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# Point Mutation of the *neu* Gene in Rat Neural Tumor RT4-AC Cells: Suppression of Tumorigenicity by s-Myc

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**Forced expression of the s-*myc* gene suppressed the tumorigenicity of rat RT4-AC tumor cells in nude mice, as reported previously. Polymerase chain reaction (PCR) analysis indicated that RT4-AC cells established from a tumor of the peripheral nervous system contain an activated *neu* gene with a T→A transversion in the transmembrane domain. Synthesis of a protein of 60 kd in RT4-AC cells was specifically inhibited by expression of the s-*myc* gene. These results strongly suggest that s-Myc suppresses the transforming activity of rat neural cells transformed by expression of the activated *neu* gene, and plays an important role in regulating expression of a cellular gene contributing to cell transformation, such as the gene that encodes the p60 protein.**

Key words: RT4-AC cells — Tumor suppression — *neu* gene — s-*myc* gene

Recently we cloned a novel *myc*-related gene named s-*myc* from a rat genomic library.[1] We also found that, when expressed in sufficient quantity, s-Myc suppressed the tumorigenicity of rat RT4-AC tumor cells in nude mice.[1] RT4-AC tumor cell lines were established from a tumor induced in the region of the sciatic nerve of a newborn male BDIX rat by subcutaneous injection of ethylnitrosourea.[2]

Perantoni *et al.* reported that in tumor cell lines established from ethylnitrosourea-induced tumors of the peripheral nervous system of rats, the cellular *neu* proto-oncogene contains a point mutation, a T→A transversion at nucleotide 2012 in the sequence encoding the transmembrane domain of the *neu* gene product.[3] Therefore, using the polymerase chain reaction (PCR) technique, we examined whether RT4-AC cells also contain a point mutation in the transmembrane domain of *neu*.[4] Based on the nucleotide sequence of *neu* cDNA reported by Bargmann *et al.*,[5,6] we prepared two oligodeoxy nucleotides with the following sequences: fragment A, 5′GTGCTGCAGGGTGACATTCATCATT3′: fragment B, 5′CACGGATCCTCTGTCTCCTTCGTTT3′. Fragments A and B contain restriction endonuclease sites for *Pst*I and *Bam*HI, respectively. Genomic DNAs from normal rat brain and RT4-AC cell lines were subjected to 50 cycles of PCR amplification with both primer A and primer B. The amplified DNA fragment of 110 bp, which contained the region between nucleotides 1986 and 2077 encoding the transmembrane domain of the Neu protein, was digested with *Pst*I and *Bam*HI, and ligated into the corresponding site of the phagemid vector pTZ18R or pTZ19R. The nucleotide sequence of the cloned DNA fragment was then determined by the dideoxy chain termination method.[7]

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As shown in Fig. 1, normal rat brain DNA had T at nucleotide position 2012, while the clones from RT4-AC DNAs had A in this position. This T→A transversion changes the valine residue present at residue 664 of the transmembrane domain of the *neu* gene product to glutamic acid.[3,5] Previous studies by others demonstrated that multiple tumor cell lines independently derived from intracranial tumors induced in rats by transplacental exposure to ethylnitrosourea also contain an activated *neu* gene.[5] It is interesting that the administration of ethylnitrosourea under different conditions leads to the same specific mutation of *neu* in the peripheral nervous system of rats. Ethylnitrosourea is a direct-acting alkylating agent and so can form various different adducts in DNA molecules. A mutation in the region outside the amplified DNA of *neu* might not be detected by this analytical method. However, Bargmann and Weinberg reported that this single base mutation, a T→A transversion in the transmembrane domain of *neu*, is sufficient to confer a potential for transformation on the *neu* oncogene.[8] An activated *neu* oncogene with the same point mutation at nucleotide 2012 was also observed in clones of RT4-AC cells transfected with s-*myc*, such as Y17 and A44 (Fig. 1). These results strongly suggest that RT4-AC cells are a rat tumor cell line transformed by the activated *neu* gene and that its tumorigenicity can be suppressed by s-Myc.

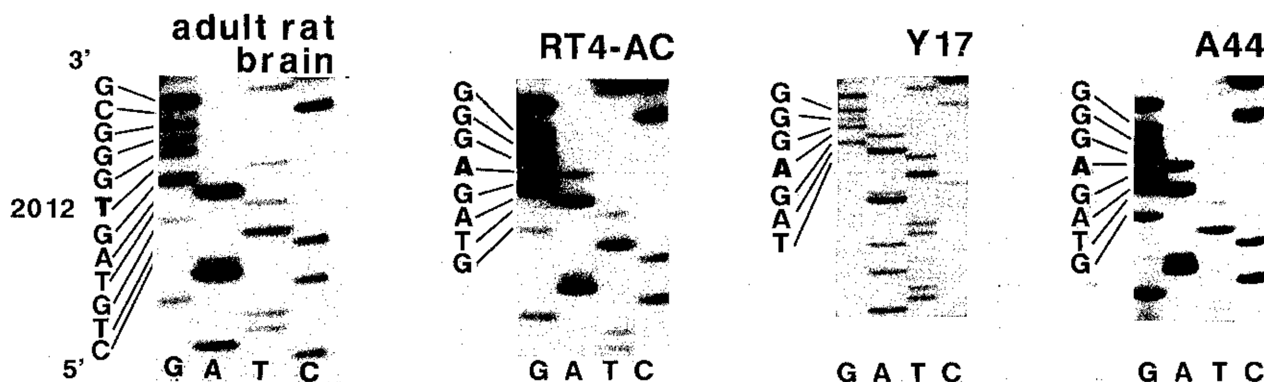


Fig. 1. PCR sequence analysis of a point mutation in the region encoding the transmembrane domain of the *neu* gene product. The reaction mixture (100 $\mu$l) for the PCR contained 50 pmol of primer DNA, 200 $\mu M$ each of dNTPs, 10 m$M$ Tris-HCl (pH 8.3), 1.5 m$M$ $MgCl_2$, 50 m$M$ KCl and 1 $\mu$g of genomic DNA. The mixture was incubated for 5 min at 95°C and then cooled to 37°C. After addition of 5 units of *Taq*I polymerase, the mixture was subjected to 50 cycles of amplification. Each cycle consisted of incubation for 1 min at 94°C, 1 min at 55°C and 2 min at 72°C. The amplified DNA fragments purified by polyacrylamide gel electrophoresis were digested with *Bam*HI and *Pst*I and then ligated into the corresponding sites of phagemid vector pTZ18R or pTZ19R. The nucleotide sequences of the antisense strands of the cloned DNAs were determined by the dideoxynucleotide method with a sequencing kit containing 7-deaza-dGTP.

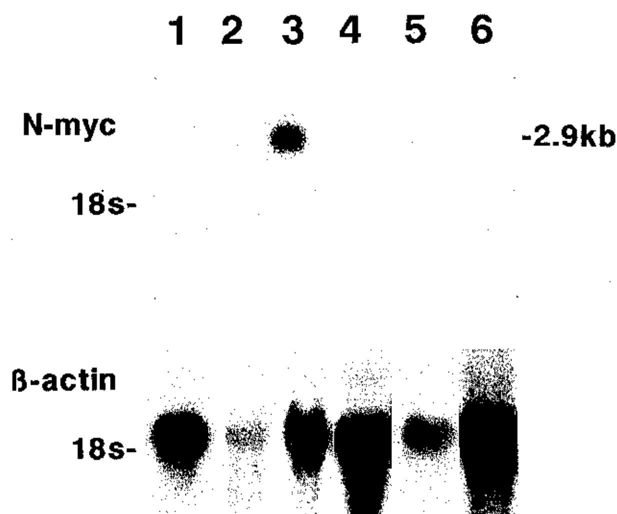


Fig. 2. Expressions of N-*myc* in rat tissues and cultured rat cells. Total RNAs were prepared from exponentially growing cultured cells and freshly prepared tissues by the guanidinium thiocyanate-cesium chloride methods.[13] Aliquots of approximately 30 $\mu$g of total RNAs from various cells were examined by Northern blot analysis with mouse N-*myc* exon 3 DNA and rat $\beta$-actin probes. The RNAs were separated in 1.0% agarose gel containing 2% formaldehyde by electrophoresis at 50 V for 3 h. Then the gels were rinsed with water, and neutralized in 10×SSC for 30 min. The RNAs were transferred to a nitrocellulose membrane and hybridized to a nick-translated $^{32}$P-labeled mouse N-*myc* exon 3 DNA probe or rat $\beta$-actin probe. Lanes: 1, rat 3Y-1 fibroblast cells; 2, rat adult brain; 3, rat embryo brain; 4, rat RT4-AC tumor cells; 5, s-*myc* transfected cell line Y17; 6, s-*myc* transfected cell line A44.

As we reported previously, the s-Myc protein has an amino acid sequence highly homologous with that of the mouse N-Myc protein, but lacks acidic amino acid-rich sequence required for cell transformation.[1] In addition, high expression of the N-*myc* gene was recently found to play a crucial role in neoplastic transformation of human neural cells.[9] From these findings, we suspected that the transforming activity of RT4-AC tumor cells was suppressed by competitive inhibition of the N-*myc* gene products by the s-*myc* gene products, if the N-*myc* gene was highly expressed in RT4-AC cells. To investigate this possibility, using Northern blot hybridization, we examined the expression level of the N-*myc* gene in RT4-AC cells with or without transfection with the s-*myc* gene. As shown in lane 3 of Fig. 2, significant expression of the N-*myc* gene was detected in rat embryo brain, as reported by Zimmerman *et al.*[10] However, unexpectedly, synthesis of the N-*myc* transcript of 2.9 kb was barely detectable in RT4-AC and its derivatives, Y17 and A44 cells, transfected with the s-*myc* gene: the levels of expression of the N-*myc* gene in these cells were almost the same as that in rat fibroblast 3Y-1 cells (Fig. 2, lanes 1, 4, 5 and 6). These results indicate that the N-*myc* gene products do not contribute to expression of neoplastic phenotypes of RT4-AC tumor cells. Moreover, these results indicate that the tumor-suppressing activity of the s-*myc* gene products in RT4-AC cells may not be caused by their direct competitive inhibition with the N-*myc* gene products.

On the other hand, the amino acid sequence of the s-Myc protein, deduced from its DNA sequence, showed

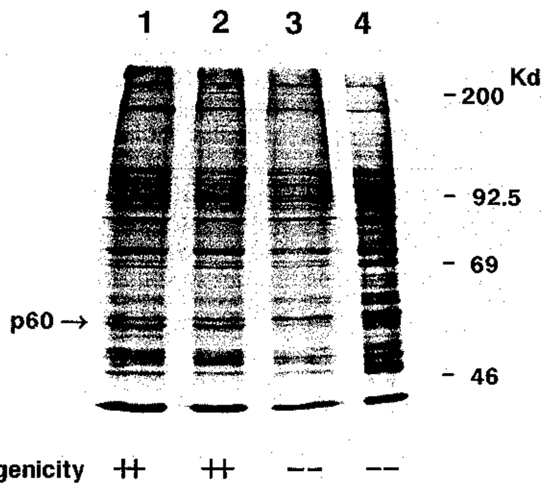


Fig. 3. Polyacrylamide gel electrophoresis of total $^{35}$S-methionine-labeled polypeptides from rat RT4-AC cells with or without transfection with the s-*myc* gene. Sparsely growing cells labeled for 6 h with $^{35}$S-methionine (10 $\mu$Ci/ml) were solubilized in RIPA buffer containing 150 m*M* NaCl, 10 m*M* Tris-HCl (pH 7.6), 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS and 2 m*M* phenylmethylsulfonyl fluoride. Samples of approximately 500,000 cpm of trichloroacetic acid-precipitable radioactivity were subjected to electrophoresis in 7.5% SDS-polyacrylamide gel at 25 mA for 4 h. The tumorigenicity of each cell line was examined by nude mouse assay as described previously.[1] Lanes: 1, RT4-AC cells; 2, B3 cells; 3, Y17 cells; 4, A44 cells.

that the DNA binding structure containing a helix-loop-helix (HLH) followed by a leucine zipper in the C-terminal region is well conserved.[1] The existence of these structural features indicates that the s-Myc protein may have the ability to form complexes with itself or other proteins with similar structural features and regulate cellular gene expression, as other Myc family proteins do.[11, 12] Accordingly, we examined the effect of the s-*myc* gene products on expression of cellular genes by comparison of the polypeptides synthesized in RT4-AC cells with those in B3, Y17 and A44 cells. Figure 3 shows a radioautogram of $^{35}$S-labeled polypeptides separated by SDS-polyacrylamide gel electrophoresis. All the polypeptides detected in RT4-AC cells were also expressed in B3, Y17 and A44 cells except a polypeptide with an apparent molecular mass of approximately 60 kd, indicated by an arrow in Fig. 3. As shown in lanes 1 and 2 of Fig. 3, synthesis of significant amounts of the p60 protein was observed in wild-type RT4-AC cells and B3 cells transfected with the *neo* gene alone. However, interestingly, in Y17 and A44 cells, which have no transforming activity, the synthesis of the p60 protein was markedly reduced. These results suggest that the s-*myc* gene products regulate the expression of a specific cellular gene such as that encoding the p60 protein. We do not yet know whether the p60 protein takes part in transformation of rat neural cells or how the s-*myc* gene products regulate the synthesis of the p60 protein. In order to obtain information on the mechanism of tumor suppression by the s-*myc* gene products, and the mechanism of transformation of rat neural cells by expression of the activated *neu* gene, it seems important to characterize the p60 protein.

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