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An Element in the Region Responsible for Premature Termination of Transcription Mediates Repression of c-*myc* Gene Expression by Thyroid Hormone in Neuroblastoma Cells*

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The thyroid hormone (T3) blocks proliferation and induces differentiation of neuroblastoma N2a-β cells that express the thyroid hormone receptor (TR) β1 isoform. c-Myc is required for cell cycle progression, and this study shows that T3-induced neuronal differentiation is preceded by a rapid decrease of c-*myc* gene expression. A negative T3 responsive element (TRE), arranged as an inverted palindrome spaced by three nucleotides, has been identified within the first exon between nucleotides +237 and +268. The TRE is adjacent to the binding site for the transcriptional repressor CCCTC binding factor and maps precisely within the region of RNA polymerase II pausing and release, suggesting a direct implication of TR on premature termination of transcription. Furthermore, the TRE confers repression by T3 to an heterologous promoter only when inserted downstream of the transcription initiation site. Binding of CCCTC binding factor and TR to their cognate sites in the region of transcriptional attenuation, as well as direct interactions between both factors, could facilitate the formation of a repressor complex and the inhibition of c-*myc* gene expression. These studies provide insight into mechanisms by which TR mediate transcriptional repression and contribute to the understanding of the important effects of thyroid hormones on growth and differentiation of neuronal cells.

The effects of the thyroid hormone (triiodothyronine, T3)[1] in cells are initiated by binding to nuclear receptors (TRs). These receptors are ligand-inducible transcription factors that exert their actions by binding, preferentially as heterodimers with the retinoid receptor RXR, to hormone response elements (TREs) located in regulatory regions of target genes (1, 2). Naturally occurring and synthetic TREs are normally composed of at least two copies of the consensus AGGTCA motif arranged as direct repeats, palindromes, or inverted palindromes (IPs) separated by a variable number of nucleotides.

The thyroid hormones are essential for brain development, although the specific mechanisms by which these hormones control neuronal proliferation and differentiation are not yet understood. It has been shown that T3 treatment of N2a-β cells (a murine neuroblastoma cell line that overexpresses the TRβ1 isoform) blocks proliferation by an arrest of cells in G_0/G_1 and induces morphological and functional differentiation (3, 4). One of the molecular events required for cell cycle progression is the inactivation by hyperphosphorylation of retinoblastoma protein (pRb) family members (5), which is catalyzed by the cyclin-dependent kinases or CDKs. We have recently shown that T3 induces a strong and sustained increase of the levels of the cyclin kinase inhibitor $p27^{Kip1}$ in N2a-β cells. As a consequence, the kinase activity associated with CDK2 complexes is inhibited and pRB proteins are hypophosphorylated in T3-treated N2a-β cells (6).

The c-*myc* proto-oncogene encodes a nuclear phosphoprotein with leucine zipper and helix-loop-helix domains, which can cause transcriptional activation as well as transcriptional repression, and also plays an important role in cell cycle progression (7–9). Although the mechanisms connecting c-Myc function to cell cycle control are not well understood, different signals that arrest growth and elicit cell differentiation suppress c-*myc* expression. Conversely, elevated levels of c-Myc prevent differentiation and lead to continued growth in many lineages. Thus, regulation of c-*myc* expression appears to play an important role in decisions of cellular growth *versus* differentiation. Therefore, it would not be surprising that T3 could repress c-*myc*. Indeed, we have observed that the effect of T3 on $p27^{Kip1}$ and N2a-β cells differentiation is accompanied by a decrease in c-Myc protein and c-*myc* mRNA levels (6).

Transcription of the c-*myc* gene is controlled by several promoters in mice, humans, and chickens. The two major promoters P1 and P2, which are positioned 164 base pairs apart in mice, contribute to more than 95% of the cytoplasmic c-*myc* mRNAs (9). Analysis of these regions has shown that numerous positive and negative regulatory elements are important for transcriptional regulation of the c-*myc* gene. One region which lies −65 to −58 base pairs upstream of the P2 promoter and appears to be essential for c-*myc* activation is bound by the cell cycle-regulated transcription factor E2F (8). In addition, a block in transcriptional elongation plays an important role in the regulation of c-*myc* gene expression (9). The regions responsible for transcriptional blockage within c-*myc* have been defined (10, 11). It has been shown that the sequences responsible for premature termination are found proximal to the P2 promoter. The site involved is known to function as a RNA polymerase II pausing region (12, 13). An 11-zinc finger transcriptional repressor CTCF binds immediately downstream of the P2 promoter at a sequence that maps precisely within the region of the polymerase II pausing and release (14). CTCF is

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[1] The abbreviations used are: T3, thyroid hormone; TR, thyroid receptor; RXR, retinoid X receptor; TRE, thyroid response element; IP, inverted palindrome; TK, thymidine kinase; CAT, chloramphenicol acetyltransferase; GST, glutathione *S*-transferase; PAGE, polyacrylamide gel electrophoresis; mmyc, murine c-*myc*; CTCF, CCCTC binding factor.

an exceptionally conserved protein that uses different combinations of the 11 zinc fingers to specifically bind to diverged regulatory DNA sequences within the promoter proximal regions of chicken, mouse, and human c-*myc* genes (15). CTCF contains two strong transcriptional repressor domains, and mutation of the CTCF binding site results in increased transcription from human c-*myc* reporter constructs (15). Very recently it has been demonstrated that CTCF is required for the enhancer blocking activity of vertebrate insulators that can act as a barrier to the influence of neighboring *cis*-acting elements preventing gene activation (16). A transcriptional repressor, negative protein 1 or NeP1, which binds to the F1 element of the chicken lysozyme silencer, has been demonstrated to be identical to CTCF (17). CTCF recognizes a sequence within the lysozyme gene silencer without any obvious sequence similarity to CTCF binding sites in the c-*myc* gene because of the different usage of zinc fingers (17). Interestingly, the lysozyme silencer is comprised of two DNA response elements (F1 and F2), which synergistically repress gene activity (18). The F1 module is bound by CTCF and the F2 is bound by TR, the homologous viral oncoprotein v-ERBA, or the retinoic acid receptor (RAR) (15). Whereas the unliganded TR acts as a repressor, in the presence of T3 the two silencer modules can synergistically activate gene transcription (18).

In this work we have analyzed the effects of T3 on c-*myc* gene expression in neuroblastoma N2a-β cells. Our results show that the hormone causes an extremely rapid decrease of c-*myc* transcripts in N2a-β cells that is independent on *de novo* protein synthesis. Transient transfection experiments and gel retardation assays showed the existence of a negative TRE located between nucleotides +239 and +268 in the region responsible for premature termination of transcription. This element is arranged as an IP with a 3-nucleotide gap (IP3), binds TR-RXR heterodimers, and is adjacent to the CTCF binding site. A promoter fragment (+140 to +266) containing binding sites for both transcription factors confers repression by T3 to an heterologous promoter but only when inserted 3′ of the promoter. These results indicate the direct implication of TR in premature termination of transcription leading in cooperation with CTCF to polymerase II pausing and release and to a decrease of c-*myc* mRNA levels.

MATERIALS AND METHODS

Cell Cultures—The clonal cell line Neuro-2a stably transfected with the β1 isoform of the human thyroid hormone receptor (N2a-β cells) was grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) T3-depleted serum as described previously (3, 6). Thyroid hormones were stripped from the serum by treatment with resin AG1 × 8.

RNA Extraction and Hybridization—Total RNA was extracted from the cell cultures with guanidine thiocyanate. The RNA was run in 1% formaldehyde-agarose gels and transferred to nylon-nitrocellulose membranes (Nytran) for Northern blot analysis. The RNA was stained with 0.02% methylene blue. The blots were hybridized with a labeled cDNA probe for c-*myc* (a 1.4-kilobase *Eco*RI/*Hin*dIII fragment from the human c-*myc* 3′-exon). Quantification of mRNA levels was carried out by densitometric scan of the autoradiograms. The values obtained were always corrected by the amount of RNA applied in each lane, which was determined by densitometry of the stained membranes.

Plasmids—Constructs containing different fragments of the mouse c-*myc* promoter were originated from genomic clone 18 (19). [-1141/+516]-mmyc-CAT was obtained by digestion with *Pst*I and *Xho*I and cloned into ΔpBLCAT3 in which a 195-base pair fragment was removed by digestion with *Nde*I and Eco0109. This treatment deletes an AP-1 element present in the plasmid backbone that can influence T3 responses (20). (+137/+516)-mmyc-CAT was obtained by digestion with *Hin*dIII and *Xho*I. The fragment *Ava*I/*Bam*HI was cloned in the *Sal*I and *Bam*HI sites of ΔpBLCAT3 to give (−140/+113)-mmyc-CAT. *Hin*dIII digestion was used to obtain (+140/+335)-mmyc-CAT. The oligonucleotides 5′-AAAGGATCCTTTTCGGGCGTT-3′ and 5′-CTCGGATCCCGAAGCTGCCTT-3′ were used as primers to obtain the fragment +140 to +269 by polymerase chain reaction. This fragment was cloned either 3′ (in the *Bgl*II site) or 5′ (in the *Bam*HI site) of the thymidine kinase promoter of ΔpBLCAT2 to give TK-(+140/+269)-CAT and [+140/+269]-TK-CAT, respectively. The same fragment with the TRE mutated was obtained using the reverse oligonucleotide 5′-CTCGGATCCCGAAGCTGGATTCTTATTTCGCCCC-3′. This fragment was cloned in the *Bgl*II site of ΔpBLCAT2 to obtain TK-(+140/+266mut)-CAT.

Transient Transfections and CAT Assays—N2a-β cells were plated 24 h prior to transfection into 60-mm dishes. The cells were transfected with calcium phosphate with 5 μg of the reporter plasmid (plus 1 μg of a luciferase internal control plasmid). After 16 h of incubation the precipitates were washed and the treatments with T3 (5 nM) were administered in medium containing 10% resin-treated serum. CAT activity was determined in the cell extracts with [^{14}C]chloramphenicol. The unreacted and acetylated [^{14}C]chloramphenicol was separated by thin layer chromatography and quantified with an Instantimager. Treatment with T3 was performed in triplicate cultures that normally exhibited less than 10% variation in CAT activity, and the experiments were repeated at least two or three times with similar relative differences in regulated expression. The results are expressed as the mean ± S.D. of the CAT values obtained.

Gel Retardation Assays—Nuclear extracts of N2a-β cells, as well as purified proteins, were used for gel retardation assays. Highly purified TR-RXR heterodimers (>80% purity) were obtained after vaccinia coinfection of HeLa cells (21). Recombinant GST-TR and GST-RXR (22) were expressed in the bacterial strain BL21 (DE3). They were grown at 37 °C in 2 XYT (16 g/liter Tryptone, yeast extract, 5 g/liter NaCl (pH 7)) until the absorbance reached 0.6. Then the induction was performed at 30 °C for 3 h with 0.4 mM isopropyl-1-thio-β-D-galactopyranoside. The expression of correctly sized proteins was monitored by SDS-PAGE. CTCF purified from HeLa cells was a kind gift from R. Arnold. The sequence of the oligonucleotides TREmmyc corresponding to sequences +239 to +268 of the murine c-*myc* gene and the mutant oligonucleotides designated as IP3 and IP8 used in the assays are shown in Fig. 4. For the binding reaction, the nuclear extracts (10 μg) or purified proteins (100 ng) were incubated at room temperature for 15 min at 4 °C in a buffer (10 mM Hepes-KOH (pH 7.9), 80 mM KCl, 1 mM dithiothreitol, 5% Ficoll) containing 3 μg of poly(dI-dC) and then for 30 min at room temperature with approximately 30,000 cpm of labeled double-stranded oligonucleotide labeled with [^{32}P]dCTP. The fragment +140 to +269, amplified with the primers indicated above, as well as the same fragment with the mutated TRE were also used in the assays. In addition, a labeled fragment of the murine c-*myc* promoter (from +137 to +335) was obtained by polymerase chain reaction using the primers 5′-AAGCTTTTCGGGCGTTTTTTTCTG-3′ and 5′-GCTGATGTTGGGTCAGTCGCAGGG-3′. For supershift experiments, 1 μl of specific antibodies against RXR (which recognizes the different receptor isoforms) and CTCF (17) were added to the binding reactions and incubated on ice for 30 min before the addition of the labeled probe. For competition experiments an excess of unlabeled double-stranded oligonucleotides or polymerase chain reaction fragment was added to the binding reaction mixture. The competitor oligonucleotide TREpal contains the palindromic element 5′-AGGTCATGACCT-3′, and the F1 probe contains the CTCF binding element of the lysozyme silencer (18). DNA-protein complexes were resolved on 6% polyacrylamide gels in 0.5× Tris borate-EDTA buffer. The gels were then fixed, dried, and autoradiographed at −70° C.

Protein-Protein Interactions—GST pull-down assays were performed with 5 μl of L-[^{35}S]methionine-labeled CTCF, and the GST fusion proteins GST-TR and GST-RXR. An N-terminally truncated TR, GST-TR-(120–408), lacking the A/B domain and the DNA binding domain (22), as well as the GST-vitamin D receptor and GST-peroxisome proliferator-activated receptor α (23, 24) were also used. A vector for CTCF (1 μg) cloned in pSG5 (15) was used for *in vitro* transcription using the TNT T7 Quick coupled Transcription/Translation System (Promega) in the presence of 40 μCi of [^{35}S]methionine. Labeled CTCF was incubated with 1 μg of GST-fusion protein or with the same amount of GST as a control immobilized in glutathione-Sepharose beads. The proteins were first incubated in the presence of 1 μM T3 or ethanol for 20 min at room temperature in glass tubes. The reaction with the beads was performed for 1 h at 4 °C in a binding buffer containing 25 mM Hepes-KOH, pH 7.9, 1% glycerol, 5 mM Mg_2Cl, 1 mM dithiothreitol, 0.05% Triton X-100, 5 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride. Free proteins were washed from the beads with a buffer containing 100 mM KCl, and the bound proteins were analyzed by SDS-PAGE and autoradiography. Whole cell extracts from N2aβ cells were prepared with 25 mM Hepes-KOH, pH 7.9, 0.1% Nonidet P-40, 150 mM KCl, 2 mM EDTA, 10 mM NaFl, 1 mM dithiothreitol, and 0.25% bovine serum albumin. GST alone or GST-TR (1 μg) was exposed to 700 μg of cell extract. Proteins were

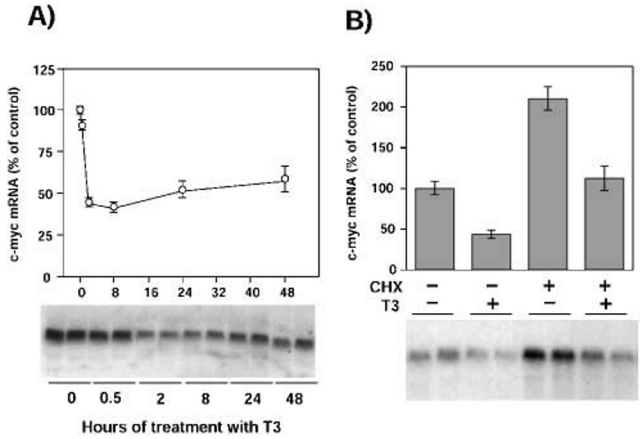

FIG. 1. **T3 decreases c-*myc* mRNA levels in neuroblastoma N2a-β cells.** *A,* Northern blot analysis were performed with 30 μg of total RNA obtained from cells incubated with the times indicated with 5 nM T3. The *lower panel* shows the autoradiogram of a representative blot. The autoradiograms from two separate experiments performed in duplicate were quantitated by densitometry, and the values obtained were corrected by the amount of RNA applied. The resulting c-*myc* RNA levels expressed as the percentage of the values obtained at time 0 are shown in the *upper panel*. *B,* the *lower panel* illustrates a representative Northern blot from cells treated for 4 h with 5 nM T3 in the presence and absence of 10 μg/ml cycloheximide (*CHX*). The *upper panel* shows the quantification of c-*myc* transcripts obtained from two independent experiments. Data are mean ± S.D. values expressed as percentages of the results obtained in control untreated cells.

eluted from the resin and resolved by SDS-PAGE. CTCF was detected by Western blot with the CTCF antibody at a 1:2000 dilution. For immunoprecipitation the cells were harvested in 400 μl of lysis buffer, and 700 μg of cell extract were incubated with 2 μg of anti-TR antibody (Santa Cruz Biotechnology) for 2 h at 4 °C. Protein A-Sepharose was added and the incubation proceed for 1 additional h. The immunocomplexes were resolved by SDS-PAGE and analyzed by Western blot with the CTCF antibody.

RESULTS

Influence of T3 on c-myc mRNA Levels in N2a-β Cells—We have previously observed that the levels of c-Myc and c-*myc* mRNA were reduced in N2a-β cells treated with T3 for 48 h (6). Fig. 1*A* shows the kinetics of reduction of c-*myc* mRNA. T3 produced a rapid decrease of this transcript with a maximal reduction found only after 2 h of incubation with the hormone. This decrease was maintained for at least 48 h. To analyze whether *de novo* protein synthesis was required for T3 repression of c-*myc* gene expression, treatment with the hormone was performed in the presence and absence of 10 μM cycloheximide. As shown in Fig. 1*B*, cycloheximide increased c-*myc* mRNA levels. Superinduction in the absence of protein synthesis is characteristic of "early response" genes, which have transcripts with short half-lives. Treatment with T3 for 4 h caused a reduction of basal c-*myc* mRNA and was able to significantly decrease the levels found in cells incubated with cycloheximide. These results indicate that the effect of the hormone does not require previous synthesis of proteins and suggest that repression of c-*myc* gene expression might represent a direct transcriptional action of the thyroid hormone receptor on the c-*myc* gene.

Proximal Promoter Sequences of the Murine c-myc Gene Mediate Repression by T3—To directly examine whether the reduction of c-*myc* transcripts following T3 treatment of N2a-β cells is exerted at a transcriptional level, reporter constructs containing different fragments of the murine c-*myc* promoter region were used in transient transfection studies. Fig. 2*A* shows that incubation with 5 nM T3 for 48 h reduced by approximately 70% the activity of (−1141/+516)-mmyc-CAT. A similar reduction was observed with the reporter (−140/+335)-mmyc-CAT. This result shows that the region between −140 and +335, which includes both the P1 and the P2 promoters, contains the sequences required for transcriptional repression by T3. In contrast, the activity of the ΔpBLCAT3 construct was very low and was not affected by incubation with T3 (not illustrated). To further map the elements responsible for this regulation, two additional plasmids (−140/+113)-mmyc-CAT and (+137/+516)-mmyc-CAT were also used. The first construct includes sequences upstream of the P1 promoter, and the second one comprises P2 and sequences downstream of this site including the premature termination region. When assayed after 48 h of treatment with T3, the activity of both promoters was similarly repressed by the hormone. However, the kinetics of repression were different. As illustrated in Fig. 2*B*, incubation with T3 prevented accumulation of CAT activity, which occurred during the period examined in the untreated cells. An inhibitory effect on the activity of (+137/+516)-mmyc-CAT was found at the first time period analyzed, namely at 3 h of incubation with T3. However, a reduction in the activity of the (−140/+113)-mmyc-CAT plasmid was not detected at 3 or 8 h of incubation and became apparent from 20 h of treatment.

A TRE Is Located within the First Exon of the c-myc Gene Overlapping Sequences Responsible for RNA Polymerase II Pausing and Release—The sequences responsible for the rapid repression of c-*myc* gene transcription by T3 were analyzed for TR binding sites by gel retardation assays. The labeled promoter fragment +140/+335 was incubated with recombinant TR and/or RXR as fusions with GST. As illustrated in Fig. 3, this region did not bind RXR and only weakly TR, but when both receptors were combined a strong retardation was observed (*lane 4*). *In vitro* incubation with T3 did not significantly alter retardation (*lane 5*). The mobility of the GST fusion proteins is slower than that obtained with highly purified TR-RXR heterodimers obtained with vaccinia infection that is shown in *lanes 14* and *15*. The promoter fragment +132 to +269 (*lanes 6–9*) also bound TR-RXR heterodimers, indicating the existence of a TRE in this region. In contrast, when the fragment +140 to +335 was excised to give +140 to +249 and +249 to +335, the resulting fragments did not bind TR-RXR (*lanes 10–13*), suggesting that the TRE is located around the excision point. Analysis of the sequences comprised between +237 and +270 revealed the existence of the three potential TRE hemisites depicted in Fig. 4*A* as TREmmyc. Fig. 4*B* shows that the TREmmyc probe, as the promoter fragments, binds TR-RXR heterodimers (*lane 4*) but not TR or RXR separately. Although with a lower affinity, this element also bound RAR-RXR heterodimers (not illustrated). The TREmmyc could be organized as an inverted palindrome separated by 3 nucleotides (IP3) or with an 8-nucleotide gap (IP8). To determine the contribution of the different hemisites to receptor binding, two additional probes were used in which either the more downstream motif or the middle one were mutated to render an IP3 or an IP8 element, respectively (Fig. 4*A*). *Lane 8* in *panel B* shows that the IP8 bound weakly TR-RXR heterodimers, whereas the IP3 (*lane 12*) bound the receptors at least as strongly as the complete TREmmyc. The competition studies illustrated in the *right panel* in Fig. 4*B* further demonstrate the IP3 configuration of the response element. Whereas the TREmmyc and IP3 probes were equally effective in competing the retardation caused by TR-RXR on the native element, a large molar excess of IP8 oligonucleotide was unable to reduce binding.

Binding of nuclear proteins to the TREmmyc is shown in Fig. 4*C*. Nuclear extracts from control N2a-β cells as well as from cells treated with T3 were subjected to gel retardation assays with the TREmmyc oligonucleotide. A predominant retarded complex with the same mobility as the purified TR-RXR het-

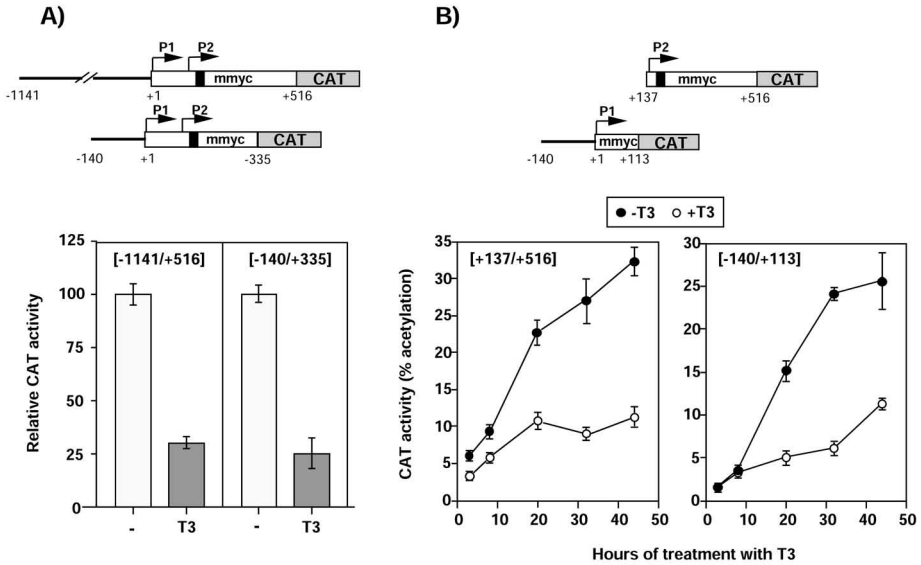

FIG. 2. **T3 represses the activity of the murine c-*myc* promoter in transient transfection assays.** A schematic representation of the reporter CAT plasmids containing fragments of the mmyc gene used to transfect N2a-β cells is shown. The *arrows* indicate the positions of the P1 and P2 transcription initiation sites, and the *black box* shows the region responsible for premature termination of transcription located downstream of P2. *A,* the cells were transfected with the constructs (−1141/+516)-mmyc-CAT and (-140/+335)-mmyc-CAT, and CAT activity was determined after 48 h of incubation in the absence and presence of 5 nM T3. Data are expressed relative to the CAT values obtained in the corresponding untreated cells. In *B* N2a-β cells were transfected with the constructs (−140/+113)-mmyc-CAT and (+137/+516)-mmyc-CAT, and CAT activity determined in cells incubated in the presence and absence of T3 at the indicated time periods. All data are mean ± S.D.

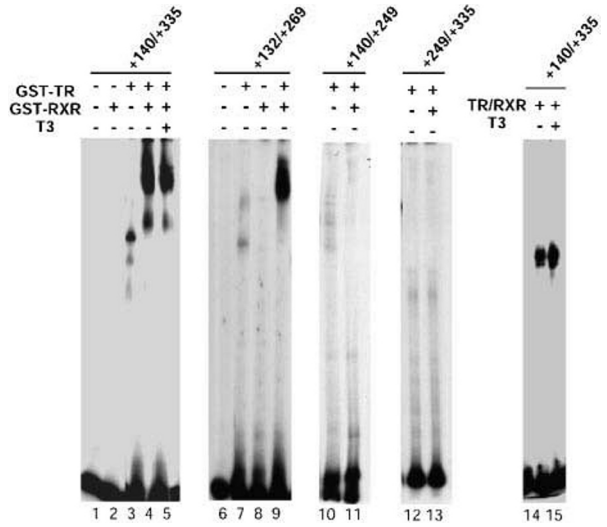

FIG. 3. **Sequences located downstream of P2 bind TR-RXR heterodimers.** The labeled regions +140 to +335 and +132 to +269 of the murine c-*myc* gene were obtained by polymerase chain reaction, and the regions +140 to +249 and +246 to +335 were obtained by digestion of the +140 to +335 fragment. The different probes were used for gel retardation assays with 100 ng of recombinant GST-TR and/or GST-RXR as indicated (*lanes 1–13*) or with an equivalent amount of purified TR-RXR heterodimers obtained with vaccinia (*lanes 14* and *15*). When indicated the assays were performed in the presence of 1 μM T3.

erodimer was formed with the cell extracts. T3 treatment did not alter the abundance of these complexes, which were competed equally well by the TREmmyc and by the palindromic element TREpal. In addition, the retarded complex was supershifted by an RXR antibody, demonstrating the presence of RXR heterodimers (most likely TR-RXR) in the protein-DNA complexes. This heterodimeric complex was not detected in nuclear extracts from parental N2a cells, which express low levels of thyroid hormone receptors (*lane 16* and *17*), confirming that TR-RXR are the major heterodimeric complexes bound to the TREmmyc.

The nuclear complexes formed with the +140 tp +266 promoter fragment are illustrated in Fig. 5*A*. Several retarded bands were observed with the nuclear extracts from either untreated or T3-treated N2a-β cells. The components of the different bands were again identified by the use of antibodies and competition with specific binding sites. The fastest complex had a mobility identical to that caused by the TR-RXR heterodimer, was displaced by an excess of TREpal, and supershifted by the anti-RXR antibody. These treatments did not affect the predominant complex, which has a slower mobility than the TR-RXR heterodimer. It has been recently described that the transcriptional repressor CTCF binds to a GC-rich region between positions +165 and +205 in the murine c-*myc* promoter (15). That the more abundant complex found in N2a-β cells corresponds to CTCF is demonstrated by the finding that this retarded band is abolished by an anti-CTCF antibody and competed by the F1 oligonucleotide. The F1 sequence of the lysozyme silencer is highly divergent from the CTCF binding site in the c-*myc* promoter but also binds this factor effectively (17). An additional complex with the slowest mobility was also detected in the nuclear extracts. This complex was disrupted in the presence of excess TREpal and F1 and by both the RXR and CTCF antibodies. Furthermore, when purified CTCF and TR-RXR were incubated with the promoter fragment, a new retarded band appeared that had the same mobility as this nuclear complex. Together, these observations demonstrate that this complex represents a ternary complex that contains both CTCF and receptor heterodimers.

That the TREmmyc within the +140 to +265 promoter region is responsible for TR-RXR binding is shown in Fig. 5*B*. This figure compares binding of the heterodimer and CTCF to the native fragment and to a fragment in which both hemisites of the TREmmyc have been mutated. TR-RXR bound to the native fragment with the expected strength and mobility, and when combined with CTCF, it produced the appearance of the super-retarded complex. In contrast, TR-RXR did not significantly bind to the promoter in which the TREmmyc sequences have been mutated. Interestingly, although TR/RXR alone did not bind this fragment, a retarded band with the mobility of the ternary complex, although weaker than that found with the

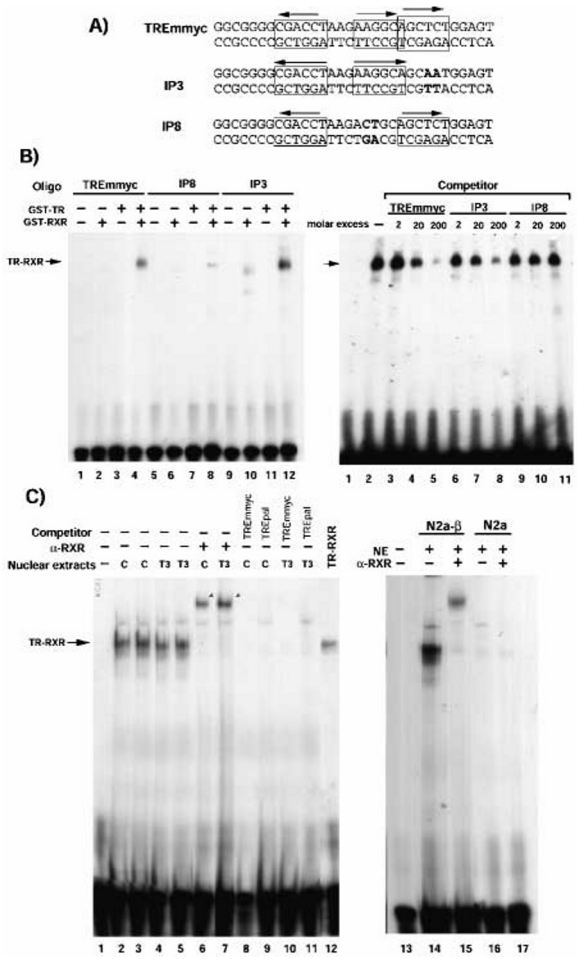

FIG. 4. **Mapping of the TRE in the murine c-*myc* gene.** *A,* sequence corresponding to the region between positions +237 to +270 was designated as TREmmyc. *Boxes* and *arrows* show the putative TRE hemisites. In IP3 and IP8 the TREmmyc sequence was mutated to eliminate the downstream or the central hemisites, respectively. Mutations are shown in *boldface*. *B,* in the *left panel* gel mobility shift assays were performed with GST-TR and/or GST-RXR and the ^{32}P-labeled TREmmyc, IP3, or IP8 probes as indicated. In the *right panel*, the TR-RXR heterodimers bound to the TREmmyc probe were competed with increasing concentrations of unlabeled TREmmyc, IP3, or IP8 oligonucleotides. The mobility of the heterodimer is shown by an *arrow*. *C,* in the *left panel* the assays were performed with nuclear extracts (10 μg of protein) of control N2a-β cells (*c*) or cells treated with 5 nM T3 for 24 h. The labeled TREmmyc oligonucleotide was used as probe. In *lanes 6* and *7*, where 1 μl of anti-RXR antibody (*α-RXR*) was added to the binding reactions, an *arrowhead* indicates the mobility of the supershifted complex. In *lanes 8–11* binding was competed with an excess of TREmmyc or TREpal oligonucleotides. In *lane 12* the mobility of the purified TR-RXR heterodimer (75 ng) is shown. In the *right panel* nuclear extracts (*NE*) from either N2a-β cells or parental N2a cells were incubated with the labeled oligonucleotide in the presence and absence of the anti-RXR antibody.

native sequence, was formed.

Interaction of CTCF with the Receptors—The results shown in Fig. 5*B* are compatible with the existence of a direct association between the receptors and CTCF. Therefore, we tested *in vitro* interaction between them in GST pull-down assays. Binding of ^{35}S-labeled CTCF to GST-TR and GST-RXR immobilized on glutathione-agarose beads is shown in Fig. 6*A*. GST protein alone did not interact with CTCF. However, ^{35}S-labeled CTCF was specifically retained by GST-TR. Binding of GST-RXR to CTCF was weak, but the presence of GST-RXR enhanced significantly the binding of GST-TR. The interaction occurred in the absence of ligand, and the ligand had little effect in the association of CTCF with the receptors. No interaction of the receptors with ^{35}S-luciferase used as a negative control was detected (not illustrated). In addition, the interaction with CTCF is not a general property of nuclear receptors as neither the vitamin D receptor nor the peroxisome proliferator-activated receptor α associated *in vitro* with this factor. As also shown in Fig. 6*A*, the interaction between TR and CTCF required the N terminus of the receptor, because a truncated TR, which contains only the ligand binding domain, did not interact with CTCF.

To test whether there was an interaction between TR and the endogenous CTCF, extract from N2a-β cells was incubated with GST-TR or GST alone. The associated proteins were subjected to electrophoresis, and CTCF was detected by Western blot. Although the size of the CTCF cDNA predicts an 82-kDa protein, in agreement with previous observations (25), our results show that CTCF migrates aberrantly in SDS-PAGE with an apparent molecular mass of 130 kDa (Fig. 6*B*, *lane 1*). *Lane 2* shows that GST did not interact with CTCF in the cell extracts. However, a band corresponding to CTCF was pulled down by GST-TR (*lane 3*). To test whether CTCF and TR can also associate *in vivo*, cell extracts were immunoprecipitated with an anti-TR antibody, and CTCF in the precipitate was detected by Western blot. As shown in Fig. 6*C*, a band corresponding to CTCF was present in the immunoprecipitates (*lane 2*). These results show that CTCF and TR can indeed associate *in vivo*. However, this interaction was weak because the amount of CTCF in the TR immunoprecipitates was below that found in *lane 1*, which represents only 3% of the input.

The Region of Premature Termination Confers Negative Regulation by T3 when Inserted Downstream but Not Upstream of the Thymidine Kinase Promoter—To test whether or not the TRE binding site in the c-*myc* promoter acts as a functional negative element conferring T3 responsiveness to an heterologous promoter, the region +140 to +266 of c-*myc* was linked either upstream or downstream of the TK promoter in ΔpBLCAT2. Fig. 7 shows that the functionality of the TRE depends on its position with respect to the initiation site. When the c-*myc* fragment, which contains the CTCF binding site besides the TRE, was cloned 5′ of the TK promoter, T3 did not cause a decrease of CAT activity. In contrast, T3 significantly decreased the activity of the reporter construct when the same fragment was linked in a 3′-position with respect to the TK promoter. This is the location of the native element in the murine c-*myc* gene. Additionally, mutation of the TRE abolished negative regulation by T3 demonstrating that this element is responsible for the repression.

DISCUSSION

Regulation of c-*myc* expression appears to play an important role in cell cycle progression and cellular differentiation. Our results show that T3-induced neuronal differentiation and growth arrest of neuroblastoma N2a-β cells is preceded by a decrease of c-*myc* gene expression. The effect of T3 was very rapid, with a maximal repression of c-*myc* mRNA being found within 2 h of hormone treatment. This decrease occurs before induction of the cyclin kinase inhibitor p27^{Kip1} (6) and is one of the most rapid effects of thyroid hormone on gene expression known. Repression by T3 does not require *de novo* protein synthesis, suggesting a direct effect of the thyroid hormone receptor on the c-*myc* gene. It has been proposed that the initial decrease of c-*myc* mRNA levels during differentiation of several cell types results from a reduction in the number of polymerases that read through sites of termination or pausing within exon 1. Even in cells with high expression of c-*myc*, the majority of polymerases on c-*myc* exon 1 pause at the P2 promoter and only a minor fraction of polymerase II actively transcribes c-*myc* exon 1 (26). The transient pausing of RNA polymerase II

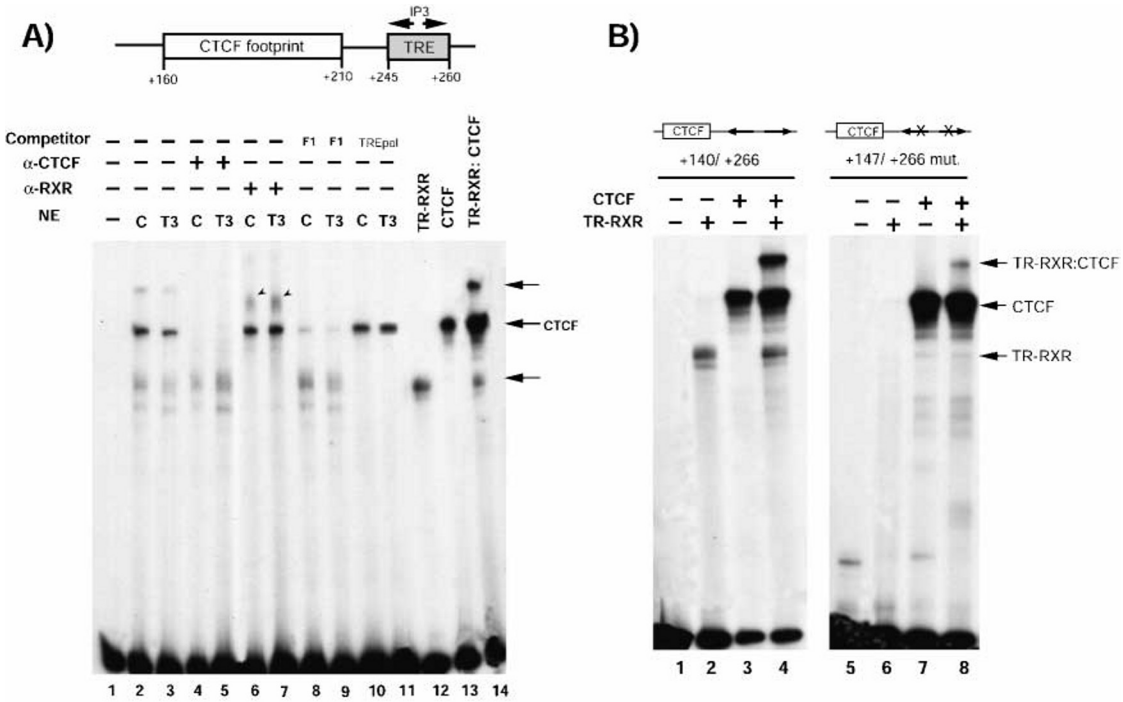

FIG. 5. **Binding of TR-RXR and CTCF to the region responsible for transcriptional block.** *A,* schematic representation of the +140 to +266 fragment showing the binding site for CTCF and the TRE organized as an IP3. This labeled region was used for mobility shift assays with 10 μg of nuclear extracts (*NE*) from control (*C*) and T3-treated cells (*lanes 1–11*) or with purified CTCF and TR-RXR (*lanes 12–14*). Specific antibodies (α-CTCF and α-RXR) were used in *lanes 4–7*. The *arrowheads* point out the supershifted complex caused by α-RXR. Binding of the nuclear extracts was competed with the F1 oligonucleotide containing the CTCF binding site in the lysozyme silencer (*lanes 8* and *9*) and with the TREpal (*lanes 10* and *11*). The mobilities of the protein-DNA complexes formed by TR-RXR, CTCF, or both are indicated by *arrows*. *B,* gel retardation assays were performed with purified CTCF and TR-RXR and the labeled probes +160/+266 and +160/+266mut. Schematic representations of the probes are depicted on the *top*. Both hemisites of the inverted palindromic TRE mutated in +160/+266mut are illustrated by an *X*. The mobilities of the different DNA-proteins complexes are indicated by *arrows*.

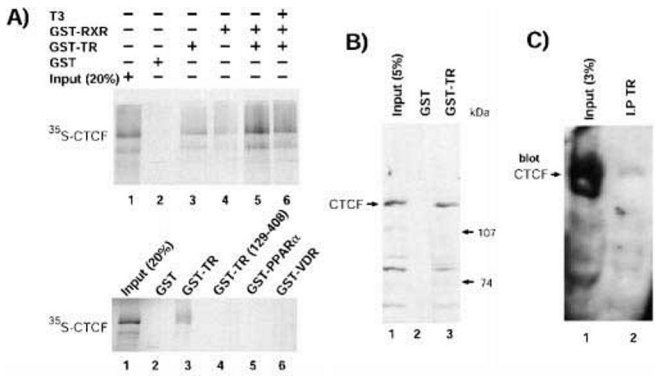

FIG. 6. **Interaction of CTCF with the receptors.** *A, in vitro* binding of CTCF to different nuclear receptors was assessed by pull-down experiments. GST-TR, GST-RXR, GST-vitamin D receptor, GST-peroxisome proliferator-activated receptor, GST-TR-(120–408) (a truncated TR), or GST alone (as a negative control) immobilized in glutathione-Sepharose beads were incubated with 5 μl of *in vitro* translated CTCF labeled with [^{35}S]methionine. When indicated incubations were performed in the presence of 1 μM T3. After incubation the beads were washed, and the labeled proteins were analyzed by SDS-PAGE and visualized by autoradiography. *B,* GST or GST-TR were incubated with 700 μg of whole cell extracts, and the bound CTCF was analyzed by Western blot. The input represents 5% of proteins used. *C,* whole cell extracts of N2a-β cells (700 μg) were immunoprecipitated with a TR antibody. The precipitates were subjected to Western blot analysis with the CTCF antibody together with 3% of the whole cell extract used.

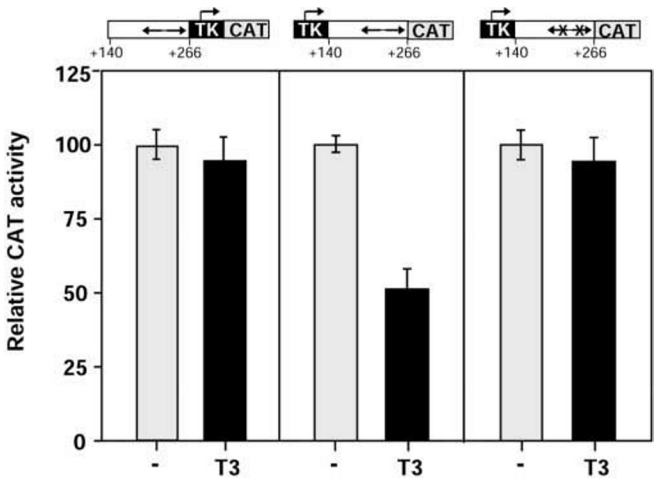

FIG. 7. **The activity of the TRE depends on its location.** The region +140 to +266 of the murine c-*myc* gene was cloned either upstream or downstream of the TK promoter to give [+140/+269]-TK-CAT and TK-[+140/+269]-CAT. The same fragment with mutations in both hemisites of the TRE was also cloned downstream of the TK promoter. A scheme of these constructs is shown at the top. N2a-β cells were transfected with the different constructs and CAT activity was determined after 24 h in cells treated in the presence or absence of 5 nM T3. The data are mean ± S.D. and are expressed relative to the values obtained in the corresponding untreated cells.

has been suggested as a prerequisite for termination (or attenuation), and it has been shown that the sequences responsible for this event map at a position proximal to the major c-*myc* promoter (P2) (10–13).

A nuclear factor, CTCF, mediating active repression of the c-*myc* promoter has been identified, and the CTCF binding sequence maps precisely within the region of polymerase II pausing (14, 15). We have shown that CTCF is the predominant nuclear factor binding to this region in N2a-β cells, and most interestingly, we have identified a functional negative TRE adjacent to the CTCF binding site. This configuration is very similar to that of the lysozyme gene silencer (18). It has been

demonstrated that binding of a protein, called NeP1, to 50 base pairs of DNA next to the TRE in the silencer is required to mediate efficient transcriptional repression by unliganded TR (27). More recently, it has been proved the identity of NeP1 with CTCF (17). Functional synergism in repression is not based on DNA-binding cooperativity to the lysozyme silencer as judged by *in vitro* binding experiments (28), and we have confirmed this lack of binding cooperativity in the c-*myc* gene element. The functional cooperation of the receptor with CTCF could involve a direct physical association, and we have been able to demonstrate by *in vitro* binding experiments the existence of a direct interaction between this two transcription factors. We have also observed an *in vivo* interaction between CTCF and TR, but this association was very weak and therefore its functional significance is still unclear.

Our results indicate that the negative TRE in the murine c-*myc* gene consists of two half-sites separated by three nucleotides and arranged as inverted palindromic repeats. This TRE shares the half-site arrangement with other TREs (18, 29–33), but to our knowledge a separation of three nucleotides has not been found before. Some negative elements have been shown to be preferentially bound by a thyroid hormone receptor homodimer in the absence of hormone, whereas in the presence of T3 a RXR/TR heterodimer is bound. However, the IP3 TRE in the mouse c-*myc* gene binds homodimers weakly, and essentially only heterodimeric binding is observed both in the presence and absence of ligand.

Despite the same arrangement the diverse TREs composed of IPs can have different functional properties. Thus, the TREs present in the Rous sarcoma virus promoter (31), the human growth hormone gene (34), or the pituitary clone 144 (29) mediate negative regulation by T3 as the TREmmyc does, whereas the elements in the malic enzyme (30), the γ-F-crystallin (32) or the F2 element in the lysozyme gene silencer (18) mediate repression by the unliganded receptor and T3-induced activation. Although at the present time the properties of the TREs governing these differences are not yet understood, location of the TRE may play a role. TREs are frequently located 5′ with respect to transcription initiation sites, but some are positioned downstream of the TATA box (31) or even have an unusual location at the 3′-untranslated region (29, 34). The TREmmyc is located downstream of the transcription initiation sites P1 and P2 and its function is position-dependent because it only confers negative regulation by T3 when placed downstream of a heterologous promoter. By contrast, the TREmmyc was inactive when placed upstream of the promoter. The finding that this sequence has properties that depend on its localization, exhibiting negative responses only when placed downstream of the transcription initiation site, strongly suggests that this TRE affects the transcriptional activity of c-*myc* by regulating the rate of release of RNA polymerase II from the c-*myc* P2 promoter.

One of the mechanisms proposed to explain how CTCF exerts its repressive effect is DNA bending. It has been shown that CTCF can induce a significant directed bend on the lysozyme silencer. Furthermore, although TR and RXR do not induce bending on their own, when all factors are bound simultaneously the RXR/TR heterodimer changes the position and orientation of the bend (28). We have observed that CTCF shows a significant bending activity on the +140 to +266 region of c-*myc* (data not shown), and although it is still not clear how bending relates with the repressive effects of CTCF, DNA bending may occur in conjunction with repositioning of nucleosomes resulting in an altered chromatin structure. The direct interaction of CTCF with the receptors could also play a role in determining this alteration and on the occupancy of this promoter region. Additionally, bending may be required for the assembly of other interacting partners. Transcriptional repression can be achieved by interaction with corepressors. Thus, corepressors such as SMRT or NCoR have been identified as interacting with TR (35, 36). It has been shown that a complex containing SMRT, mSin3, and histone deacetylases mediates transcriptional repression (37, 38). The finding that CTCF contains at least two repressor domains (15) suggests that this factor could also associate with still unidentified nuclear corepressors. The simultaneous binding of CTCF and the thyroid hormone receptor to their close cognate sites in the region of transcriptional attenuation, as well as the direct interactions between them and with corepressors could facilitate the formation of the repressor complex and the inhibition of c-*myc* gene expression.

Our data also show that two distinguishable modes of regulation operate on c-*myc* during T3-induced differentiation of neuroblastoma cells. During the early stages, the TRE at the site responsible for transcriptional attenuation could contribute to increase termination or pausing of transcriptional elongation near the end of the first exon, thus preventing formation of full-length c-*myc* transcripts. However, at later stages, other control mechanism mediated by sequences contained upstream of the P1 initiation site occurs, because T3 also decreases the activity of plasmids containing these sequences in the absence of P2. A later acting mechanism of down-regulation, besides the early increased blocking of c-*myc* transcripts elongation, also occurs during differentiation of HL60 or U937 cells (39, 40). The time course of the later mechanism of inhibition by T3 suggests a loss of promoter function because of previous changes in the abundance or activity of other trans-acting factors binding to the promoter region −140 to +113. For instance, it has been described that E2F factors appear to be essential in determining the rate of transcription of the c-*myc* gene (8). We have shown that CDK2 activity is inhibited and pRB proteins are hypophosphorylated in T3-treated N2a-β cells (6). Therefore, T3 maintains pRb family proteins in their active form a condition in which they associate with E2F factors and could repress transcription of the c-*myc* gene. Therefore multiple mechanisms can contribute to the long term maintenance of c-*myc* repression by T3.

In conclusion, our studies provide evidence of a novel role of the thyroid hormone on premature termination of transcription of the murine c-*myc* gene during neuronal differentiation and give insight into the mechanisms by which TR and CTCF mediate transcriptional repression. Given the crucial role of c-Myc in the regulation of cell growth, differentiation, and survival, the rapid down-regulation of c-*myc* gene expression on the neuroblasts could be one of the initial events responsible for the effects of thyroid hormones during brain development.

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