then processed for the MNase digestion assay. Again, T_3 and TR/RXR together led to chromatin disruption (compare lanes 13 to 15 to lanes 1 to 3). In contrast, TR/RXR alone (lanes 7 to 9), TSA alone (lanes 4 to 6), and TSA plus TR/RXR (lanes 10 to 12) failed to alter the ordered nucleosomal array structure. (B) DNA topology analysis demonstrates that T_3 but not TSA induces gross alterations of the structure of the LTR minichromosome. Oocytes were injected and treated as described for panel A, and LTR plasmid DNA was isolated for the supercoiling assay. After electrophoresis on a chloroquine-containing gel to separate the DNA with different numbers of negative superhelical turns (the higher the number of negative superhelical turns, the slower the DNA migration on the gel), the DNA was detected by Southern blot hybridization with a labeled LTR probe. Note that the average number of negative superhelical turns (star) was reduced by two to three when both TR/RXR and T_3 were present (compare lanes 3 and 1). In contrast, TSA had little effect on the number of superhelical turns on the plasmid (compare lanes 2 and 1).

Thus, both assays showed that liganded TR binds to the LTR to disrupt chromatin and activate the transcription while TSA can activate the LTR promoter without altering the gross structure of the minichromosome in vivo.

Transcription repression by unliganded TR is associated with histone deacetylation at the LTR promoter. Based on biochemical and tissue culture cell studies, unliganded TR is presumed to recruit corepressor complexes containing histone deacetylases to facilitate transcriptional repression. The addition of T_3 leads to the release of the corepressor complexes and recruitment of coactivator complexes, many of which contain histone acetyltransferase activity (see the introduction). Thus, to investigate how TR represses HIV-1 LTR in the absence of the ligand, we analyzed local histone acetylation at the LTR by using ChIP assays with antibodies against acetylated histone H_4 or H_3. The results showed that the presence of either TR or TR/RXR led to a reduction in the acetylation levels of both histone H_4 and H_3 at the LTR (Fig. 6, compare lanes 3 and 6 to lane 1), and the addition of T_3 reversed this reduction (Fig. 6, lanes 5 and 8). Similarly, inhibiting histone deacetylases with TSA also eliminated the deacetylation caused by unliganded TR or TR/RXR. These results support the view that a histone deacetylase complex(es) was recruited by unliganded TR either by itself or as a heterodimer with RXR to the LTR to repress the promoter.

Unliganded TR recruits corepressors and a histone deacetylase to the LTR. To investigate the mechanism responsible for the histone deacetylation caused by the unliganded TR, we carried out ChIP assays using antibodies against putative corepressors that may be recruited to the LTR. We first analyzed the recruitment of the two related corepressors, N-CoR and SMRT, that bind to the unliganded TR. ChIP assays clearly showed that unliganded TR recruited both to the LTR while the addition of T_3 dissociated the binding of both corepressors to the LTR (Fig. 7). Both N-CoR and SMRT are known to form multisubunit deacetylase complexes and interact with the corepressor Sin3, which in turn binds to the deacetylase Rpd3 (15, 16, 22, 26, 32). ChIP assays using antibodies against *Xe-*

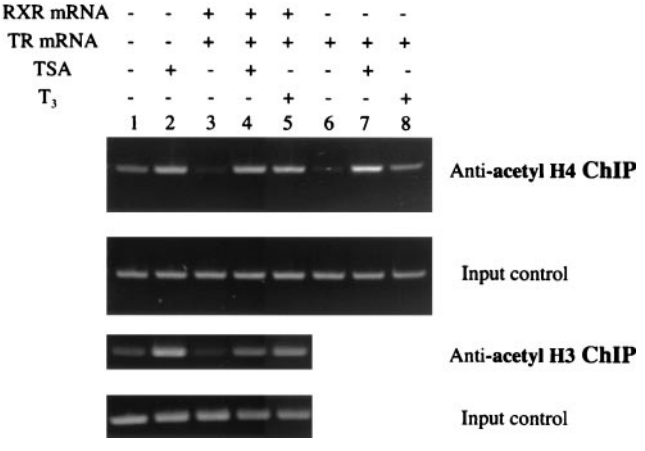

FIG. 6. The ChIP assay shows that the transcription regulation by TRs leads to alterations of histone acetylation at the LTR promoter. Oocytes were injected and treated as described for Fig. 3 and were then harvested for ChIP assay with an antibody against acetylated histone H_4 or histone H_3 acetylated at position K9. Note that the acetylation levels of both H_4 and H_3 at the LTR were decreased in the presence of unliganded TR or TR/RXR (compare lanes 6 and 3, respectively, to lane 1). The addition of TSA or T_3 treatment prevented the deacetylation of H_4 by unliganded TR/RXR (lanes 4 and 5 for TR/RXR and 7 and 8 for TR). (H_3 ChIP was not done for TR alone, as similar results were expected based on the data in the figure.) Equal amounts of DNA were present prior to immunoprecipitation (LTR input control).

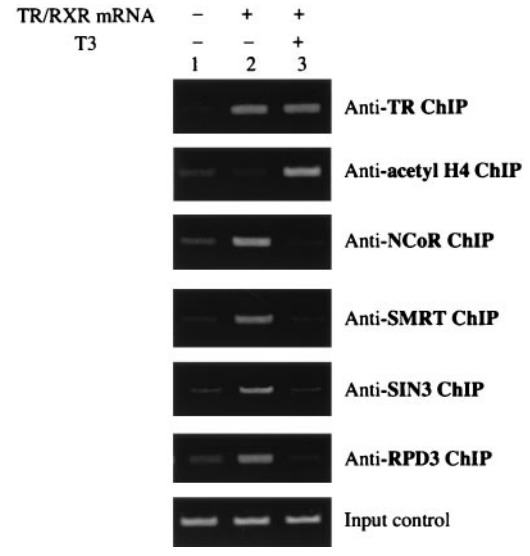

FIG. 7. Unliganded TR/RXR recruits corepressors to the LTR in a T_3-dependent manner. ChIP assays were performed as described for Fig. 6 with the indicated antibodies. As controls, anti-TR and -acetyl-H_4 ChIP assays were done to show the binding of TR to the LTR and changes in histone acetylation in the samples used for the assays of the corepressors. Note that unliganded TR/RXR recruited N-CoR, SMRT, Sin3, and Rpd3 to the LTR (compare lanes 2 and 1). T_3 treatment eliminated this recruitment (compare lane 3 to lanes 2 and 1). The LTR input control indicated that equal amounts of DNA were present during immunoprecipitation.

nopus Sin3 and Rpd3 showed that both Sin3 and Rpd3 were also recruited by unliganded but not liganded TR and their recruitment as well as that of the N-CoR and SMRT correlated with the deacetylation at the LTR (Fig. 7). Thus, unliganded TR may repress the LTR at least in part by recruiting histone deacetylase complexes through the binding to N-CoR and/or SMRT.

DISCUSSION

The HIV-1 LTR directs the expression of the proviral message. Many diverse cellular transcription factors have been shown to bind to the LTR in vitro and/or regulate LTR activity in vitro or in tissue culture cells (35). On the other hand, the roles of these factors in vivo largely remain to be demonstrated. Using the frog oocyte system, which allows us to study LTR promoter activity in the context of chromatin that mimics somatic cells, where the proviral DNA resides, we have shown here (i) that TR either alone or as a heterodimer with RXR binds to the LTR in chromatin constitutively in vivo and (ii) that transcriptional activation of the LTR by T_3 is associated with two levels of chromatin remodeling, chromatin disruption and histone acetylation.

Constitutive binding and transcriptional regulation of the HIV-1 LTR by TR in vivo. Several studies, including our earlier work, have shown that in vitro-purified TR/RXR is capable of binding to the two putative TRE sequences in the HIV-1 LTR (NF-κB TRE and SP1 TRE) (Fig. 1), although no sequence similarity to the consensus TRE can be found in the LTR (8, 17, 41, 67). On the other hand, the results in reference 41 contrasted with others' as well as ours by showing that unliganded TR activated the LTR and T_3 eliminated this activation. The different conclusions from the studies might be due to the use of different cell systems where different levels or combinations of host factors may influence how TR regulates the LTR. In addition, it is possible that TR may influence LTR activity indirectly in tissue culture cells when transient-transfection studies are carried out as in the earlier studies, since direct demonstration of TR binding to the LTR in vivo was not reported in any of the earlier studies. TR may accomplish this by regulating the expression of some host factors that are implicated to play a role in LTR activity (35, 56). Such a mechanism may also account for the contradictory findings with different tissue cultures (8, 41, 67).

The ChIP assays used here show for the first time that TR is bound to the LTR in chromatin in vivo under conditions that mimic somatic cells. In the absence of RXR, TR may bind to the LTR as either a monomer or homodimer, although our assays could not differentiate the two possibilities. In the presence of RXR, TR is likely bound to the LTR as a heterodimer with RXR, as RXR is associated with the LTR in vivo only when TR is present. The ChIP assay, on the other hand, does not have sufficient resolution to determine whether both TREs or only a single TRE is bound by TR in vivo. Our supercoiling assay suggests that only one TR is bound to the LTR under our experimental conditions (see below).

In the absence of T_3, TR or TR/RXR represses the LTR in the oocytes. The addition of T_3 relieves the repression and leads to further activation of the LTR above the basal expression level observed in the absence of both T_3 and TR. Such a regulation pattern for TR is similar to what has been found for other T_3-inducible promoters in various model systems, including the *Xenopus* TRβA gene promoter in frog oocytes (63, 65), and thus is consistent with the model that TR or TR/RXR regulates the LTR through direct binding to the LTR but not through indirect regulation of the levels and/or the functions of other host factors.

Two types of chromatin modification participate in the regulation of HIV-1 LTR by T_3. Increasing evidence indicates that chromatin structure plays important roles in regulating gene expression (23, 25, 49, 61, 66). It is known that transcriptionally active chromosome domains have structure and protein compositions distinct from those of repressed chromatin (61). Thus, it is very likely that regulation of the proviral LTR promoter may require chromatin remodeling. Our data here reveal that transcriptional regulation of HIV-1 LTR by TR involves at least two types of chromatin remodeling: histone acetylation and chromatin disruption.

Our results indicate that the transcriptional activation by T_3 alters the nucleosomal organization on the LTR plasmid. This observation was evidenced by MNase digestion and DNA topology assays. The MNase digestion assay reveals that the ordered nucleosomal array is disrupted by T_3-bound TR in an active process that is independent of transcriptional elongation. However, the LTR appears to remain associated with nucleosomes, as indicated by the presence of LTR sequence in the mononucleosomal and higher-molecular-weight DNA bands on the Southern blot of the digested DNA. This observation is similar to the finding from the in vitro study of (48), where co-occupancy of other transcription factors, including Sp1 and NF-κB, and histones at the LTR assembled into chro-

matin in vitro was observed. The TR-mediated chromatin disruption is also independently supported by our supercoiling analyses, which showed that liganded TR/RXR results in a topological change in the minichromosomes equivalent to the loss of two to three nucleosomes, although it is unlikely that the nucleosomes are actually lost. Our earlier studies on the *Xenopus* TRβA promoter, which is also regulated by T_3, showed that the binding of each TR/RXR heterodimer to a plasmid DNA leads to loss of two to three superhelical turns (64). Thus, it is likely that at steady state, only one TR or TR/RXR is bound to the LTR even though two TREs are present in the LTR. This may also be preferred physiologically because (i) multiple host factors have been implicated in LTR regulation (35, 56) and (ii) the TREs overlap with Sp1 and NF-κB binding sites and in vitro studies indicate that binding by TR/RXR and that by Sp1 or NF-κB are mutually exclusive (8). Thus, it is quite possible that the existence of multiple binding sites for these different factors allows the simultaneous binding of at least one molecule of each factor to the LTR for the promoter. This will allow the virus to respond properly and efficiently to the host environment. Such a mechanism might be advantageous and effective for the virus considering our earlier studies showing that a single binding site for TR/RXR is as effective as multiple TREs at a single location, such as in the LTR promoter, in terms of transcriptional activation by T_3 (64).

How liganded TR disrupts chromatin structure of LTR promoter remains to be investigated. Possible mechanisms include the involvement of chromatin remodeling complexes, such as SNF/SWI, NURF, and CHRAC, etc. (6, 19, 24, 34, 47, 50, 53, 54, 57, 72). It has been shown that SWI/SNF complexes are required for transcriptional activation by glucocorticoid receptor, which belongs to the nuclear receptor superfamily that includes TR (14, 31, 33, 72). Similar complexes may be involved in chromatin disruption by liganded TR/RXR. In addition, a number of TR-interacting coactivators have been identified. Some have histone acetyltransferase activities, while others do not (4, 20, 30, 39, 68, 69). Some of these cofactors and associated complexes may contribute to chromatin disruption, which may in turn facilitate downstream steps in the activation of the LTR by T_3 in vivo.

In addition to chromatin disruption, we also found that in the absence of T_3, the binding of TR or TR/RXR to the LTR leads to a reduction of local histone deacetylation level at the LTR. Exactly how this occurs remains to be determined. It is most likely due to the recruitment of a corepressor complex(es) to the promoter by unliganded TR. TR is known to bind several corepressors, such as N-CoR and SMRT, in the absence of T_3 (2, 69, 73). These corepressors are known to associate with histone deacetylases in large multisubunit complexes. Recently, several groups have biochemically purified histone deacetylase complexes containing the TR-binding corepressors SMRT and N-CoR (15, 22, 26). Three N-CoR complexes in the *Xenopus* oocytes have been reported, and two of them have histone deacetylase activities (22). One member of the histone deacetylase-containing complex has Sin3 and Rpd3 or histone deacetylase 1, both of which have been known to associate with N-CoR or SMRT (16, 32). Although a similar complex(es) has not been identified in mammals, and immunoprecipitation studies have so far failed to detect an association of TR with Sin3 or Rpd3 in the frog oocyte under our assay conditions (P. Jones and Y.-B. Shi, unpublished observation), our ChIP assays clearly demonstrated an enhanced association of Sin3 and Rpd3 with the LTR in the presence of unliganded TR, possibly as part of the N-CoR or SMRT complex recruited by TR. This apparent discrepancy between the ChIP assay and coimmunoprecipitation may be due to differences in assay sensitivities. Alternatively, Sin3 and Rpd3 may be more stably associated with the TR in the chromatin context of the repressed promoter, as measured by the ChIP assay, than in solution, as measured by coimmunoprecipitation. Regardless, the recruitment of Sin3/Rpd3 containing deacetylase complex and/or another deacetylase complex(es) may be responsible for the deacetylation of the LTR by unliganded TR.

Upon the addition of T_3, all the corepressors were released from the LTR, which may be responsible for the increase in histone acetylation caused by T_3. In addition, T_3-bound TR can also recruit histone acetyltransferase complexes, as many TR-binding coactivators are themselves histone acetyltransferases (3, 30, 39, 68, 69, 73). While we are unable to determine if any of these acetyltransferase complexes are recruited by liganded TR to the LTR due to the lack of good antibodies recognizing their *Xenopus* homologs (the vast majority of them are not yet cloned in *Xenopus*), some may contribute to the T_3-induced histone acetylation at the LTR.

While histone acetylation is clearly important for the regulation of the LTR by TR, it is not responsible for the chromatin disruption that we have observed when both T_3 and TR are present. This is clearly demonstrated by the lack of chromatin disruption of the TR-bound LTR minichromosome in oocytes treated with TSA, which induces histone acetylation and transcriptional activation. While it is possible that T_3-induced chromatin disruption is not required for transcriptional activation of the LTR repressed by unliganded TR, it is more likely that TSA may play additional roles than simply reversing the deacetylation brought about by unliganded TR, thus activating the LTR even though it does not induce alterations in chromatin structure that can be detected by our assays. Indeed, histone acetylation has also been shown to alter DNA accessibility to nucleases and enhance the activity of the HIV-1 LTR assembled into chromatin in vitro in the absence of TR and RXR (45, 46). Thus, it is likely that T_3-induced activation of the LTR bound by TR in a native chromatin environment involves a multistep mechanism that requires the participation both of gross chromatin reorganization and disruption and of histone acetylation. Such a conclusion is also consistent with studies on the regulation of other promoters by TR or other nuclear receptors, where both histone acetylation and chromatin disruption were found to contribute to promoter activity (9, 10, 62, 64). The consequence of these chromatin modifications is the facilitation of the access of the other transcription factors and/or transcriptional machinery as well as the subsequent steps leading to the activation of the promoter. It is clearly important to determine the participants in these processes and their functional pathways.

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