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Thyroid hormone receptors bind to the promoter of the mouse histone $H1^0$ gene and modulate its transcription

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ABSTRACT

It has been shown that the mouse histone $H1^0$ promoter contains a DNA element, composed of a direct repeat of the sequence GGTGACC separated by 7 nt, which is able to bind retinoic acid receptors and to modulate transcription of reporter genes following treatment with retinoic acid. We have now investigated whether this DNA motif is also responsive to thyroid hormone. We co-transfected CV-1 monkey kidney cells with chloramphenicol acetyltransferase (CAT) expression plasmids containing either 740 bp of the $H1^0$ wild-type promoter or five copies of the repeat element cloned in front of the thymidine kinase promoter and expression vectors for human thyroid hormone receptors (TRs) α or β and retinoid X receptor α (RXRα). Treatment of transfected cells with triiodothyronine led to a dose-dependent increase in CAT activity. Transfection experiments with increasing amounts of expression vectors for either TRα or RXRα resulted in up to 6-fold enhancement of CAT transcription. Furthermore, point mutations within the half-sites of the response element of the $H1^0$ promoter, as well as deletions within the interspace region, lowered CAT activity to 60–80% of that of the wild-type control. Electrophoretic mobility shift assays showed that the repeat element was able to form retarded complexes with TRα homodimers, as well as with TRα–RXRα heterodimers. Our results suggest that thyroid hormone receptors are involved in the regulation of mouse histone $H1^0$ expression.

INTRODUCTION

Histone H1 proteins are known to interact with the DNA connecting the nucleosomal core particles, each of which is composed of two molecules of histones H2A, H2B, H3 and H4 and 146 bp of DNA (1). The H1 linker histones have been shown to participate in the control of transcription and may influence the accessibility of regulatory DNA regions at the chromatin level (2–3). One of the H1 subtypes, $H1^0$, has been found to accumulate in terminally differentiated cells and may be implicated in fixation and maintenance of differentiated phenotypes (4–6).

Treatment of mouse F9 teratocarcinoma stem cells with retinoic acid (RA), which leads to differentiation of the cells to parietal endoderm, is accompanied by an early increase in $H1^0$ mRNA transcription (7–8). Furthermore, it has been reported for other embryonal carcinoma cell lines that induction with RA leads to an increase in $H1^0$ mRNA and $H1^0$ protein (9). These data suggest that this linker histone is associated with commitment of cells to differentiation.

Molecular cloning of the $H1^0$ promoter region revealed a high conservation between rodents and humans (10–11), suggesting that important control motifs have been conserved throughout evolution. The mouse $H1^0$ promoter contains multiple *cis*-acting elements, one of which is composed of a direct repeat of the sequence GGTGACC, separated by 7 nt and found ~540 bp upstream of the transcription start point (12–13). We have recently shown that this DNA motif is able to bind retinoic acid receptors (RARs) and to confer RA responsiveness on a heterologous thymidine kinase (*tk*) promoter in F9 and HeLa cells (14). In addition, *in vivo* studies on transgenic mice demonstrated a strong correlation between the expression patterns of a transgene containing a 3.2 kb fragment of the mouse $H1^0$ promoter region cloned in front of the β-galactosidase (β-Gal) reporter gene and thyroid hormone receptors (TRs; 15,16). Furthermore, it has been reported that hypophysectomy of rats is accompanied by loss of $H1^0$ protein in the thyroid gland and that treatment with thyrotropin, which stimulates thyroid hormone production, results in reappearance of the protein (4). Thus it seems likely that $H1^0$ transcription is modulated by different steroid hormones.

TRs are ligand-inducible members of the superfamily of nuclear hormone receptors, which regulate expression of target genes by binding to specific response elements located within regulatory regions of these genes (17;18, and references therein). Current opinion suggests that TRs bind to thyroid hormone response elements (TREs) consisting of two half-sites of a consensus hexanucleotide sequence AGGTCA arranged as direct repeats with a 4 bp spacer region (19–20). TRs have been shown to bind to TREs as monomers, homodimers or heterodimers with retinoid X receptors (RXRs), thereby modulating transcription of responsive genes (21–26). It has also been shown that some

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response elements are co-regulated by both RA and thyroid hormone (3,5,3′-triiodothyronine, T3) (27–29). It seems likely that several members of the steroid hormone receptor family would bind to the retinoic acid responsive element (RARE) described for the mouse histone $H1^0$ promoter, thus differentially modulating histone $H1^0$ transcription. Here we present evidence that TRs bind to the tandem repeat motif in the promoter region of the mouse $H1^0$ gene, either as monomers or as heterodimers with RXRs, and that T3 enhances transcription of CAT reporter constructs in transient transfection experiments in CV-1 cells.

MATERIALS AND METHODS

Construction of plasmids

Recombinants used in this study were derived from a construct that contains 740 bp of the mouse $H1^0$ promoter cloned in front of the CAT gene (13; hereafter referred to as $H1^0$-wt). By PCR-based site-directed mutagenesis we generated two plasmids containing point mutations in either the first (5′-GGTGACC-3′→5′-AATGACC-3′) or second half-site (5′-GGTGACC-3′→5′-GGTGAAA-3′) of the $H1^0$ RARE, which is composed of the sequence GGTGACCCCCGAGAGGTGACC. Taking advantage of the fact that the 7 bp spacer region (CCCGAGA) contains an *Ava*I restriction site (CCCGAG), a recombinant that contained both mutations could be generated by standard cloning procedures. Constructs $H1^0$–1, $H1^0$–2 and $H1^0$–3, containing 1, 2 and 3 bp deletions within the 7 bp spacer region of the RARE respectively, were created by eliminating the RARE wild-type sequence that contains two *Bst*EII restriction sites (GGTGACC) and by inserting one of the double-stranded oligonucleotides RAREΔ1–RAREΔ3 (given below) by standard cloning procedures. All constructs were verified by DNA sequencing. In addition, we used a construct containing five copies of the RARE wild-type sequence cloned head-to-tail in front of the *tk* promoter into the vector pBLCAT2 (14). Oligonucleotides used for generation of $H1^0$ clones containing 1, 2 or 3 bp deletions within the 7 bp spacer region of the RARE were: RAREΔ1, 5′-GTGACCCCCAGAG-3′; RAREΔ2, 5′-GTGACCCCCGAG-3′; RAREΔ3, 5′-GTGACCCCGAG-3′.

Cell culture and transient transfections

CV-1 and COS-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. In order to eliminate T3, fetal calf serum used for CV-1 cell culture was incubated for 30 min with Norit A (50 mg/ml), followed by centrifugation and sterile filtration. Unless otherwise noted, CV-1 were plated at ~3×10^5 cells/6 cm dish and co-transfected the following day with 3 μg pCMVβ-Gal DNA, 0.5 μg both TRα and RXRα expression vectors and 3.5 μg $H1^0$–CAT reporter construct using the calcium-phosphate protocol described by Chen and Okayama (30). After incubating for 16–20 h at 35°C/3% CO_2 the medium was replaced by fresh medium with or without 10^{-7} M T3 and incubated at 37°C/5% CO_2 for 48 h. Thereafter cell extracts were prepared with cell lysis buffer (Promega, Madison, WI). An aliquot of 10 μl of each cell extract was used to determine β-Gal activity, which was used to normalize for differences in transfection efficiency. Cell extracts were heated at 60°C for 10 min before CAT analysis. CAT assays were performed at 37°C for 1 h in a total volume of 50 μl containing 0.1 mCi [^{14}C]chloramphenicol and acetyl CoA at a final concentration of 0.4 mM. Reaction products were separated by thin layer chromatography. CAT activity was determined by liquid scintillation counting of the product spots. In some experiments CAT assays were performed using the Quan-T-CAT™ assay system following the recommendations of the supplier (Amersham International, Little Chalfont, UK).

Electrophoretic mobility shift assay (EMSA)

Whole cell extracts from COS-1 cells transfected with TRα or RXRα expression vectors were incubated in a 20 μl reaction mixture containing 10 mM Tris–HCl, pH 7.5, 50 mM NaCl, 5% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 2 μg poly(dI–dC) and ~50 000 c.p.m. 5′-^{32}P-labelled $H1^0$-wt or mutated $H1^0$ oligonucleotides (see Table 1) for 20 min at room temperature. Protein–DNA complexes were resolved on a 6% polyacrylamide gel in 0.5× TBE at 4°C. After drying the gels were exposed to X-ray films at –70°C using an intensifying screen.

Table 1. DNA oligonucleotides used for electrophoretic mobility shift assay[a]

Name	DNA sequence (5′→3′)
$H1^0$-wt:	TGGTCG**GGTGACC**CCCGAGA**GGTGACC**GGACCCT
MB1	----------AA----------
MB2	----------aa----------
MB1/2	----------aa----------aa----------
$H1^0$–1	---------- ----------
$H1^0$–2	---------- ----------
$H1^0$–3	---------- ----------
DR4	TACTTAT**AGGTCA**CATG**AGGTCA**AGTTAC

[a]The two half-sites of the $H1^0$ RARE are underlined within the sequence of the $H1^0$ wild-type oligonucleotide ($H1^0$-wt). Oligonucleotides MB1, MB2 and MB1/2 contain point mutations within the upstream half-site, the downstream half-site and both half-sites respectively. Oligonucleotides $H1^0$–1, $H1^0$–2 and $H1^0$–3 have 1, 2 and 3 bp deletions respectively within the 7 bp spacer region of the RARE. Oligonucleotide DR4 represents a TRE with two half-sites (underlined) arranged as a direct repeat with a 4 bp nucleotide gap (20).

RESULTS

Thyroid hormone receptors up-regulate transcription from the $H1^0$ promoter in a T3-dependent manner

Since *in vivo* experiments with transgenic mice and hypophysectomized rats suggested a correlation between the expression of linker histone $H1^0$ and TRs (4,16), we designed a series of experiments in order to analyse whether $H1^0$ expression is controlled by thyroid hormone receptors and thyroid hormone. For this purpose we used a reporter construct containing 740 bp of the proximal $H1^0$ promoter region cloned in front of the *CAT* gene ($H1^0$-wt; see 13). In addition, we used a recombinant containing five copies of a DNA element, composed of the sequence GGTGACCCCCGAGAGGTGACC and located ~540 bp upstream of the transcription start point of the $H1^0$ gene (multimer). This element has been shown to bind RARs and to modulate transcription of reporter genes (14). Sequence similarity of this DNA motif to canonical TREs suggested that it may not

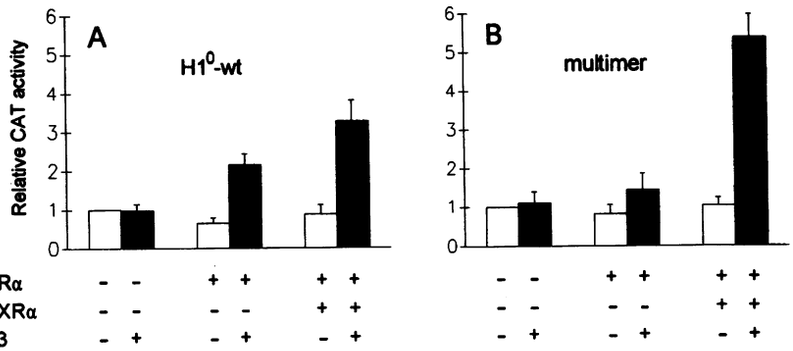

Figure 1. Thyroid hormone stimulates transcription from the mouse $H1^0$ promoter in transiently transfected CV-1 cells. CV-1 cells were co-transfected with 2.5 μg pCMVβ-Gal DNA and 3.5 μg $H1^0$-wild-type or multimer CAT reporter plasmid in the presence (+) or absence (–) of 0.5 μg TRα and RXRα expression vectors and carrier DNA (Bluescript) to a final amount of 7.5 μg DNA. After incubation for 16–20 h at 35°C/3% CO_2 the medium was replaced by fresh medium with (+) or without (–) 10^{-7} M T3 and incubated at 37°C/5% CO_2 for 48 h. Thereafter cell extracts were prepared. Aliquots of 10 μl of each cell extract was used to determine β-Gal activity, which was used to normalize for differences in transfection efficiency. CAT assays were performed at 37°C for 1 h. CAT activities obtained for constructs $H1^0$-wt (**A**) and multimer (**B**) in the absence of T3 as well as TRα and RXRα expression vectors were set to 1.

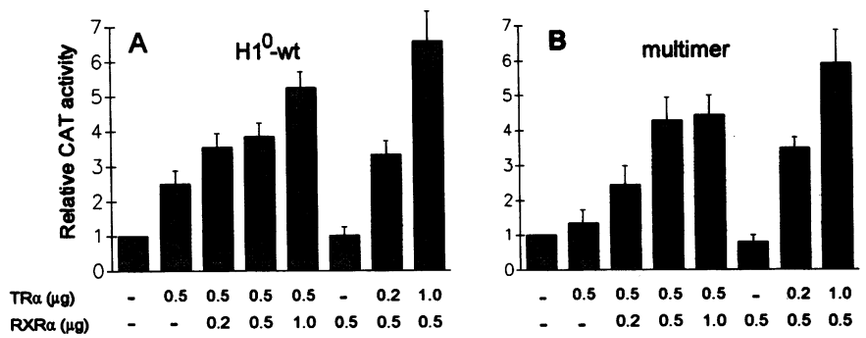

Figure 2. Effect of increasing amounts of TRα and RXRα expression vectors on the promoter activity of $H1^0$-wt and multimer CAT constructs. CV-1 cells were co-transfected with 2.5 μg β-Gal DNA, 3.5 μg $H1^0$-wt or multimer *CAT* reporter plasmids, different amounts of TRα and RXRα expression vectors and carrier DNA (Bluescript) to a final amount of 7.5 μg DNA in the presence of 10^{-7} M T3. CAT analysis was performed as described in the legend to Figure 1. CAT activities of the samples were normalized to that obtained for $H1^0$-wt (**A**) or multimer (**B**) constructs in the absence of TRα and RXRα expression vectors.

only be responsive to RA, but also to thyroid hormones. To test this hypothesis we transiently transfected the $H1^0$ plasmids into CV-1 cells together with constructs expressing human TRα and RXRα. After transfection the cells were cultured for 48 h either in the presence (+) or absence (–) of 10^{-7} M T3. Thereafter cell extracts were prepared and CAT activity was determined.

As shown in Figure 1, addition of T3 to the transfected cells did not increase CAT activity of $H1^0$-wt and multimer constructs in the absence of co-transfected receptors. Co-transfection of TRα resulted in an ~1.5–3-fold increase in CAT transcription. Furthermore, when both TRα and RXRα were co-transfected an ~3.5-fold induction of CAT activity was observed using the $H1^0$-wt construct (Fig. 1A). Similar results were obtained when TRβ was used instead of TRα (data not shown). The stimulatory effect was even more pronounced when using the multimer construct (Fig. 1B). Enhanced transcription after co-transfection with TRs and RXRs has been reported for other regulatory sequences as well and seems to reflect inceased binding of TRs to the respective elements in the presence of RXRs (21–26). These findings suggest that the RARE identified in the mouse $H1^0$ promoter is also activated by T3 upon binding of TRs.

Transcription from the $H1^0$ promoter is dependent on the amount of transfected thyroid hormone receptor

If transcription of the $H1^0$ gene is dependent on TRs an increase in the amount of DNA encoding the receptor should increase the transcription rate, since in the standard transfection experiments described above an excess of CAT reporter plasmids was used compared with co-transfected receptors. To test this hypothesis we performed co-transfection experiments on CV-1 cells using either $H1^0$-wt or the multimer recombinant and different molar concentrations of TRα and RXRα expression vectors. As shown in Figure 2, increasing amounts of expression vectors resulted in an up to 6-fold increase in CAT activity compared with control transfections. Enhancement of CAT activity was observed for both types of receptors, suggesting that complex formation between the receptors is necessary for efficient transcription.

As demonstrated in Figure 1, induction of transcription from the $H1^0$ promoter occurs only in the presence of hormone. We therefore analysed whether different concentrations of T3 would influence the transcriptional efficacy of the promoter. Incubation of CV-1 cells co-transfected with the $H1^0$-wt clone and equimolar

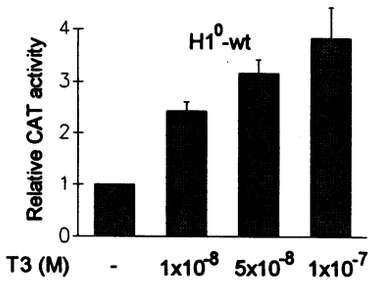

Figure 3. Thyroid hormone stimulates transcription from the mouse $H1^0$ promoter in transiently transfected CV-1 cells in a dose-dependent manner. CV-1 cells were co-transfected with 3 μg β-Gal DNA, 3.5 μg $H1^0$-wt plasmid and 0.5 μg both TRα and RXRα expression vectors. CAT analysis was as decribed in the legend to Figure 1. CAT activity obtained in the absence of T3 (–) was set to 1.

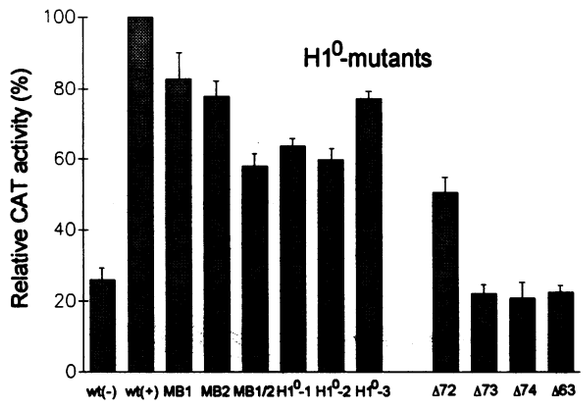

Figure 4. CAT analysis of mouse $H1^0$ promoter mutants. CV-1 cells were co-transfected with 3 μg β-Gal DNA, 0.5 μg both TRα and RXRα expression vectors and 3.5 μg CAT reporter plasmids containing point mutations within one (MB1 and MB2) or both (MB1/2) half-sites of the RARE or 1–3 bp deletions within the 7 bp spacer region of the RARE ($H1^0$–1 to $H1^0$–3; see Table 1 in Materials and Methods) in the presence of 10^{-7} M T3. In addition, deletion mutants Δ72, Δ73, Δ74 and Δ63, containing 483, 449, 398 and 314 nt of the proximal $H1^0$ promoter respectively (13), were analysed. The $H1^0$-wt construct (wt) was analysed in the presence (+) and absence (–) of T3. CAT analysis was performed as described in the legend to Figure 1. CAT activity obtained for the $H1^0$-wt construct in the presence of T3 (wt, +) was set to 100%.

amounts of TRα and RXRα expression vectors with T3 in concentrations of 1×10^{-8} to 1×10^{-7} M led to a dose-dependent 2–4-fold increase in CAT activity compared with control transfections without T3 (Fig. 3).

Taken together these results suggest that $H1^0$ transcription is not only dependent on the amount of receptor present in the cell, but also on the concentration of added ligand.

Mutations in the response element of the $H1^0$ promoter affect transcription

In order to further analyse the role of the response element of the $H1^0$ promoter in controlling transcription we generated recombinants containing point mutations in either one or both half-sites of the element (see Materials and Methods). These mutants were designed on the basis of dimethylsulfate methylation interference analyses using nuclear extracts from F9 cells, which have been found to respond to RA treatment with an early increase in $H1^0$ transcription (7,31), indicating that these cells contain all the factors necessary for up-regulation of $H1^0$ expression. In addition, in order to investigate the role of the unusually long spacer sequence separating the half-site sequences, we produced mutants containing 1, 2 or 3 bp deletions within the 7 bp spacer region (see Materials and Methods). We performed co-transfection experiments on CV-1 cells using these constructs and equimolar amounts of TRα and RXRα expression vectors (Fig. 4). Point mutations within both half-sites of the element (MB1/2) lowered the relative CAT activity to 58% of the value obtained for the $H1^0$-wt construct (wt). Point mutations in either one of the half-sites resulted in a reproducible decrease of ~20% in CAT transcription. Transfection of deletion mutants showed a reduction in transcriptional activity of 20–40%.

In order to assess the relevance of the reduction in CAT activity observed with these RARE mutants and to investigate the possible involvement of other regulatory sequences outside the response element of the $H1^0$ promoter in TR-mediated transcription we performed co-transfection experiments using $H1^0$ deletion mutants that were devoid of the element (13). As illustrated in Figure 4, the use of deletion mutant Δ72, which contains 483 nt of the proximal $H1^0$ promoter, resulted in a reduction in CAT activity comparable with that obtained with mutant MB1/2. Interestingly, a further shortening of the $H1^0$ promoter of only 34 bp up to nt –449 (Δ73) decreased the relative CAT activity to ~20% of the value obtained for the $H1^0$-wt construct in the presence of T3. Further deletions up to nt –314 (Δ73) did not impair transcriptional activity.

Thyroid hormone receptors bind to the response element of the $H1^0$ promoter

To investigate whether TRs can bind to the response element of the $H1^0$ promoter we performed EMSAs using oligonucleotides diagnostic for either the $H1^0$-wt sequence or for sequences mutated in either one or both half-sites of the element. In addition, oligonucleotides containing 1–3 bp deletions within the 7 bp gap were analysed (Table 1). The radioactively labelled probes were incubated with whole cell extracts from COS-1 cells transiently transfected with TRα or RXRα expression vectors or with both. A retarded complex was observed when incubating the $H1^0$-wt probe with whole cell extract isolated from COS-1 cells transfected with TRα (Fig. 5, lane 2, marked with arrow a). Interestingly, mutations within the half-sites of the element did not cause a marked reduction in formation of this complex, although the signals obtained with oligonucleotides MB2 and MB1/2 were somewhat weaker (see lanes 4 and 5). Heterodimerization of TRs and RXRs has been reported to have a synergistic effect on binding of TRs to TREs (21–26). Incubation of extracts from COS-1 cells transfected with equimolar amounts of TRα and RXRα expression vectors with either the $H1^0$-wt or the point-mutated probes yielded more slowly migrating complexes (marked with arrow b), ~4-fold stronger in intensity than those observed after incubation with TRα extract alone (compare lanes 2–5 with lanes 7–10). In this case also no major differences in signal intensity were found between the $H1^0$-wt and the mutated probes. No signals were observed when using cell extracts from COS-1 cells transiently transfected with RXRα alone (lanes 11–15). When extracts from COS-1 cells transfected with TRβ expression vector were tested in the EMSA similar results were

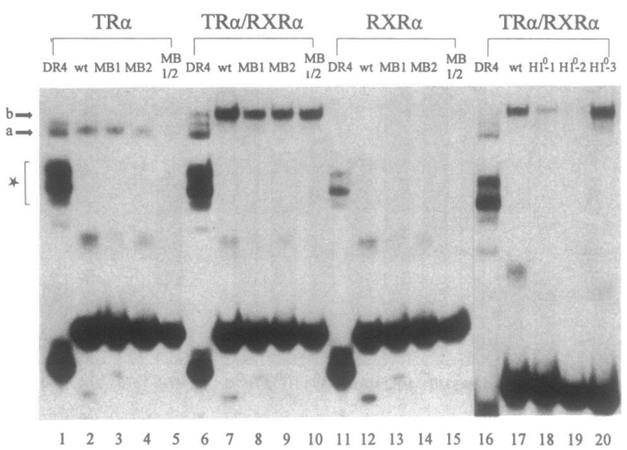

Figure 5. TRα and TRα–RXRα complexes bind to the H1^0 promoter response element. Whole cell extracts from COS-1 cells transfected with TRα or RXRα expression vectors were incubated in a 20 µl reaction mixture containing 50 000 c.p.m. 5′-^{32}P-labelled oligonucleotides corresponding to the H1^0-wt sequence (wt) or to the H1^0-mutated oligonucleotides given in Table 1. Lanes 1–5, 10 µg cell extract from COS-1 cells transfected with TRα expression vector; lanes 6–10 and 16–20, 10 µg extract from COS-1 cells transfected with both TRα and RXRα; lanes 11–15, 10 µg cell extract from COS-1 cells transfected with RXRα. Arrows a and b indicate the positions of retarded complexes obtained after incubation of the given oligonucleotides with TRα and TRα–RXRα respectively. Note that the incubation buffer used in this experiment was found to yield maximal binding efficiency of the H1^0 oligonucleotides. We took into account that under these conditions the DR4 oligonucleotide showed a rather strong non-specific signal, denoted by a star.

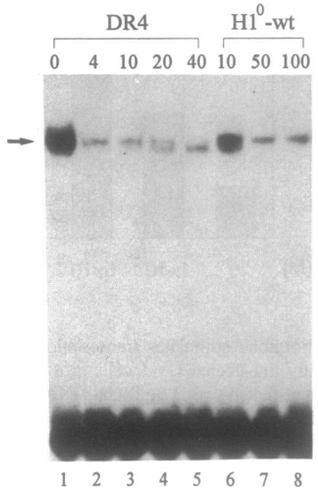

Figure 6. The response element of the H1^0 promoter competes for receptor proteins binding to the thyroid hormone responsive element DR4. Increasing molar concentrations of unlabelled oligonucleotides DR4 (4–40-fold, lanes 2–5) or H1^0-wt (wt, 10–100-fold, lanes 6–8) were incubated with ~50 000 c.p.m. 5′-^{32}P-labelled oligonucleotide DR4 and 10 µg both TRα and RXRα COS-1 cell extracts. Lane 1, no unlabelled competitor added. Please note that the buffer used in this experiment differed from that used in the experiment described in Figure 5. Here we used 0.8 µg salmon sperm DNA instead of 2 µg poly(dI–dC) per 20 µl reaction volume (33).

obtained (data not shown), indicating that different TR isoforms can bind to the response element in the mouse H1^0 promoter. Interestingly, changing the spacing between the two half-sites of the element clearly affected the intensity of the complex formed between the respective oligonucleotides and TRα–RXRα extracts (lanes 17–20). Whereas the signal gradually decreased when 1 or 2 nt were deleted (lanes 18 and 19), a 2-fold increase in signal intensity was observed with the 3 bp deletion mutant (lane 20).

To demonstrate that the H1^0-wt oligonucleotide was able to bind the same factors as the thyroid hormone response element DR4 we performed a competition experiment, where increasing amounts of either DR4 or H1^0-wt unlabelled double-stranded oligonucleotides were incubated with ^{32}P-labelled DR4 probe and both TRα- and RXRα-transfected COS-1 cell extracts. The intensity of the complex formed in the absence of unlabelled competitor was strongly reduced when a 50-fold molar excess of unlabelled H1^0-wt oligonucleotide was present in the incubation mixture. Self-competition was at least 10 times more efficient, since a similar reduction in signal intensity was seen with a 4-fold excess of unlabelled DR4 oligonucleotide (Fig. 6, compare lanes 2 and 7). These results demonstrate that receptor proteins that bind to the DR4 motif also bind to the H1^0 element, although more weakly.

Taken together our EMSA data indicate that: (i) TRs bind to the response element in the mouse H1^0 promoter either as homodimers or as heterodimers with RXRs; (ii) spacing between the half-sites of the element affects the intensities of the complexes formed with TRα–RXRα heterodimers.

DISCUSSION

In this report we demonstrate that the promoter of the mouse H1^0 gene, which has been found to be regulated by RA (14), is also modulated by T3. The results presented in Figure 1A show that CAT activity of a construct containing 740 nt of the proximal mouse H1^0 promoter is enhanced >3-fold in the presence of co-transfected TRα. Induction of CAT transcription was even stronger when the cells were co-transfected with expression vectors for both TRα and RXRα. Furthermore, our data clearly indicate that up-regulation of CAT expression is dependent on the presence of T3, since no increase in CAT activity was observed in the absence of hormone. The ratio between T3-induced and basal CAT expression in the presence of TRs was similar to that reported for other elements responsive to both RA and T3 (32). An even more pronounced stimulatory effect was observed when performing analogous experiments using a construct that contained five copies of the H1^0 promoter response element. These results suggest that transcription from the mouse H1^0 promoter is regulated by binding of TRs to the GGTGACC direct repeat motif and that the H1^0-wt sequence is a dual responsive element induced by both RA and T3. This assumption is supported by the results presented in Figure 2, which show a clear correlation between the amount of co-transfected receptor and the relative CAT activities, indicating that the observed transcriptional modulation is dependent on the amount of receptor present. A similar increase in transcription rate was observed with the multimer construct, further demonstrating that this effect was due to binding of receptors to the response motif. Interestingly, we reproducibly observed a T3 dose-dependent increase in CAT expression from the wild-type construct, indicating that modulation of transcriptional activity of the H1^0 promoter is not only

dependent on the presence of receptors, but also on the amount of ligand added.

The transfection experiments with constructs containing $H1^0$ mutated sequences showed a low but reproducible reduction in reporter gene expression. For the half-site mutants a reduction of ~20% was consistently observed, whereas with the recombinant containing mutations in both half-sites of the element a reduction to ~50% of the value obtained with the $H1^0$ wild-type construct was found. These values are similar to that found by Brent *et al.* (19), who analysed a series of recombinants containing mutations within the rat growth hormone promoter, which has been shown to be responsive to both T3 and RA (32). Furthermore, the results of our band shifting experiments suggest that the G and C residues mutated within the $H1^0$ wild-type sequence had only a minor effect on the capacity of the $H1^0$ element to bind TRs. Indeed, chemical modification interference analysis of a T3 responsive element complexed with TRα and RXRα receptors (33) suggests that the G nucleotides present within the upstream half-site of the $H1^0$ motif are probably not directly involved in receptor binding. Additionally, since at least in some cases other proteins seem to be involved in complex formation between receptor proteins and response elements, activation of a given responsive element is probably a more complex process than simply binding of two receptor molecules (34). Thus larger DNA–protein complexes may be less disturbed by mutation of certain nucleotides in the respective element. Finally, we cannot exclude the possibility that other regulatory sequences in the $H1^0$ promoter upstream or downstream of the responsive element may act as TR binding sites. Indeed, co-transfection experiments on CV-1 cells using $H1^0$ deletion mutants (13) completely devoid of the element analysed in this and in the study of Mader *et al.* (14) suggest the presence of DNA sequences between nt –483 and –449 of the proximal $H1^0$ promoter implicated in TR-dependent transcription (see Fig. 4), although no TRE-related motif can be found within this region. On the other hand, EMSA experiments with a restriction fragment covering the DNA region between nt –596 and –340 of the proximal $H1^0$ promoter did not reveal signals indicative of additional TR binding sites (data not shown).

Steroid hormone receptors affect transcription of target genes by binding to responsive elements located within regulatory regions of these genes (18, and references therein). Our EMSA results indicate that the $H1^0$ element is able to bind TRs (see Figs 5 and 6). This binding is enhanced in the presence of RXRs, as already described for various other RA and T3 responsive elements (21–26). Interestingly, mutations within the half-sites of the response element did not markedly reduce complex formation. This correlates with the results obtained in the CV-1 transfection experiments, but contrasts with the data presented by Mader *et al.* (14), who found a clear decrease in complex formation when testing nuclear extracts from F9 cells with oligonucleotides mutated in either one or both $H1^0$ motifs. However, in the experiments presented here whole cell extracts of receptor transfected COS-1 cells were used. Furthermore, in contrast to the oligonucleotides analysed in our study, Mader *et al.* used mutated sequences that differed essentially from the $H1^0$-wt sequence. The small differences in TR binding efficiency observed for the $H1^0$ mutants in our experiments are in agreement with data presented by Williams *et al.* (32) and Katz *et al.* (34), who found no quantitative correlation between receptor binding and hormone response.

Interestingly, altering the spacing between the two half-sites of the responsive element clearly affected the intensity of the complex formed between the respective oligonucleotides and TRα/RXRα extracts (Fig. 5, lanes 17–20). Whereas deletion of 1 or 2 nt resulted in a decrease in the retarded complex compared with the $H1^0$-wt oligonucleotide, deletion of 3 bp produced a 2-fold increase in the intensity of the complex. This is an interesting observation, since deletion of 3 nt of the 7 bp spacer region converts the response element of the $H1^0$ promoter into a canonical TRE according to the 3-4-5 rule suggested by Umesono *et al.* (20). Nevertheless, the transfection experiments demonstrated that the CAT activity of all three mutants was lower than that found for the wild-type construct. Moreover, the $H1^0$–3 mutant showed only a slightly higher activity than that observed for the $H1^0$–1 and $H1^0$–2 mutants, despite the great differences seen in complex formation in the band shifting experiments. Thus, in agreement with the data reported by Williams *et al.* (32) and Katz *et al.* (34), our results show that TR–RXR complex formation cannot be taken as a direct measure of the transcriptional activity of the $H1^0$ element and that other proteins are possibly involved in binding and activation of this element.

Taken together, the results presented in this report indicate that the repeat element in the promoter region of the mouse $H1^0$ histone gene is a dual element responsive to RA and thyroid hormone. The interplay between hormone-induced TRs and RARs and other putative proteins involved in complex formation at the responsive element may allow refined modulation of $H1^0$ expression in response to external hormonal stimuli, leading to concomitant modification of the chromatin structure.

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