MOLECULAR AND CELLULAR BIOLOGY, Apr. 1997, p. 1860–1867 Vol. 17, No. 4
0270-7306/97/$04.00+0

Differential Regulation of the N-*myc* Proto-Oncogene by RORα and RVR, Two Orphan Members of the Superfamily of Nuclear Hormone Receptors

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Received 22 October 1996/Returned for modification 22 November 1996/Accepted 10 January 1997

RORα1 and RVR are orphan members of the superfamily of nuclear hormone receptors which constitutively activate and repress, respectively, gene transcription by binding to a common DNA sequence. In an attempt to understand the physiological functions of these two transcription factors, we aimed to identify target genes. We have identified a consensus binding site for RORα1 and RVR in the first intron of the N-*myc* gene that we designated N-*myc* RORE (ROR response element). Unlike most of the intronic sequence, the region encompassing the N-*myc* RORE is highly conserved between human and mouse, underscoring its importance. Our studies revealed that RORα1 and RVR specifically bind to the human and mouse N-*myc* ROREs and transactivate and transrepress, respectively, reporter constructs containing the ROREs. Moreover, Northern blot analysis demonstrated a direct modulation of an exogenously introduced N-*myc* gene by RORα1 and RVR in COS-1 cells. This effect is mediated through the N-*myc* RORE, since mutation of this site abolished the regulatory effects of both receptors. While transfection of RORα1 in P19 embryonic carcinoma cells had no effect on the levels of endogenous N-*myc* mRNA, RVR down-regulated its expression. The regulatory function of the N-*myc* RORE was further demonstrated by the rat embryonic fibroblast (REF) transformation assay. Mutation of the RORE increased the oncogenic potential of the N-*myc* gene in the REF assay. The foci were more numerous and significantly larger with the mutated than with the wild-type N-*myc* gene, regardless of RORα1 or RVR expression. Moreover, concomitant expression of RORα1 and wild-type N-*myc* resulted in a twofold increase in the number of transformed foci. In contrast, RVR expression resulted in the formation of foci that could be established as permanent clones with a very low frequency compared to foci transformed in its absence. These observations show that ablation of the RORE results in a more oncogenic form of N-*myc* and suggest that deregulation of the activity of the RORα1 and RVR could contribute to the initiation and progression of certain neoplasias.

N-Myc is a member of a family of nuclear phosphoproteins consisting of products of three well-characterized genes, c-*myc*, N-*myc*, and L-*myc*, which possess oncogenic activity (27). Deregulated expression of all three *myc* genes has been implicated in the genesis or progression of naturally occurring tumors as well as in the transformation of cultured cells in cooperation with activated Ha-*ras* (3, 24, 38, 49). In normal tissues, the precise function of the N-*myc* gene product remains unclear. The gene is primarily expressed in the developing embryo (5, 17, 32, 51) and is essential for normal organogenesis (2, 40). N-*myc* expression is absent from normal adult tissues, but high levels of transcript have been observed in tumors of neuroendocrine and embryonic origin. These include Wilms' tumor, neuroblastoma, retinoblastoma, and small-cell lung carcinoma (SCLC) (20, 21, 26, 33, 37). Moreover, this tumor distribution correlates strongly with N-*myc*-specific expression during normal development (51), suggesting that neoplasia may result from a deregulation of N-*myc* in tissues that expressed it during organogenesis.

Amplification of the gene appears to be the predominant mechanism by which N-*myc* is activated in most tumors (20, 21, 26, 33, 37). However, a subset of aggressive neuroblastomas lack amplification but express high levels of N-*myc* (42, 43). Furthermore, deregulation of N-*myc* gene expression in SCLC cell lines not only has been attributed to gene amplification but also may be secondary to increased promoter activity (27). Therefore, the lack of a strict correlation between high levels of N-*myc* expression and gene amplification suggests that overexpression of N-*myc* may also result from changes in the activity of specific transcriptional regulators.

In contrast to the wealth of information accumulated during the last 10 years on the regulation of c-*myc* transcription, the molecular basis for the control of N-*myc* expression remains to be elucidated. The structure of the N-*myc* gene is closely related to that of the c-*myc* gene, including the utilization of multiple transcription initiation sites (22). However, the N-*myc* gene has a single major promoter that contains a TATA box and Sp1 and AP2 binding sites (4). It has been demonstrated that major regulatory elements are located within 1 kb upstream of the major transcriptional start site of the human N-*myc* gene and in the first exon and intron of the gene (16). Furthermore, murine and human regulatory elements are functionally equivalent, as the human gene was appropriately expressed in transgenic mice (16). The regulatory elements present in the first intron of N-*myc* are involved in the down-regulation of the gene (16, 31, 43, 44, 46–48), and transcriptional attenuation was proposed as one of the mechanisms responsible for this effect (46). It remains possible, however, that the negative regulatory elements located in the first intron act as binding sites for transcriptional repressors (48). To date,

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no transcriptional factor known to regulate N-*myc* expression has been identified and/or functionally characterized.

Orphan nuclear receptors now constitute a large subgroup of the superfamily of steroid/thyroid/retinoid receptors for which no ligand has been identified. The ROR (also termed RZR) subfamily of receptors is encoded by three different genes: α, which produces four isoforms; β; and γ (1, 14, 18, 35). The Rev-Erb subgroup contains two members, Rev-Erbα (25, 30, 39) and RVR (or Rev-Erbβ) (6, 8, 36). RORα1 and RVR bind DNA as monomers to a common sequence (referred to as a ROR response element [RORE]) composed of a single half site PuGGTCA preceded by a 6-bp AT-rich region (6, 8, 12, 14, 29, 36). While RORα1 appears to act as a constitutive transcriptional activator in the absence of exogenously added ligand, RVR lacks the activation function (AF-2) normally present at the carboxy-terminal end of the ligand binding domain and, through direct interaction with the transcriptional corepressor N-CoR, represses basal as well as RORα1-mediated promoter activity (8, 15, 36, 50). This finding suggests that RORα1 and RVR may differentially regulate transcription in cells where they are coexpressed depending on their respective levels of expression. However, target genes responsive to both RORα and RVR remain to be identified. In this study, we report the identification of a binding site for these orphan receptors in the first intron of the N-*myc* gene, a region of the gene thought to be involved in its transcriptional regulation. We demonstrate that RORα1 and RVR differentially control the transcription of the N-*myc* gene, an observation that identifies these proteins as the first known modulators of N-*myc* expression.

MATERIALS AND METHODS

Oligonucleotides and plasmid construction. Oligonucleotides used in this study were designed with *Sal*I and *Bam*HI cohesive ends. The following oligonucleotides and their complements were used as probes for electrophoretic mobility shift assay (EMSA): ROREα2, 5′-TCGACTCGTATAACTAGGTCA AGCGCTG-3′; human N-*myc* RORE, 5′-TCGACTCGTCAATCTGGGTCAA GCGCTG-3′; mouse N-*myc* RORE, 5′-TCGAGAGTGATAATGTAGGTCAC GGCTG-3′; and mouse mutant m1 N-*myc* RORE with 4-bp substitutions (underlined), 5′-TCGAGAGTGATACTGGAGATTACGGCTG-3′. These oligonucleotides were introduced into TKLUC (41) in one or three copies to create reporter genes ROREα2TKLUC, human N-mycRORETKLUC, mouse N-mycRORETKLUC, and mouse mN_{m1}-mycRORETKLUC. In constructs containing three copies of the ROREs, each binding site was separated by an 18-bp spacer in a head-to-tail configuration. Plasmid TKLUC contains sequences −105 to +51 of the thymidine kinase promoter linked to the firefly luciferase gene. All constructs were confirmed by sequencing. RSVmN-myc and $RSVmN_{m1}$-myc were constructed as follows. The entire transcriptional unit of the N-*myc* gene (a 7.3-kb *Eco*RI genomic fragment; gift of C. Asselin, Sherbrooke University) and mN_{m1}-myc (see below) were digested with *Nar*I, blunted with the Klenow fragment of DNA polymerase, and ligated to *Not*I linkers. After digestion with *Not*I, the fragments were introduced into pRC/RSV (InVitrogen).

Site-directed mutagenesis. The 7.3-kb *Eco*RI N-*myc* genomic fragment was subcloned into the *Eco*RI site of plasmid pBluescript II KS (Stratagene) in the reverse orientation and transformed into competent *Escherichia coli* CJ236 (*dut ung*) cells on a 2×YT plate containing 0.25 μg of uracil and 50 μg of ampicillin per ml. CJ236 cells containing the N-*myc* plasmid were used to synthesize single-stranded DNA with the M13 helper phage. The mutant N-*myc* RORE oligonucleotide was used for mutagenesis by the method of Kunkel et al. (23). The mutant constructs were identified by sequencing.

In vitro synthesis of proteins and EMSA. Plasmids pCMX-hRORα1 (14) and pCMX-mRVR (36) were used to synthesize human RORα1 and mouse RVR proteins in vitro, using rabbit reticulocyte lysates (RRL) with a TNT-T7 kit (Promega, Madison, Wis.). Probes for EMSA were radiolabeled by end filling with the Klenow fragment of DNA polymerase. Approximately 0.1 ng of probe was used in each reaction with a total of 1 μl (RORα1) and 10 μl (RVR) of programmed RRL in a buffer containing 10 mM Tris-HCl (pH 8.0), 40 mM KCl, 6% glycerol, 1 mM dithiothreitol, and 0.05% Nonidet P-40 in a final volume of 25 μl. To prevent single-stranded binding, 10 ng of a nonspecific oligonucleotide was included in the binding reaction. As a control, probes were also incubated with the same amount of unprogrammed lysate. Complexes were resolved on a 4% nondenaturing polyacrylamide gel in 0.5× Tris-borate-EDTA.

Cell culture and transfection assays. COS-1 and P19 embryonic carcinoma (EC) cells were maintained in alpha minimal essential medium in 7.5% fetal calf serum. COS-1 cells were transfected by a calcium phosphate coprecipitation technique, and P19 EC cells were transfected with liposomes (Lipofectamine; Gibco BRL) as indicated in the appropriate figure legends. β-Galactosidase and luciferase assays were performed as described previously (13).

REE transformation assay. Primary rat embryonic fibroblasts (REFs) were purchased from BioWhittaker and maintained in Dulbecco modified Eagle medium containing 10% fetal calf serum. After one passage, secondary REFs were transfected by the calcium phosphate method with 2 μg of Ha-*ras*, 2 μg of RSVN-myc wild type or mutant as indicated, 5 μg of appropriate receptor, and pBluescript II KS as a carrier for a total of 20 μg per 100-mm-diameter tissue culture dish. The transfected REFs were washed, fresh medium was added 16 h after transfection, and cultures were split 1:3 24 h later. Numbers of foci were determined 10 days after the transfection.

Northern blot analysis. Total RNA was collected from cells transiently transfected with high efficiency (monitored by measuring β-galactosidase activity) by using a Pharmacia RNA extraction kit or TRIZOL (Gibco BRL) according to the manufacturer's instructions. RNA samples (10 μg of total RNA) were electrophoresed through a 1% formaldehyde–1× MOPS (morpholinepropanesulfonic acid)–1% agarose gel and transferred to a nylon membrane in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). RNA was UV cross-linked to the membrane and hybridized at 42°C to the appropriate random-primed probe (10^6 cpm ml^{-1}) in 50% formamide–5× Denhardt's solution–5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7])–0.1% sodium dodecyl sulfate (SDS)–100 μg of denatured salmon sperm DNA per ml. The membranes were washed once for 20 min at room temperature with 2× SSC–0.1% SDS and twice at 65°C in 0.2× SSC–0.1% SDS for 15 min each time. Autoradiography was carried out at −70°C with an intensifying screen. An oligonucleotide hybridizing to the 18S rRNA (5′-ACGGTATCTGATCGTCTT CGAACC-3′) was used to control for RNA loading.

Nucleotide sequence accession numbers. GenBank accession numbers for human and mouse N-*myc* are Y00664 and M12731, respectively.

RESULTS

RORα1 and RVR bind to the N-*myc* RORE. Our molecular studies of orphan receptor function have led us to identify consensus DNA binding sites for RORα1 and RVR (referred to as a RORE) (10, 36). One approach to identify target genes for nuclear receptors consists of scanning databases for the presence of consensus DNA binding sites in regulatory sequences of cloned transcription units. This strategy led to the localization of a RORE within a region of the first intron of the N-*myc* gene implicated in the regulation of its expression (Fig. 1) (16). Although the first introns of the human and mouse N-*myc* genes have only a very limited degree of homology, the consensus RORE (hereafter referred to as human or mouse N-*myc* RORE) is extremely well conserved in the two species, suggesting an important regulatory function (Fig. 1C).

To test whether RORα1 and RVR can interact with the N-*myc* RORE, we performed EMSA using in vitro-synthesized RORα1 and RVR and synthetic ^{32}P-labeled oligonucleotides representing ROREα2, the element originally described as the consensus binding site for RORα1 (14), as well as the human and mouse N-*myc* ROREs. As shown in Fig. 2, lane 2, RORα1 binds strongly to the positive control, ROREα2, and the binding is specifically competed by addition of a 100-fold excess of unlabeled synthetic oligonucleotides representing ROREα2 and human and mouse N-*myc* ROREs (lanes 4 to 6). Similarly, RORα1 binds to the human (lane 8) and mouse (lane 14) N-*myc* ROREs, and in each case binding is competed specifically by unlabeled ROREα2 and by human and mouse N-*myc* RORE oligonucleotides (lanes 10 to 12 and 16 to 18). RORα1 bound to the mouse N-*myc* RORE with higher affinity than to the human N-*myc* RORE (compare lanes 8 and 14). Moreover, the human N-*myc* RORE was a less efficient competitor than either the ROREα2 or the mouse N-*myc* RORE (for example, compare lane 17 with lanes 16 and 18). Figure 2 also demonstrates the binding of RVR to the mouse N-*myc* RORE (lane 20) as well as specific competition with 100-fold molar excess of unlabeled mouse N-*myc* RORE (lane 22).

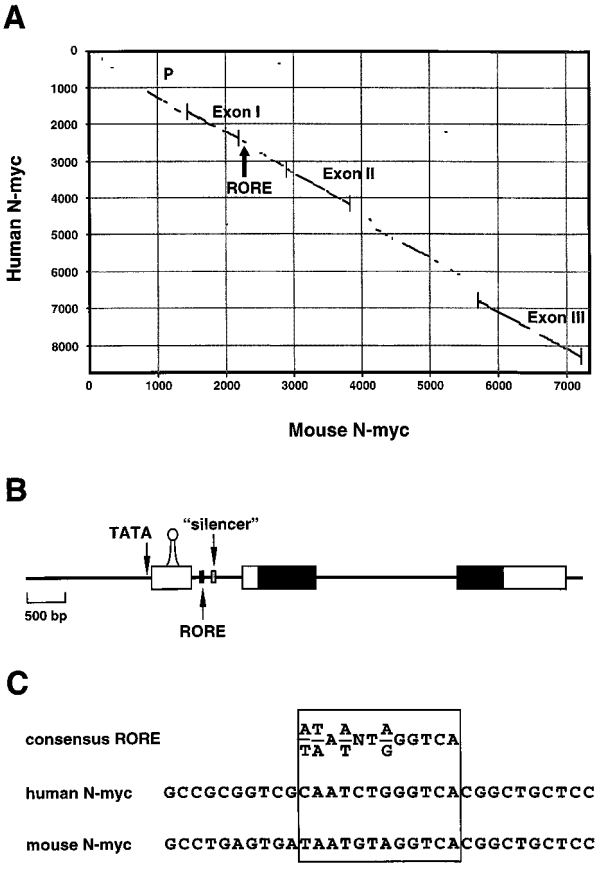

FIG. 1. The mouse and human N-*myc* genes contain a RORE. (A) Schematic comparison of the sequences of the human and mouse N-*myc* genes. Solid lines represent regions of homology, whereas gaps represent divergence between the two genes. (B) Schematic representation of the transcriptional unit of the murine N-*myc* gene used in this study. The three exons are represented by boxes; translated regions are shown in black. The locations of the TATA box, a putative stem-loop, a silencer region, and the RORE are also indicated. (C) Sequence surrounding the N-*myc* ROREs and comparison between consensus ROREα2 and the human and mouse N-*myc* ROREs.

RORα1 and RVR have opposite transcriptional activities on N-*myc* RORE. To confirm that the human and mouse N-*myc* ROREs confer responsiveness to RORα1 and RVR, we linked one or three copies of the N-*myc* ROREs upstream of the thymidine kinase promoter and the luciferase reporter gene. These reporter constructs were then tested by transfection in COS-1 cells together with RORα1 and RVR expression vectors (Fig. 3). Transfection of the RVR expression vector resulted in significant repression of basal promoter activity with mouse (Fig. 3A and B) and human (Fig. 3C) N-*myc* RORE reporter constructs. As expected, when RORα1 expression vector was cotransfected, a fourfold induction of luciferase activity over basal levels was observed with the human N-*myc* RORE construct (Fig. 3C). Higher levels of RORα1 responsiveness (>10-fold) were observed when the mouse N-*myc* RORE was tested (Fig. 3B). To investigate whether RVR could repress RORα1-induced transcriptional activity on the human and mouse N-*myc* elements, we transfected increasing amounts of RVR with constant levels of RORα1 together with the human or the mouse N-*myc* RORE reporter. We found that RVR could efficiently decrease RORα1-induced transcriptional activity from both human and mouse N-*myc* ROREs (Fig. 3).

To further assess the specificity of these interactions, we generated a pair of oligonucleotides harboring point mutations in the mouse N-*myc* RORE (Fig. 4A). Figure 4B demonstrates that both RORα1 (lane 3) and RVR (lane 6) failed to interact with the mutated RORE in EMSA and also failed to repress (RVR) or activate (RORα1) transcription from it in transient transfection experiments (Fig. 4C).

RORα1 and RVR regulate N-*myc* gene expression. To test the hypothesis that the N-*myc* RORE is functional and that the N-*myc* gene is responsive to RORα1 and RVR, we transfected into COS-1 cells the entire mouse N-*myc* transcription unit (a 7.3-kb *Eco*RI genomic fragment) together with RORα1 and RVR expression vectors, singly or in combination. Northern blot analysis revealed that cotransfection of RORα1 causes a significant increase in N-*myc* transcript levels (Fig. 5, lane 2). In contrast, introduction of the RVR expression vector led to a reduction in N-*myc* mRNA levels (Fig. 5, lane 3) and antagonized the stimulatory effects of RORα1 (Fig. 5, lane 4). These effects were mediated by the N-*myc* RORE, since mutation of this site abolished both the RORα1- and RVR-mediated responses (Fig. 5, lanes 5 to 8).

To study the effects of RORα1 and RVR on the expression of the endogenous N-*myc* gene in vivo, we took advantage of the P19 cell line, which expresses the N-*myc* gene. We used Lipofectamine to transfect a large proportion (>50%) of P19 cells and coexpressed RORα1 and RVR, alone or in combination. Northern blot analysis demonstrated that transiently transfected RVR can repress the endogenous N-*myc* gene (Fig. 6, lane 3). However, RORα1 alone did not seem to influence N-*myc* transcription (Fig. 6, lane 2) but could reverse the inhibitory effect of RVR (Fig. 6, lane 4). Since P19 EC cells

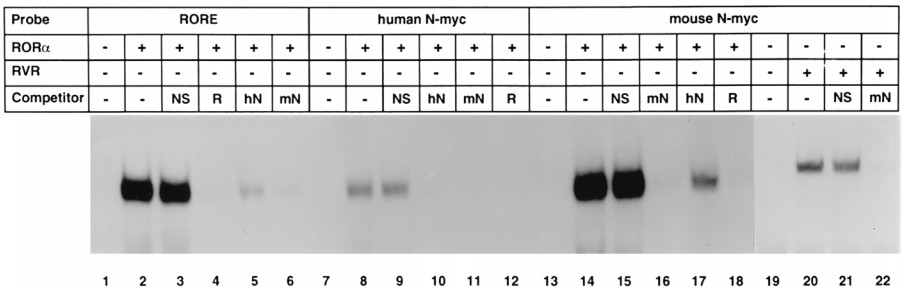

FIG. 2. RORα1 and RVR bind specifically to the N-*myc* RORE. Radiolabeled oligonucleotides encoding consensus ROREα2 and human and mouse N-*myc* ROREs were incubated with RRL programmed with RORα1 (lanes 2, 8, and 14). Radiolabeled mouse N-*myc* RORE was incubated with RRL programmed with RVR (lane 20). Probes were also incubated with unprogrammed lysate as a control (lanes 1, 7, 13, and 19). Unlabeled ROREα2 (R; lanes 4, 12, and 18), human N-*myc* RORE (hN; lanes 5, 10, and 17), and mouse N-*myc* RORE (mN; lanes 6, 11, 16, and 22) as well as a nonspecific competitor (NS; lanes 3, 9, 15, and 21) were used at 100-fold molar excess.

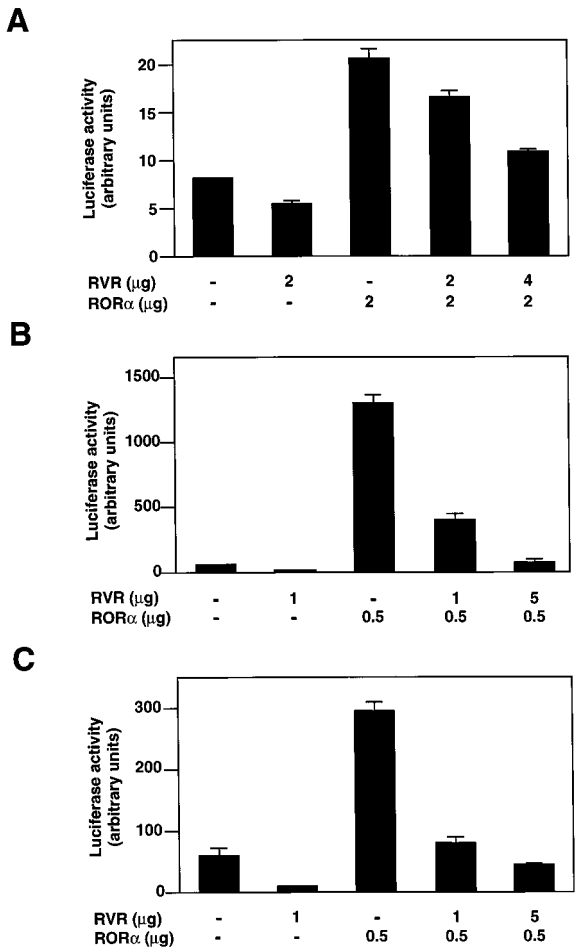

FIG. 3. RORα1 and RVR differentially modulate transcription from the mouse (A and B) and human (C) N-*myc* ROREs. COS-1 cells were transfected with 2 μg each of reporters carrying one (A) or three (B) copies of the mouse N-*myc* RORE or three copies of the human N-*myc* RORE (C) and pCMX (control), pCMXRVR, pCMXRORα1, pCMXRVR plus pCMXRORα1, and pCMXRVR plus pCMXRORα1 expression vectors as indicated. pRSVβgal (1 μg) was cotransfected as an internal control. The cells were harvested 36 h later and assayed for luciferase and β-galactosidase activities. The experiments were repeated three times. Standard deviations were less than 10%.

express RORα (7, 28), it is possible that N-*myc* expression is at or near its highest level, and therefore only repression can be observed. Taken together, these results indicate that RORα1 and RVR can compete to regulate the expression of the N-*myc* gene in the context of its own regulatory sequences.

RORα1 potentiates N-*myc* oncogenic potential. To further demonstrate that RORα1 and RVR are functional regulators of N-*myc* transcription, we used a quantitative biological assay based on the ability of N-*myc* to transform REFs in cooperation with activated Ha-*ras* (3, 38, 49). We therefore decided to compare the numbers of foci generated with wild-type and RORE-deficient N-*myc* genes. We show that transformation in the presence of the RORE-deficient N-*myc* gene results in a fourfold increase in the number of foci compared to the wild-type N-*myc* (Fig. 7A). Furthermore, these foci were significantly larger than those produced by wild-type N-*myc* (data not shown). To test whether this increase in foci number with RORE-deficient N-*myc* was due to the loss of a site for a transcriptional repressor, we decided to verify if RORα1 and/or RVR could play a role in N-*myc* transformation in the REF assay. Activation and/or inhibition of N-*myc* activity was assessed by comparing the numbers of transformed foci generated in cotransfections containing wild-type or mutated N-*myc* and activated Ha-*ras* in the presence of RORα1 and/or RVR. We observed that when RORα1 was coexpressed with wild-type N-*myc*, a twofold increase in the number of foci was detected compared to REFs transformed with Ha-*ras* and N-*myc* alone (Fig. 7B). In contrast, when RVR was transfected with wild-type N-*myc*, the same number of foci was formed as in cells not expressing RVR (Fig. 7B). In this assay, RVR only slightly altered RORα1 action. However, unlike foci generated with Ha-*ras* and wild-type N-*myc* in the absence or presence of RORα1, very few foci expressing RVR could be expanded in culture (Fig. 7C). When RVR was coexpressed with Ha-*ras* and the RORE-deficient N-*myc* gene, the foci produced established cell lines with greater efficiency than foci transformed with wild-type N-*myc* (Fig. 7C). These results indicate that the effects of RVR on cellular activities are via N-*myc* through its RORE.

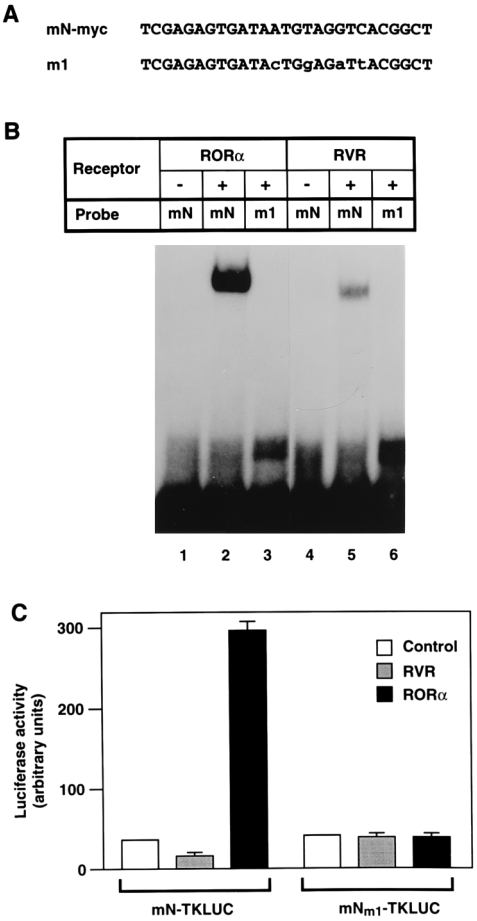

FIG. 4. RORα1 and RVR fail to bind to and modulate transcription via a mutant mouse N-*myc* RORE. (A) Wild-type (mN-myc) and mutated (m1) mouse N-*myc* ROREs. (B) Radiolabeled wild-type (mN) and mutated (m1) mouse N-*myc* RORE probes were incubated with RRL programmed with RORα1 (lanes 2 and 3) and RVR (lanes 5 and 6). The probes were also incubated with unprogrammed lysate (lanes 1 and 4). (C) COS-1 cells were transfected with 1 μg of expression vector and 2 μg of reporters containing three copies of either the wild-type (mN) or mutated (mN_{m1}) N-*myc* RORE upstream of TKLUC. Luciferase values were normalized to β-galactosidase activity. The experiments were repeated three times. Standard deviations were less than 10%.

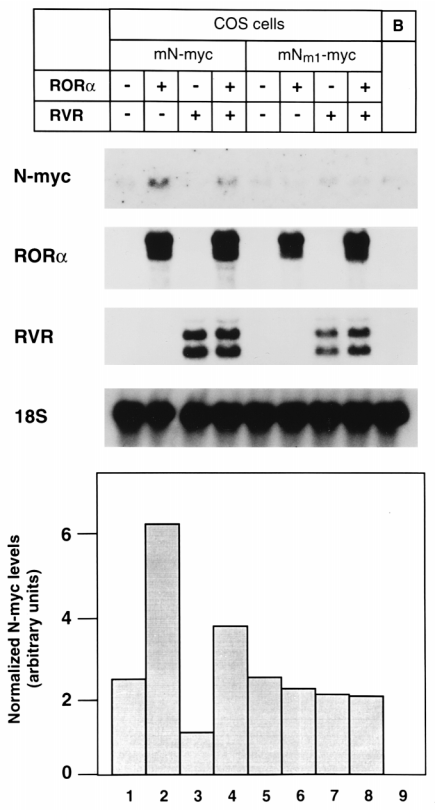

FIG. 5. RORα1 up-regulates and RVR down-regulates the transcription of the murine N-*myc* gene. Northern blot analysis of RNA isolated from COS-1 cells transfected, using the calcium phosphate coprecipitation technique, with 2 μg of pCMX (lanes 1 and 5), 2 μg of pCMXRORα1 (lanes 2 and 6), or 2 μg of pCMXRVR (lanes 3 and 7) alone or in combination (lanes 4 and 8) and 6 μg of a 7.3-kb wild-type (mN-myc) or mutated (mN_{m1}-myc) mouse N-*myc* genomic fragments. Ten micrograms of total RNA from each group was loaded on an agarose gel. An equal amount of mouse newborn brain RNA (B) was used as a positive control (lane 9). The blot was probed with a 643-bp *Pvu*II/*Spe*I fragment encompassing the second exon of the mouse N-*myc* gene. Expression of transfected RORα1 and RVR was also monitored. An 18S rRNA probe was used as a control for equal loading and transfer. Levels of N-*myc* mRNA relative to the amount of 18S RNA were determined by quantitation with a Fuji BAS1000 PhosphorImager. A representative experiment is shown.

DISCUSSION

RVR has been shown to interfere with RORα1-induced transcriptional activity in transient transfections using synthetic response elements linked to reporter genes, suggesting that these two orphan receptors may regulate differentially a common set of genes (36). In an attempt to understand their physiological functions, we aimed to identify natural target genes. Using a database search and interspecies DNA sequence comparisons, we have identified a binding site for these two orphan receptors in the first intron of the N-*myc* gene. Our results have clearly demonstrated that the N-*myc* RORE is functional in vivo and that RORα1 and RVR differentially regulate the expression of N-*myc*. These observations identify for the first time transcription factors, members of the superfamily of nuclear hormone receptors, that specifically interact with the N-*myc* gene. In addition, this work firmly establishes the existence of an orphan nuclear receptor-based signaling pathway that can regulate the expression of specific gene networks through competition between transcriptional activators and repressors for the same recognition site.

We have shown that RORα1 and RVR directly modulate N-*myc* expression. An exogenous N-*myc* gene transiently introduced into COS-1 cells was up-regulated by RORα1 and down-regulated by RVR. More importantly, RVR was also able to down-regulate the endogenous N-*myc* gene in P19 EC cells. The transcriptional effects of RORα1 and RVR on the N-*myc* gene must be mediated through the N-*myc* RORE, since mutation of this element abolished the transcriptional activity of both transcription factors in COS-1 cells. The N-*myc* RORE is configured as a monomeric nuclear receptor binding site, containing a single PuGGTCA core motif preceded by a 6-bp AT-rich sequence. That RVR can repress the expression of a gene containing a single copy of a monomeric nuclear receptor binding site is an important observation. It has recently been suggested that Rev-Erbα, whose structure and mechanisms of action are similar to those of RVR, acts efficiently as a basal repressor of a promoter linked to a hormone response element (HRE) configured as a direct repeat spaced by two nucleotides (DR-2) (15). RVR also shares with Rev-Erbα the ability to bind as a homodimer and function as a repressor on a DR-2 (7). However, our data clearly indicate that RVR, in the context of a natural and functional transcription unit, can also act as an active repressor when bound to a monomeric HRE. This is demonstrated by the observation that expression of RVR in COS-1 cells down-regulates N-*myc* but that ablation of the N-*myc* RORE has no effect on its expression (Fig. 5). If squelching or simple competition for binding site occupancy of an activator were responsible for RVR action in these cells, we would predict lower basal N-*myc* expression in the absence of the RORE. That RVR can function as an active repressor is further supported by the recent finding that ligand-independent transcriptional repression by Rev-Erbα is potentiated by the corepressor N-CoR through direct protein-protein interaction (50). However, competition for binding site occupancy remains an important mechanism of action for ROR and RVR in cells where the two receptors are coexpressed. Taken together, these observations define Rev-Erbα and RVR as a class of transcriptional repressors that can function in both the monomeric and the dimeric state. The specific configuration of the RORE, a single PuGGTCA motif preceded by a 6-bp AT-rich region, also suggests that the interactions between this element and nuclear receptors may be specific to members of the ROR and Rev-Erb subfamilies. To date, no other members of the superfamily of nuclear receptors

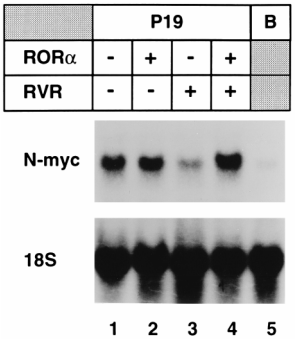

FIG. 6. RVR down-regulates the endogenous N-*myc* gene. P19 cells were transiently transfected, using liposomes, with 10 μg of control plasmid pCMX (lane 1), pCMXRORα1 (lane 2), pCMXRVR (lane 3), or the latter two together (lane 4) as recommended by the manufacturer. An equal amount of mouse newborn brain RNA (B) was used as a positive control (lane 5). Twenty micrograms of total RNA was loaded, and the blot was hybridized with the same N-*myc* probe as described in the legend to Fig. 5. An 18S rRNA probe was used as a control for equal loading and transfer. A representative experiment is shown.

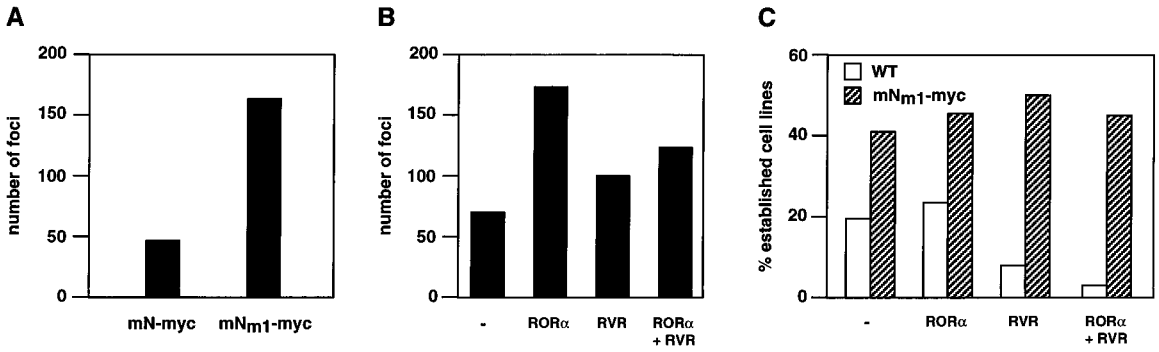

FIG. 7. The N-*myc* RORE is important for the oncogenic activity of N-*myc*. (A) REFs were transfected with 2 μg of wild-type (mN-myc) or mutated (mN_{m1}-myc) N-*myc* gene and 2 μg of activated Ha-*ras*. The number of foci was recorded from three individual experiments that included 10 100-mm-diameter plates/group/experiment. (B) REFs were transfected with 2 μg of N-*myc* gene, 2 μg of activated Ha-*ras*, 5 μg of pCMXRORα1, 5 μg of pCMXRVR, or 5 μg of each expression vector. The total number of foci from six individual experiments that included 10 plates/group/experiment is represented. (C) Percentage of permanent clones established from foci transformed with wild-type (WT) and RORE-deficient (mN_{m1}-myc) N-*myc* genes. Twenty-four foci were picked from each group, trypsinized, and plated into 24-well tissue culture plates. The clones were passaged every other day for 1 week and were subsequently transferred to 60-mm-diameter dishes. Permanent clones were those that grew at a high rate in 60-mm-diameter dishes for an additional 10 days.

have been demonstrated to recognize this form of monomeric HRE.

The importance of the RORE in the control of expression of the N-*myc* gene is also strengthened by its conservation across species. Our results have demonstrated that both RORα1 and RVR specifically bind to the human and mouse N-*myc* ROREs. However, RORα1 has a slightly greater affinity for the mouse N-*myc* RORE than for the human counterpart. This may be due to the G residue at position +1 of the hexamer PuGGTCA and/or to the C at position −6 of the human N-*myc* RORE, since these are the only two changes with respect to the ROREα2, the ideal consensus site originally defined for RORα1 (Fig. 1) (14). Although a G at position +1 permits binding of RORα1 to the RORE, the site most often selected for binding contains an A at this position. Furthermore, a T or an A at position −6 results in higher-affinity binding (14). The stronger binding ability of RORα1 to mouse N-*myc* RORE was reflected by a stronger transactivation of the mouse N-*myc* luciferase reporter construct by RORα1. However, although the mouse N-*myc* RORE gave better responsiveness, the human N-*myc* RORE behaved identically; i.e., RORα1 activated and RVR repressed transcription from both human and mouse N-*myc* RORE reporter constructs. In addition, RVR interfered with RORα1-induced transcriptional activity on both reporters, consistent with previous studies by us (14) and others (8) using an ideal synthetic element.

That the N-*myc* RORE acts as an essential regulatory element of the N-*myc* gene in vivo comes from our observation that when the RORE was functionally impaired by mutations, the N-*myc* gene became more oncogenic. This phenomenon has previously been observed in the REF assay using N-*myc* gene deletion constructs (46). In this study, an increase in steady-state mRNA after the removal of a stem-loop structure present in the first exon was observed, and therefore a loss of transcriptional attenuation was proposed to be partially responsible for this phenomenon. To observe a large increase in the number of foci, the entire first exon and intron had to be removed, suggesting the presence of other *cis*-acting elements (46). The N-*myc* RORE is also distinct from a silencer element, located in the first intron of human N-*myc*, that dictates cell-type-specific activity to the N-*myc* promoter (45). In contrast to the RORE, the short DNA sequence encoding the proposed silencer (AGCCTCTCC) is not conserved between humans and rodents. These previous studies as well as our present observations suggest multiple modes of regulation of the N-*myc* gene.

That the RORE appears to play an important role in N-*myc* regulation may have important implications for the normal and abnormal biology of both orphan nuclear receptors and N-*myc*. It will be of interest to compare the patterns of expression of all members of the ROR and Rev-Erb subfamilies with that of N-*myc* during early embryonic development as well as to investigate whether RVR and Rev-ErbAα contribute to the silencing of N-*myc* expression in adult tissues. It is also conceivable that deregulation of RORα1 and/or RVR can directly affect N-*myc* expression. Overexpression of RORα1, or loss of RVR, could lead to inappropriate expression of N-*myc* such as is the case in retinoblastoma, neuroblastoma, and SCLC. For example, N-*myc* gene amplification and overexpression are common events in SCLC. Genetic alterations associated with SCLC are numerous but always include deletions in the short arm of chromosome 3 (9). Deletion of this region is a very early event in the pathogenesis of lung cancer as it is detected in hyperplastic lung tissue (19). However, N-*myc* gene amplification and expression are often observed later in the course of the disease (34), suggesting that loss of 3p may be a prerequisite for N-*myc* expression. We have recently localized RVR to a region of mouse chromosome 14 which is in synteny with human chromosome 3p24 (11), suggesting that the RVR gene may be nonfunctional in SCLC and therefore can contribute to neoplastic transformation. The REF assay also demonstrated that RVR can repress N-*myc* expression. In all cases, when RVR was coexpressed with Ha-*ras* and wild-type N-*myc*, very few foci could be passaged in culture. In contrast, RVR had no effect on the number or morphology of the foci when coexpressed with Ha-*ras* and N-*myc* with a mutated RORE. Taken together, these observations suggest that RVR can be considered a candidate tumor suppressor gene, a possibility that warrants further studies on the status of the RVR gene in tumors overexpressing N-*myc*.

In conclusion, we have identified two transcription factors, RORα1 and RVR, that specifically interact with a novel regulatory element in the N-*myc* gene and differentially regulate its expression. Moreover, the *cis*- and *trans*-acting elements that we have identified may have physiological relevance to the development and progression of tumors expressing N-*myc*. These findings raise the possibility that in the event that natural and/or synthetic ligand/activators specific for these orphan

receptors are discovered, pharmacological agents could be used as therapeutic modalities for malignancies in which the N-*myc* transcript is overexpressed.

ACKNOWLEDGMENTS

We thank members of the Giguère laboratory for critical reading of the manuscript, J. Trasler for the 18S oligonucleotide sequence and hybridization protocol, and C. Asselin for providing the mouse N-*myc* gene.

This research was supported by grants from the Cancer Research Campaign Inc. and the Medical Research Council of Canada (MRC). V.G. is an MRC Scientist, and I.D. is a recipient of a fellowship from the Fonds de la Recherche sur la Santé du Québec.

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