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N-myc Transactivates RCC1 Gene Expression in Rat Fibroblast Cells Transformed by N-myc and v-ras[1]

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Oncogenic cooperation was found between the N-myc and v-ras oncogenes in a rat fibroblast cell line, 3Y1. To investigate the specific role of N-myc in the transformation, we established transformed cell lines that expressed N-myc under a controllable promoter. Using these cells, we found that constitutive expression of N-myc is necessary to maintain the transformation, and that the expression level of N-myc is closely correlated with the transformation. Since another myc family gene, c-myc, directly activates expression of RCC1, which has important functions for eukaryotic cell proliferation, we focused on the relationship between N-myc and RCC1. Cells transformed by N-myc and v-ras expressed several times more RCC1 mRNA than the parent 3Y1 cells, and the expression of RCC1 changed in a parallel with the expression of N-myc. Gel retardation analysis and experiments with reporter plasmids constructed from a DNA fragment of the RCC1 gene indicated that the N-Myc protein controls expression of RCC1 by binding directly to CACGTG elements in the RCC1 gene. These results suggest that N-myc can directly transactivate expression of RCC1, a c-myc target gene.

Key words: E box element, gene expression, N-myc, RCC1, transformation.

The N-myc gene was originally isolated as an amplified DNA sequence with limited homology to c-myc in a subtype of neuroblastomas (*1*). Amplification of the N-myc gene had also been found in tumor cells derived from retinoblastomas (*2*) and small-lung cell carcinomas (*3*). N-myc amplification (gene dosage) and expression are highly correlated with advanced stage of diseases (*4-6*). Ectopic expression of N-myc with a second oncogene, *e.g.* an activated ras, can efficiently induce tumors in range of many type of tissues (*7*). These results clearly showed that N-myc is a causal factor in tumorigenesis, but the mechanism of malignant transformation by N-myc remains poorly understood.

Myc genes constitute a small gene family, of which the best studied member is c-myc, which is also a causal factor in tumorigenesis. The mechanism of the transformation by c-myc is also unclear. The c-myc gene encodes c-Myc protein, which forms a heterodimer with a partner protein Max to bind an E box element (mainly CACGTG), then to act as a sequence-specific transcription factor. Tumorigenic activity of c-myc is at least in part attributed to the transcriptional activity (*8*). Recently we found that c-myc directly stimulates expression of RCC1 (*9*). The RCC1 gene is necessary for mammalian and yeast cells to proliferate (*10, 11*). The RCC1 gene product is a chromatin-bound protein (*12*) that acts as a guanine nucleotide exchange factor for Ran, a ras-related nuclear GTPase (*13, 14*), and is required for many important nuclear functions, including DNA replication (*15-17*) and nuclear-cytoplasm transport of proteins and RNAs (*18-23*). It has also been shown that c-Myc can directly control several genes (*24*), including ornithine decarboxylase (ODC) (*25*), p53 (*26*), ECA39 (*27*), prothymosin α (*28*), cad (*29*), Cdc25A (*30*), eIF4E (*31*), MrDb (*32*), and LDH-A (*33*). It is plausible that stimulated expression of some of these genes by c-myc contributes to the transformation.

Although N-Myc protein forms a heterodimer with Max and binds to E-box elements as c-Myc protein does, its amino acid sequence is only 40% identical to that of c-Myc (*8*). Differences are even found in the DNA-binding region, suggesting that N-Myc may not bind to all the same E-boxes in the genome to which c-Myc binds. The amino acid sequence of the transactivation domain in N-Myc is also different from that of c-Myc. Other transcription factors, UBF and TEF, which bind to E-box elements but have different sequences in transactivation domains, do not transactivate expression of genes which are transactivated by c-myc (*34, 35*). Therefore, it is not clear whether N-Myc and c-Myc share the same target genes; certainly no genes that are directly stimulated by N-Myc have yet been reported.

Poor understanding of the mechanism of transformation by myc is, in part, due to lack of a proper experimental system to observe biological events during tumorigenesis.

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; eIF4E, eukaryotic Initiation Factor 4E; FBS, fetal bovine serum; LDH-A, lactate dehydrogenase-A; ODC, ornithine decarboxylase; RCC1, regulator of chromosome condensation-1.

Introduction of myc together with a second oncogene, *e.g.*, an activated ras, into mice, causes tumorigenesis in many types of tissues. It is, however, difficult to investigate biological events during tumorigenesis using this system, because they happen inside mice. In another system, rodent primary embryonic fibroblasts are transfected with myc and a second oncogene, resulting in the transformation. These embryonic cells, however, originate from many tissues, making the system unsuitable to investigate molecular events during transformation. The myc oncogene transforms a rat fibroblast cell line Rat-1 (*36*), but a second oncogene is not required for this transformation, which thereby differs from myc transformation in animal or primary cells.

In this paper, we show the oncogenic cooperation between N-myc and v-ras in a rat fibroblast cloned cell line, 3Y1. We also report the isolation of transformed cell lines that express N-myc under a controllable promoter, in which expression level of N-myc is well correlated to the transformation, and N-myc directly stimulates expression of RCC1 in the transformed cells.

MATERIALS AND METHODS

Plasmids—The 2 kb *Bam*HI fragment of mouse N-myc cDNA from pNc-1 (*37*) was inserted into the *Bam*HI sites of Bluescript KS(+) (Stratagene, La Jolla, CA). A plasmid having a *Hin*dIII site at the 5′ end of N-myc cDNA was selected and named pN-myc/BS. The 2 kb *Hin*dIII-*Xba*I fragment of mouse N-myc cDNA from pN-myc/BS was inserted into pCDM8 (Invitrogen Corp, San Diego, CA) which had been digested with *Hin*dIII and *Xba*I, to produce pN-myc/CDM8. The 2 kb *Bam*HI fragment of mouse N-myc cDNA from pNc-1 was also inserted into the *Bam*HI sites of pUHC10-3, which harbors a tetracycline-responsive promoter (*38*), to produce ptetN-myc. To express GST-N-Myc fusion protein, the cDNA encoding 101 amino acids from the carboxyl terminal of N-Myc protein was amplified by PCR with 5′-CCCGGGATCCCAGCAAAAGCGAAGAGCCTGAGC (adding *Bam*HI site) and 5′-CGGGGAATTCGTTTAGCAAGTCCGAGCGTGTTCG (adding *Eco*RI site) as primers. The amplified 0.3 kbp fragment was inserted into the 4.5 kb *Eco*RI-*Bam*HI fragment of pGEX-3X (Pharmacia Biotech) to produce pGEX-N-mycC101. The fusion protein, GST-N-MycC101, was produced according to the instruction manuals of Pharmacia Biotech. The plasmid structures were confirmed by DNA sequencing (*39*). pUHD 15-1 encodes a tetracycline-sensitive transcription factor (*38*). The v-ras expression vector was p4E, a Ki-MSV provirus clone, in which v-Ki-ras was driven by the Ki-MSV LTR promoter (*40*). pXb is a derivative of p4E which lacks the part of the DNA sequence coding v-Ki-ras and has lost the activity of transformation. pRSVlacZ, which expressed β-galactosidase under the RSV promoter was a kind gift of Dr. Okazaki. pAct Hyg, which contains a hygromycin-resistance gene under the control of an actin promoter, was a kind gift of Dr. Nakanishi.

Cell Lines, Cell Culture, and DNA Transfection—Rat 3Y1 fibroblasts (*41*) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). DNA transfection of 3Y1 fibroblasts was performed by the calcium-phosphate procedure as described previously (*42*). To analyze their capacity for anchorage-independent growth, the transfected cells were grown in soft agar for 2-3 weeks, then the number of visible colonies was counted. To select 3Y1 cells transformed with v-ras and N-myc, whose expression is regulated by tetracycline, 3Y1 cells were transfected with 3 μg of p4E, a v-ras expression vector, 3 μg of ptetN-myc, and 4 μg of pUHD 15-1, and the transformed cells were picked up, dissociated into single cells, grown in soft agar, and established. 3Y1 cells were transfected with pN-myc/CDM8 and p4E to establish a control transformed cell line, NR3a whose N-myc expression is not affected by tetracycline.

Preparation of Probe DNAs—The DNA probes for RCC1, N-myc, and β-actin were prepared as follows: RCC1, 1.7 kb *Bam*HI fragment excised from pcD40, which contains human RCC1 cDNA (*43*); N-myc, 2 kb *Hin*dIII-*Xba*I fragment from pN-myc/CDM8 (described in this paper); β-actin, 2.0 kb *Bam*HI-*Pvu*II fragment from pβActA1, in which a human β-actin gene is inserted into pBR322 (a kind gift of Dr. Nakanishi).

RNA Preparation and Northern Blot Analysis—RNA was isolated from cells by the acid guanidinium thiocyanate-phenol-chloroform extraction method described previously (*44*). RNA was electrophoresed in a gel containing formaldehyde, transferred to hybond-N (Amersham), and detected with ^{32}P-labeled cDNA probes as described previously (*45*). Probes were labeled with [α-^{32}P]dCTP using a Multiprime labeling kit (Amersham). The results were quantified using a BAS2000 image analyzer (Fuji Photo Film).

Gel Retardation Experiments—Gel retardation assays were performed essentially as described previously (*9, 46, 47*). Recombinant GST-N-Myc and GST-Max were mixed and incubated for 30 min at room temperature. The mixture was analyzed for binding to a synthetic double stranded oligonucleotide encompassing a CACGTG element (MB4) of the human RCC1 gene. Double stranded oligonucleotide probe (MB4) was generated by annealing synthetic oligonucleotides tcgaTTAGTC**CACGTG**TCCCG and tcgaTCGGGA**CACGTG**GACTAA, and labeling the product with [^{32}P]dCTP using Klenow Enzyme. Final conditions for 20 μl of reaction mixture were: 1 ng of ^{32}P-labeled MB4 oligonucleotide, 1 μg of poly(dI-dC)·poly(dI-dC) (Pharmacia Biotech), 1 μg of a nonspecific single-stranded oligonucleotide (5′-GAGTCGACGAACACACCAGGTCTTGGAGCG), 98.5 mM NaCl, 2.1 mM KCl, 4.8 mM sodium phosphate, 2 mM Tris (pH 7.4), 0.1 mM EDTA, and 1% glycerol. The reaction was allowed to proceed at room temperature for 20 min. For antibody experiments, affinity-purified rabbit anti-Max (C-17) (Santa Cruz Biotechnology) antibody, anti-N-Myc antibody (C-19) (Santa Cruz Biotechnology), or nonspecific rabbit antibody was added during the DNA-binding reaction. Double-stranded oligonucleotides used as competitors included MB4, mMB4 (generated by annealing of tcgaTTAGTC**CACCTG**TCCCG and tcgaTCGGGA**CAGGTG**GACTAA), MB5 (tcgaCGGC-CC**CACGTG**AAGCCC and tcgaGGGCTT**CACGTG**GGGCCG), mMB5(tcgaCGGCCC**CACCTG**AAGCCC and tcgaGGGCTT**CAGGTG**GGGCCG), MB6 (tcgaTTCGAC**CACGTG**TGACTT and tcgaAAGTCA**CACGTG**GTCGAA), mMB6 (tcgaTTCGAC**CACCTG**TGACTT and tcgaAAGTCA**CAGGTG**GTCGAA), MB7 (tcgaGGATTG**CACGTG**CAACGG and tcgaCCGTTG**CACGTG**CAATCC), and mMB7 (tcgaGGATTG**CACCTG**CAACGG and tcgaCCGTTG**CAGGTG**-

CAATCC).

Reporter Assay Using Luciferase—To establish cells carrying the RCC1 reporter plasmid, tetNR7b1 cells were transfected with 2 μg of the reporter plasmid, 3 μg of pRSVlacZ and 0.16 μg of pActHyg by the calcium-phosphate method (*42*). The cells were cultured for 2 weeks in selection medium (DMEM supplemented with 10% FBS and 200 μg/ml hygromycin) to select stable transfectants. Cells were stored frozen in LN_2. Twenty-four hours after tetracycline was added to the medium, cells were collected and analyzed for luciferase activity and β-galactosidase activity. Luciferase activity was determined using PicaGene™ (ToyoInki) according to the manufacturer's instructions. Galactosidase activity was determined as described previously (*48*).

RESULTS

N-myc Dependent Transformation of a Rat Fibroblast Cell Line, 3Y1—A rat fibroblast cell line, 3Y1, was tested to determine whether it is transformed by N-myc. The cell line retains the *in vivo* characters with a regulated growth property and the diploid karyotype (*41, 49*). As shown in Fig. 1 and Table I, transfection of rat 3Y1 fibroblasts with N-myc and v-ras produced colonies in soft agar, while transfection with N-myc or v-ras alone did not. Thus, oncogenic cooperation between N-myc and v-ras, and that N-myc is necessary for the transformation.

To investigate the specific role of N-myc in the transformation, 3Y1 cells were transfected with v-ras and N-myc, which is controlled by a tetracycline-sensitive promoter. Gene expression under this promoter is suppressed when tetracycline is added to culture medium (*38*). Two cell lines, tetNR2a2 and tetNR7b1, were isolated and characterized. A control transformed cell line, NR3a, whose N-myc expression is under a constitutive promoter, was also isolated. Twenty-four hours after tetracycline was added to culture medium, expression of N-myc was analyzed by Northern blotting. As shown in Fig. 2 and Table II, the treatment of tetNR2a2 and tetNR7b1 with tetracycline reduced expression of N-myc to 12–15% in a tetracycline-dose-dependent manner. On the other hand, tetracycline treatment of the control cell line NR3a did not reduce the expression of N-myc.

When tetNR2a2 and tetNR7b1 cells were plated in soft agar, tetracycline reduced the number of colonies to 0% in a dose-dependent manner (Fig. 3). Colony formation of the

TABLE I. **Transformation of rat 3Y1 fibroblasts by N-myc and v-ras.** Rat 3Y1 fibroblasts were transfected with N-myc and/or v-Ki-ras, then cultured in soft agar. Numbers of visible colonies are shown.

Oncogene	Exp. 1	Exp. 2	Exp. 3
N-myc & v-ras	43	101	57
N-myc	0	0	0
v-ras	0	0	0
Control	0	0	0

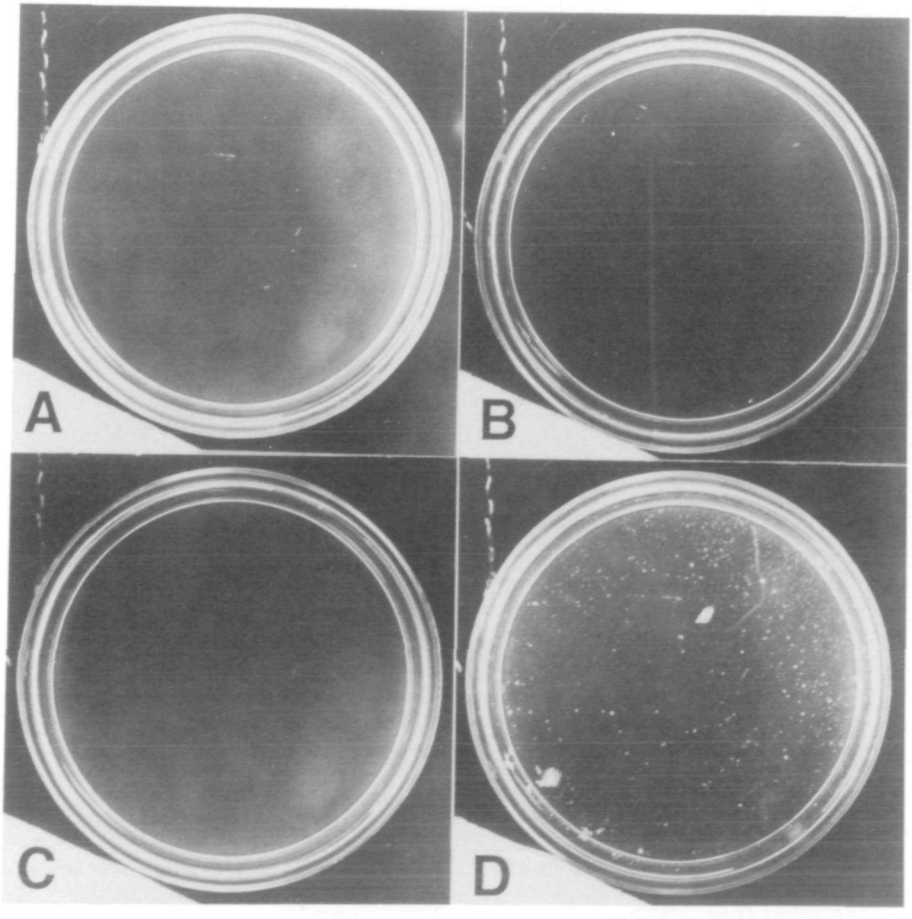

Fig. 1. **Oncogenic cooperation between N-myc and v-ras in rat 3Y1 fibroblasts.** 3Y1 cells transfected with pCDM8 and pXb (A), pN-myc/CDM8 and pXb (B), pCDM8 and p4E (C), or pN-myc/CDM8 and p4E (D) were grown in soft agar. pN-myc/CDM8 is the N-myc expression vector. pCDM8 is an empty vector. p4E is the v-ras expression vector. pXb is a derivative of p4E which lacks the part of DNA encoding v-Ki Ras protein and has lost the transforming activity.

control clone, NR3a, was not reduced by tetracycline. These results show that even after their establishment as a transformed cell line, these cells need a continued high level of expression of N-myc to maintain the transformation. These cell lines, 3Y1, tetNR2a2, tetNR7b1, and NR3, provide us with a good system to investigate biological events during the transformation.

N-myc Dependent High Expression of RCC1—RCC1 has important functions for eukaryotic cell proliferation (*23*). We recently found that another myc family gene, c-myc, directly activates the expression of RCC1 (*9*), and here we examined the expression of RCC1 in the cells transformed by N-myc and v-ras in comparison to that in the parental 3Y1 cells by Northern blotting. As shown in Fig. 2, all of the three transformed clones, tetNR2a2, tetNR7b1, and NR3a, highly expressed RCC1 compared to the parental 3Y1 cells. Quantitative analysis showed that expression of RCC1 in the transformed cells was several times more than that in parent cells. This elevated expression of RCC1 could be due to N-myc or v-ras. Twenty-four hours after tetracycline treatment of tetNR2a2 and tetNR7b1, which suppressed expression of N-myc, expression of RCC1 was decreased to 20% in a tetracycline-dose-dependent manner (Fig. 2 and

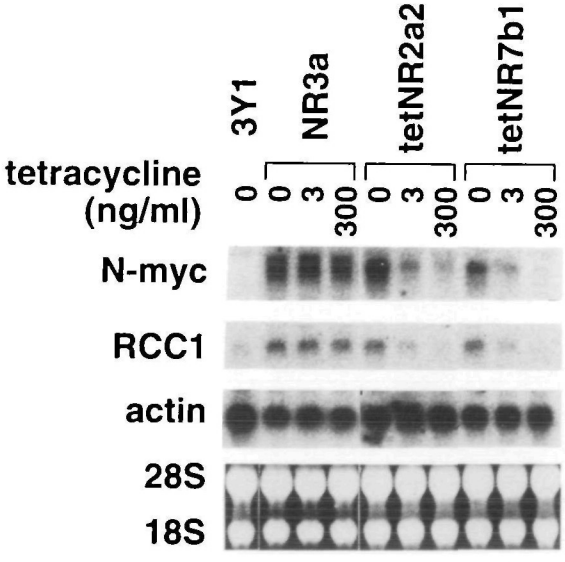

Fig. 2. **Gene expression in the cells transformed by N-myc and v-ras.** tetNR2a2 and tetNR7b1 express N-myc under a tetracycline-sensitive promoter, while NR3a does so under a tetracycline-resistant promoter. 3Y1 is the non-transformed parent cell line. One day after the addition of the indicated concentration of tetracycline, RNA was isolated from the cells and analyzed by Northern blotting using cDNAs for N-myc, RCC1, or actin.

TABLE II. **Effect of tetracycline on expression of genes.** Expression of N-myc, RCC1, and actin in cells transformed by N-myc and v-ras were detected by Northern blotting.

	Tetracycline (ng/ml)	N-myc	RCC1	Actin
NR3a	0	100%	100%	100%
	3	108	99	103
	300	90	97	102
tetNR2a2	0	100%	100%	100%
	3	25	43	101
	300	15	24	113
tetNR7b1	0	100%	100%	100%
	3	39	43	96
	300	12	19	92

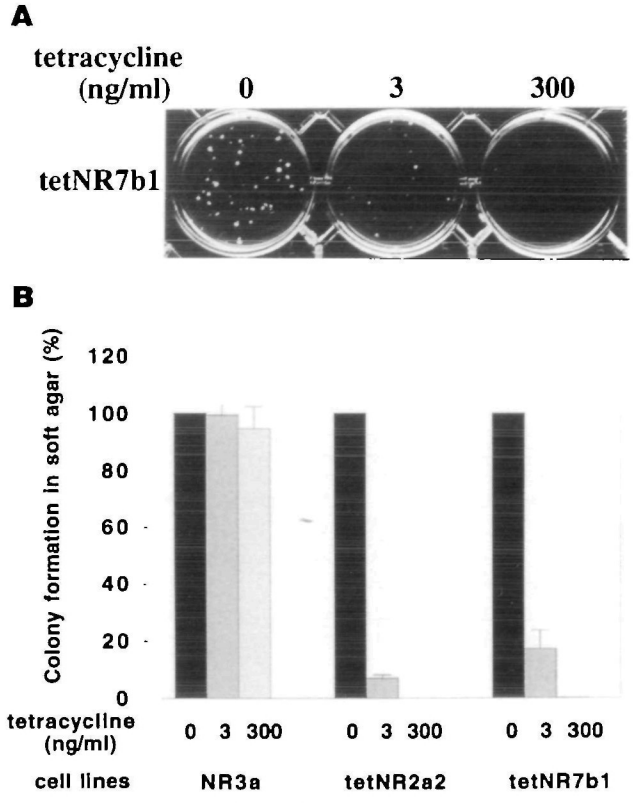

Fig. 3. **N-myc-dependent colony formation of the established transformed cell lines in soft agar.** A: Two weeks after tetNR7b1 cells were seeded into soft agar in the presence of the indicated concentration of tetracycline, the visible colonies were photographed. B: Two weeks after cells were seeded into soft agar in the presence of the indicated concentration of tetracycline, the visible colonies were counted. Efficiency of colony formation is expressed as the percentage to that without tetracycline, and values are the means of at least three different experiments. The bars indicate the standard error. tetNR2a2 and tetNR7b1 are tetracycline-sensitive in regard to expression of N-myc, while NR3a is resistant.

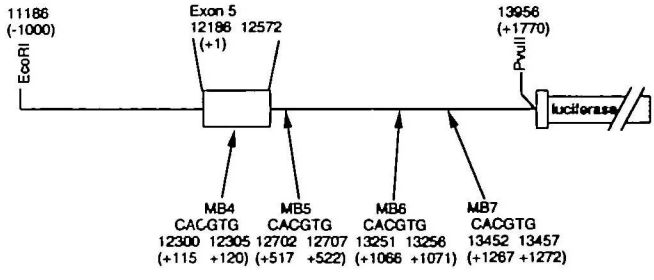

Fig. 4. **A reporter plasmid to investigate the effect of N-myc on RCC1 expression.** The gene fragment of the RCC1 gene around the major transcription start site is joined to firefly luciferase to construct a reporter plasmid [pHgRCC1-II(W)luci] as described before (*9*). The numbers without parentheses indicate the distance from the start nucleotide of the entire RCC1 genomic DNA and the numbers in the parentheses indicate the position relative to the major transcription start site. The locations of four potent Myc protein-binding sites are indicated by MB.

Table II). Expression of actin was not affected by tetracycline. On the other hand, expression of RCC1 in a control clone, NR3a, was not affected by tetracycline. Expression of v-ras was not decreased by tetracycline in tetNR2a2 and tetNR7b1 cells (data not shown). These results indicate that N-myc stimulates expression of RCC1 in the transformed cells.

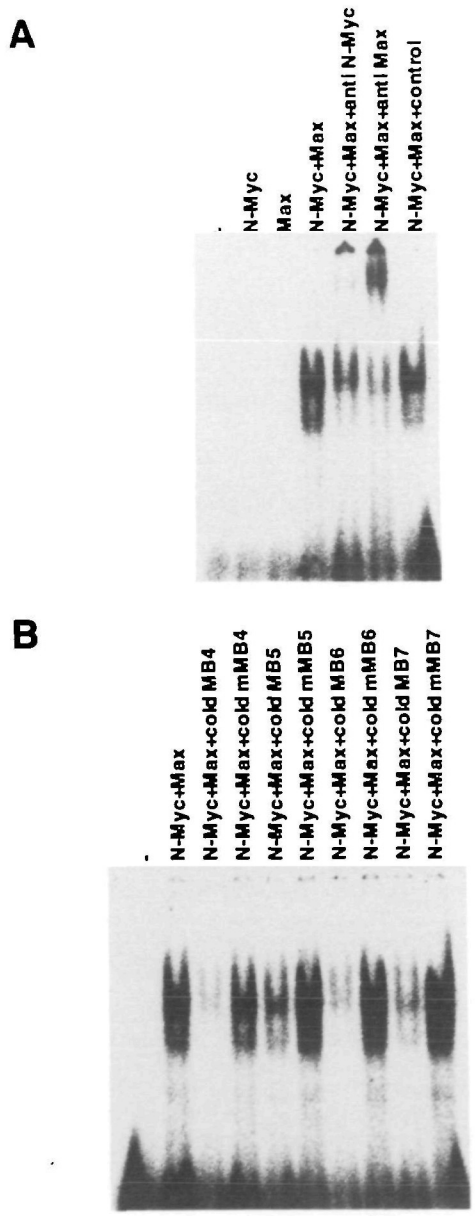

Fig. 5. **N-Myc protein binds to CACGTG elements in the RCC1 gene.** A: Binding of N-Myc with Max to MB4 oligonucleotide. Recombinant GST-N-MycC101 and/or GST-Max were incubated with ^{32}P-labeled MB4 oligonucleotide and analyzed by a gel retardation assay as described in "MATERIALS AND METHODS." To test the specificity of the shifted band, affinity-purified rabbit anti-N-Myc antibody, affinity-purified rabbit anti-Max antibody, or control monoclonal antibody was added to the incubation mixture containing GST-N-MycC101, GST-Max, and ^{32}P-labeled MB4 oligonucleotide before analysis by gel retardation assay. B: N-Myc protein bound to the CACGTG elements of MB4, 5, 6, and 7 oligonucleotides. The complex of GST-N-MycC101 and GST-Max was incubated with ^{32}P-labeled MB4 oligonucleotide plus a 200-fold excess of the unlabeled MB4, MB5, MB6, or MB7 oligonucleotides or plus the same oligonucleotides in which the CACGTG sequence was replaced with CACCTG (mMB4, mMB5, mMB6, and mMB7). The reaction mixtures were then analyzed by gel retardation as described in "MATERIALS AND METHODS."

Binding of N-Myc Protein to the CACGTG Elements in the RCC1 Gene In Vitro—To investigate whether N-Myc protein binds to the CACGTG elements in the RCC1 gene, gel retardation experiments were performed using recombinant N-Myc and Max. We designated the CACGTG elements from 12300 to 12305, 12702 to 12707, 13251 to 13256, and 13452 to 13457 in the RCC1 gene as MB4, MB5, MB6, and MB7, respectively (Fig. 4) (9). In a gel retardation experiment using radiolabeled MB4 oligonucleotide, the combination of the recombinant N-Myc and Max produced a shifted band, but neither N-Myc nor Max alone did so under the experimental conditions used here (Fig. 5A). In the presence of anti-N-Myc antibody or anti-Max antibody, the intensity of the shifted band decreased and supershifted bands were detected. The intensity of the shifted band by N-Myc and Max was decreased when an excess of unlabeled MB4 oligonucleotide was added, while it was barely affected by addition of an excess of unlabeled mutant MB4 oligonucleotide (mMB4) in which CACGTG was changed to CACCTG (Fig. 5B). These results indicate that N-Myc and Max specifically recognize the CACGTG element in MB4.

Excess unlabeled MB5, MB6, or MB7 oligonucleotides in the reaction mixture decreased the intensity of the band shifted by N-Myc and Max (Fig. 5B). Unlabeled mutant mMB5, mMB6, or mMB7 oligonucleotides that contain CACCTG instead of CACGTG did not have this effect, suggesting that complex of N-Myc and Max also binds MB5, MB6, and MB7 through CACGTG elements.

N-myc Controls Expression of RCC1 through CACGTG Elements in the RCC1 Gene—To investigate whether CACGTG elements are involved in control of RCC1 expression by N-myc, tetNR7b1 cells were transfected with

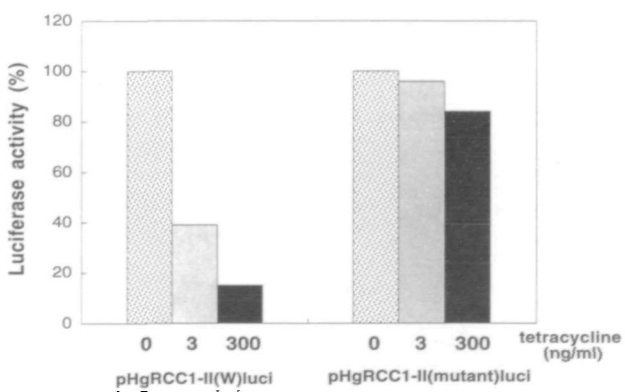

Fig. 6. **CACGTG elements in the RCC1 gene are involved in control of the RCC1 gene expression by N-myc.** tetNR7b1 cells carrying the reporter plasmids and pRSVlacZ (for normalization of cell harvesting) were obtained as described in "MATERIALS AND METHODS." One reporter plasmid, pHgRCC1-II(W)luci, has four CACGTG elements. In the other one, pHgRCC1-II(mutant)luci, CACGTG were all mutated to CACCTG to which N-Myc protein does not bind. Twenty-four hours after addition of tetracycline to the medium, cells were harvested and enzyme activities were measured as described in "MATERIALS AND METHODS." After normalization to β-galactosidase activities, luciferase activities were expressed relative to expression without tetracycline.

reporter plasmids constructed from a RCC1 gene fragment and firefly luciferase cDNA as described in "MATERIALS AND METHODS" (Fig. 4) (*9*). One reporter plasmid, pHgRCC1-II(W)luci, has 4 CACGTG elements. In the other one, pHgRCC1-II(mutant)luci, all four CACGTG elements were mutated to CACCTG, to which N-Myc protein does not bind (Fig. 5). As shown in Fig. 6, 24 h after addition of tetracycline, luciferase activity expressed from pHgRCC1-II(W)luci decreased to 15%. On the other hand, the tetracycline treatment hardly affected luciferase activity from pHgRCC1-II(mutant)luci. These results indicate that N-myc controls the expression of pHgRCC1-II(W)luci through CACGTG elements.

DISCUSSION

N-myc Dependent Transformation in a Rat Cell Line—We found that 3Y1 cells are transformed by N-myc and v-ras. In these cells, both oncogenes are necessary for the transformation as in primary embryonic fibroblasts. In order to study the specific role of N-myc in the transformation, we isolated transformed cells in which N-myc expression was under a controllable promoter. In these cells, the level of N-myc expression is closely correlated with the transformation. This property resembles that of certain human neuroblastomas, in which N-myc is highly expressed and the level of N-myc expression is highly correlated with advanced stage of diseases (*4, 5*). Because 3Y1 is a cloned cell line, it is possible to study the biological events of transformation by comparing transformed cells to parental 3Y1 cells.

Strong reduction of N-myc expression completely suppressed the colony formation in soft agar, showing that constitutive expression of N-myc is necessary to maintain the transformation. These results indicate that N-myc is necessary for the maintenance of the transformation even after the transformed cell line is established, and suggest that medical treatment to decrease the level of N-myc expression will be effective in human neuroblastomas expressing high levels of N-myc.

What does N-myc do in the transformed cells? We showed here that N-myc functions as a transcription factor to stimulate expression of RCC1 in the transformed cells (see below). Expression of RCC1 and N-myc was closely correlated with the efficiency of colony formation in soft agar. These results suggest that N-Myc functions as a transcription factor in the transformed cells, and that this function may be important for the transformation.

N-myc Stimulates Expression of RCC1—We showed here that N-myc stimulates expression of RCC1. (i) 3Y1 cells transformed by N-myc and v-ras highly expressed RCC1 compared to parental 3Y1 cells. (ii) The expression of RCC1 changed in a parallel with the expression of N-myc in the transformed cells. (iii) N-Myc protein with Max binds to CACGTG elements in the RCC1 gene *in vitro*. (iv) Expression from the reporter plasmids containing the RCC1 gene fragment changed in parallel with expression of N-myc. Expression from the reporter plasmids without CACGTG elements was hardly affected by the change of N-myc expression. Together, these results show that N-Myc stimulates expression of RCC1 by binding directly to the RCC1 gene. We showed previously that c-myc directly stimulates expression of RCC1 (*9*). Therefore both c-myc and N-myc can directly transactivate expression of RCC1.

Stimulation of RCC1 expression by N-myc is closely correlated with colony formation in soft agar, suggesting that elevated expression of RCC1 may be involved in the transformation. However, the possibility remains that elevation of RCC1 expression by N-myc may be involved not in the transformation but in other functions of N-myc, including suppression of differentiation and induction of apoptosis. Further studies are required to clarify these points.

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