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Biochemistry

Isolation and characterization of s-*myc*, a member of the rat *myc* gene family

(oncogene/gene transfer/tumor suppression)

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ABSTRACT **A 9-kilobase-pair clone containing a *myc*-related gene that we have designated s-*myc* was isolated from a rat genomic library. The entire nucleotide sequence of the cloned DNA was determined, showing that the s-*myc* gene contains an open reading frame consisting of 1287 base pairs without introns. *In vitro* transcription–translation analysis of s-*myc* indicated that this gene produces a protein of ≈50 kDa. The amino acid sequence predicted from the DNA sequence showed that the s-Myc protein is closely related to the murine N-Myc protein but lacks an acidic amino acid-rich sequence commonly present in the Myc-family proteins. Studies on transfection of s-*myc* into rat RT4-AC tumor cells revealed that the gene produces a polyadenylylated transcript of ≈4.7 kilobases and that its high-level expression suppresses the tumorigenicity of RT4-AC tumor cells in nude mice.**

The c-, N-, and L-*myc* genes constitute a group of cellular *myc*-family genes and have a well conserved three-exon structure (1–5). These *myc* genes have a single large translatable open reading frame beginning at the first ATG codon in exon 2 and extending to exon 3. The proteins encoded are nuclear phosphoproteins with similar molecular weights.

In general, factors activating eukaryotic transcription, such as AP-1, c-Fos, c-Myb, and adenovirus E1A protein, contain two domains: a DNA-binding domain, consisting of a basic region followed by a leucine-zipper structure, and a transcription-activating domain, which is highly acidic (6, 7). The Myc-family proteins contain a leucine-zipper structure preceded by a basic amino acid-rich sequence in their C-terminal region and an acidic domain in the region encoded from the exon 2/3 junction region of the genes. Together, these two domains should control the expression of the physiological functions of the Myc proteins in regulation of cell proliferation and transformation.

Recently, we reported that transcription of the c-*myc* gene in rat 3Y-1 fibroblasts is activated by Rous sarcoma virus infection (8). During studies of the relationship between the activation of transcription of the rat c-*myc* gene and cell transformation in Rous sarcoma virus-infected rat cells, we found that rat cells contain a *myc*-related gene encoding a protein of ≈47 kDa that is closely related to the mouse N-Myc protein but lacks the acidic domain. In this paper, we report the nucleotide sequence of this *myc*-related gene isolated from a rat genomic library.** The gene product has a suppressive effect on the tumorigenicity of rat RT4-AC tumor cells in nude mice. As a result of the suppressive effect, this *myc*-related gene has been designated s-*myc*.

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MATERIALS AND METHODS

Molecular Cloning. A rat genomic library supplied by T. Sargent (9) was screened by plaque hybridization using a 0.8-kilobase-pair (kbp) *Sal* I–*Dra* I fragment of v-*myc* exon 3 DNA labeled with ^{32}P by nick-translation as a probe. Plaque hybridization was carried out at 42°C in the presence of 40% (vol/vol) formamide. Sixteen positive clones were obtained from about 10^6 recombinant phages. Restriction mapping and Southern hybridization of phage DNAs showed that these 16 clones could be grouped into two classes. A typical clone of each class, named λNM3 and λNM5, contained 9- and 6.9-kbp *Eco*RI DNA inserts, respectively. The *Eco*RI fragments in these two phage recombinants were independently subcloned into the *Eco*RI site of plasmid vector pUC19 to obtain pNM3 and pNM5.

DNA Sequencing. DNA restriction fragments from the plasmid clones pNM3 and pNM5 were subcloned into vector M13, pUC, or pTZ and then sequenced by the dideoxynucleotide method with sequencing kits containing 7-deaza-dGTP (Takara Shuzo, Kyoto, Japan).

***In Vitro* Transcription and Translation.** The 4.9-kbp *Eco*RI–*Kpn* I, 2.9-kbp *Eco*RI–*Dra* I, 2.3-kbp *Eco*T22I–*Dra* I, and 2-kbp *Dra* I–*Dra* I fragments from the insert DNA of pNM3 were subcloned into the appropriate site of phagemid vector pTZ18R, resulting in formation of pTEK, pTED, pTETD, and pTDD, respectively (see map in Fig. 3). Each DNA fragment was inserted downstream from the bacteriophage T7 promoter and in the orientation intended to generate RNA transcripts with the same polarity as natural mRNA. The phagemid DNAs from pTEK, pTED, pTETD, and pTDD were linearized by digestion with *Hin*dIII and transcribed *in vitro* by T7 RNA polymerase in the presence of 7-methyl-GTP according to the manufacturer's protocols. The capped RNAs were translated *in vitro* with a rabbit reticulocyte lysate in the presence of [^{35}S]methionine. The labeled proteins were subjected to electrophoresis in a SDS/12% polyacrylamide gel and revealed by fluorography.

Gene Transfection. Exponentially growing NIH mouse 3T3 fibroblasts and RT4-AC rat tumor cells were seeded at 10^6 cells per 10-cm plate and incubated for 20 hr in 10 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with either 10% bovine calf serum or 12.5% horse serum and 2.5% fetal bovine serum, respectively. Before transfection, the 4.6-kbp *Eco*RI–*Sca* I DNA fragment of

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**The sequence reported in this paper has been deposited in the GenBank data base (accession no. M29069).

pNM3 (positions 3146 to 7701 in Fig. 1) was subcloned into pSV2 expression vector, containing the simian virus 40 early promoter, to obtain pSVNM3. Then, 18 μg of pNM3 or pSVNM3 plasmid DNA and 2 μg of pSV2neo plasmid DNA were cotransfected by the calcium phosphate precipitation method (10). G418-resistant cells were selected for 2–3 weeks in growth medium containing the neomycin analogue G418 at 400 μg/ml.

Rat RT4-AC tumor cells were kindly supplied by N. Sueoka (11). Rat GH3 pituitary tumor cells, established by Tashjian *et al.* (12), were obtained from Japanese Cancer Research Resources Bank (JCRB)-Cell. Plasmid pSV2neo, carrying the neomycin-resistance gene, and plasmid pMC3, carrying the v-*myc* gene, developed by Southern and Berg (13) and Bishop and coworkers (14), respectively, were obtained from JCRB-Gene. Female athymic nude mice [BALB/c (*nu/nu*)] were purchased from CLEA Japan Inc. (Tokyo) and were housed under specific pathogen-free conditions.

RESULTS

Cloning of the *myc*-Related Gene. Complete digestion of the 9-kbp insert of pNM3 with *Eco*RI resulted in the formation of 3- and 6-kbp fragments (Fig. 1 *Lower*). The 6.9-kbp DNA insert in pNM5 hybridized strongly to mouse N-*myc* exon 3

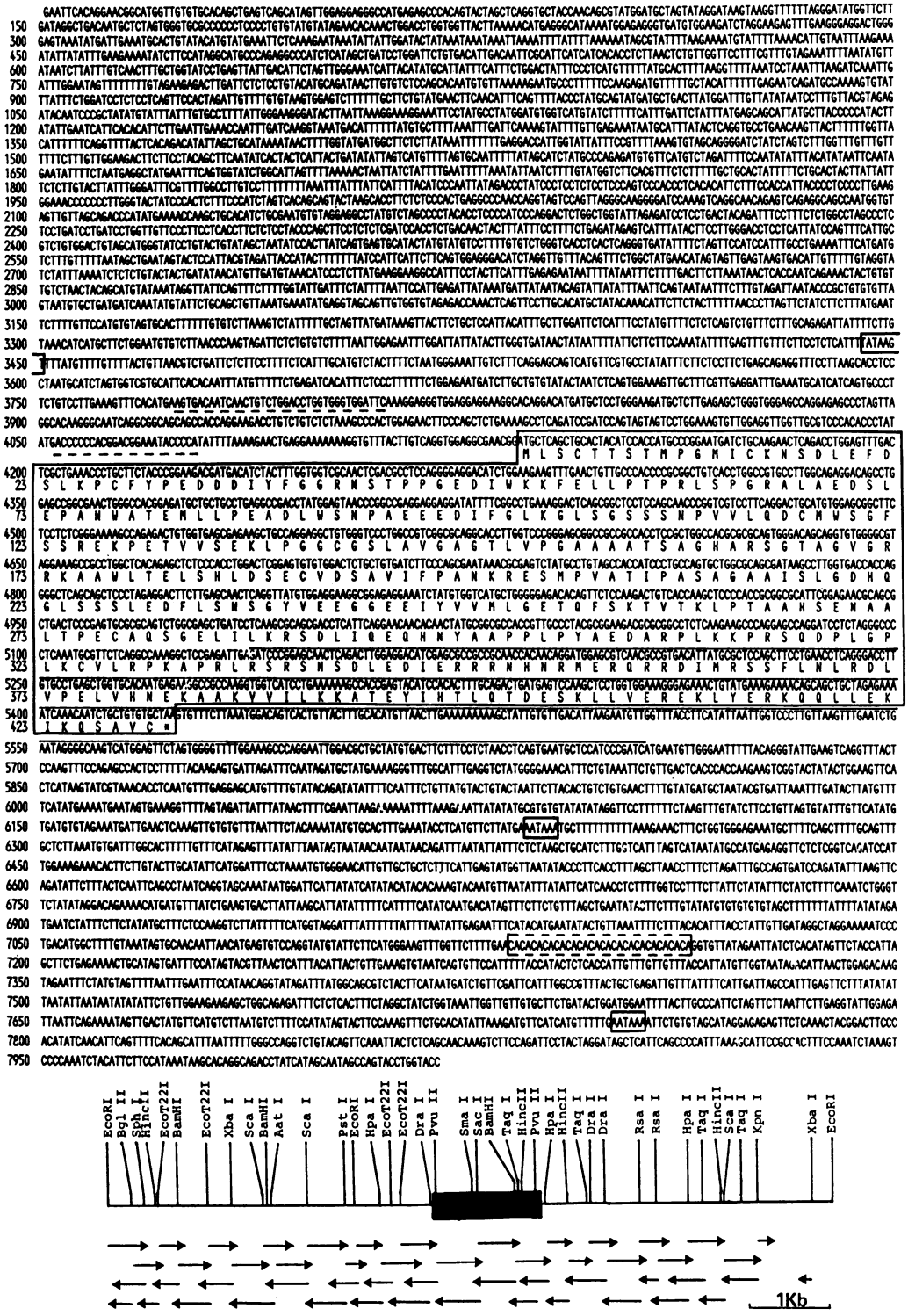

FIG. 1. Restriction map and nucleotide sequence of the cloned rat s-*myc* gene. (*Upper*) The large box indicates the predicted position of the translatable region of the s-*myc* gene. The predicted amino acid sequence of the s-Myc protein is shown by single-letter symbols. The small boxes indicate the putative TATA sequence and polyadenylylation signals. Overlining shows the nucleotide sequence of the region that hybridizes with the v-*myc* exon 3 probe. Broken underlines show the regions containing sequences highly homologous with those of mouse N-*myc* exons 1 and 2. The region boxed with a broken line is a CA repeat region. (*Lower*) Solid bar indicates the protein-coding region of the s-*myc* gene. Horizontal arrows below the restriction map indicate direction and extent of dideoxy sequencing. Scale bar represents 1 kilobase (kb).

probe, but the 6-kbp DNA insert of pNM3 hybridized only weakly with the same probe. Both insert DNAs in pNM3 and pNM5 did not hybridize to the rat and mouse c-*myc* genes. To further characterize the genes present in pNM3 and pNM5, the nucleotide sequences of the 519-bp fragment derived from pNM3 by *Sau*3A1 digestion and the 750-bp fragment derived from pNM5 by *Hin*cII digestion, both of which hybridized to v-*myc* exon 3 probe, were determined. Preliminary sequence analysis revealed that the 750-bp *Hin*cII fragment of pNM5 contains high sequence homology (≈98%) with the mouse N-*myc* exon 3 region. In addition, this 750-bp *Hin*cII fragment was found to hybridize with the expected 6.9- and 7.7-kbp *Eco*RI fragments containing the N-*myc* genes of rat and mouse genomic DNA, respectively. The 519-bp *Sau*3A1 DNA fragment of pNM3 also showed high sequence homology (≈85%) with the mouse N-*myc* gene. However, this fragment cross-hybridized with a 6- and a 5.8-kbp *Eco*RI fragment of rat and mouse genomic DNA, respectively. The respective sizes of the *Eco*RI fragments containing the different rat and mouse *myc* genes are 17 and 23 kbp for c-*myc*, 7 and 7.7 kbp for N-*myc*, and 18 and 14 kbp for L-*myc*, respectively (1–5). Ingvarsson *et al.* (15) reported that the rat and the mouse B-*myc* gene, which is related to the c-*myc* gene, hybridizes to 6.1- and 17-kbp *Eco*RI fragments of rat and mouse DNA, respectively (15). These results indicate that the DNA insert of pNM3 contained a novel *myc*-related gene (s-*myc*) and that the DNA insert of pNM5 contained the rat N-*myc* gene.

Nucleotide Sequence of the s-*myc* Gene. The restriction endonuclease map of the 9-kbp DNA insert of pNM3 (Fig. 1 *Lower*) differed markedly from those of the mouse, c-, N-, and L-*myc* genes and rat c- and B-*myc* genes (1–5, 15). Various subfragments of the DNA insert of pNM3 were subcloned into M13 phage vector or pTZ phagemid vector and their nucleotide sequences were determined by the strategy shown in Fig. 1 *Lower*. The nucleotide sequence of 8 kbp of the 9-kbp DNA insert of pNM3 is shown in Fig. 1 *Upper*. The longest open reading frame found started with an ATG codon at position 4134 and terminated with a TAA at position 5421. The nucleotide sequence homologies of this 1287-bp coding sequence with the coding sequences of the mouse N-, c-, and L-*myc* genes and the rat c-*myc* gene are approximately 62%, 48%, 45%, and 48%, respectively (1–5). The TAA termination codon at position 5421 is followed by two consensus polyadenylylation signals, AATAAA, at positions 6235 and 7750. Between the two polyadenylylation signals, there is a stretch of 16 CA repeats.

Structural Features of the s-*myc* Gene for Transcription. In the region upstream of the translation initiation codon, especially in the regions starting at positions 3774 and 4054, the sequences are highly homologous with those of mouse N-*myc* exons 1 and 2, respectively (Fig. 1 and ref. 1). Moreover, the region from position 3759 to 3877 contains a sequence homologous with the 240-bp fragment of mouse c-*myc* exon 1, which is known to act in cis as a translational inhibitor (4, 16). The presence of sequences that are homologous with those of mouse N- and c-*myc* exon 1 regions suggested that the region from position 3759 to 4054 might be transcribed *in vivo*. In the region upstream of the homologous sequences, three or four potential TATA sequences are present. Among them, the TATA sequence at position 3444 is followed by an AC dinucleotide sequence located about 30 bp downstream. This motif is frequently used as a transcription start site in eukaryotic mRNA. Similar circumstances for transcription initiation have been found in the mouse and rat c-*myc* genes and the human N- and c-*myc* genes (4, 5, 17).

Expression of the s-*myc* gene was barely detectable in naturally occurring sources such as rat liver, kidney, thymus, and whole embryo and in clonal rat cell lines such as 3Y-1 fibroblasts, GH3 pituitary tumor cells, and RT4-AC tumor cells, when total RNA isolated was examined by Northern blot analysis (Fig. 2). Then, in order to exclude the possibility that the cloned s-*myc* gene is a transcriptionally inactive pseudogene, pNM3 plasmid DNA was transfected into RT4-AC tumor cells and the level of s-*myc* mRNA was analyzed. Y17, an RT4-AC cell line stably transfected with pNM3 plasmid DNA having no artificial expression promoter, produced significant amounts of a polyadenylylated RNA of about 4.7 kb (Fig. 2). This polyadenylylated RNA hybridized with the 0.9-kbp *Eco*RI–*Dra* I fragment of the s-*myc* gene and its size was as predicted from the DNA sequence of the s-*myc* gene. Therefore, it is very likely that s-*myc* is a functional gene in *in vivo*; it appears that the TATA box at position 3444 may act as a promoter and that polyadenylylation of s-*myc* transcripts occurs near the AATAAA sequence at position 7750.

***In Vitro* Translation of s-*myc* RNA.** In order to prove that the open reading frame of s-*myc* actually codes for a 47-kDa protein, sense RNAs containing the predicted open reading frame with 5′ and 3′ untranslated regions of various lengths were synthesized *in vitro* by using T7 RNA polymerase (see *Materials and Methods*) and then used as templates for protein synthesis in a rabbit reticulocyte lysate system (Fig. 3). All the RNAs synthesized *in vitro* were able to specify a protein of ≈50 kDa.

Suppression of Tumorigenicity by s-*myc* Expression. To obtain information on the physiological properties of the s-*myc* gene product, we cotransfected pNM3 or pSVNM3

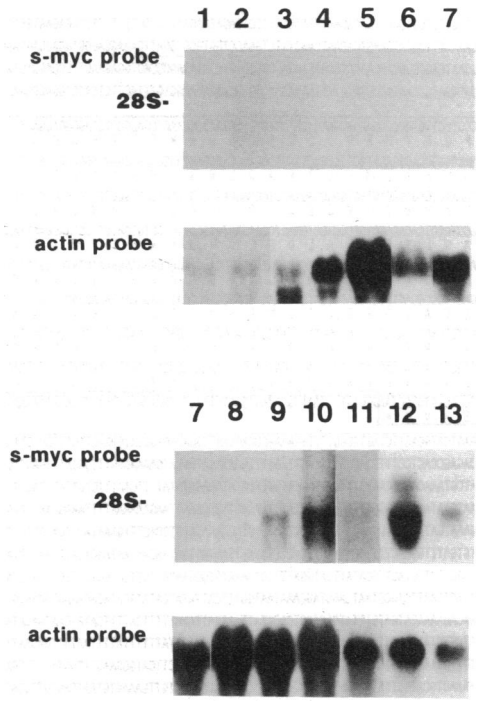

FIG. 2. Expression of s-*myc* in rat tissues and cultured rat cells. Approximately 20 μg of total RNA or 5 μg of poly(A)$^+$ RNA was assayed for s-*myc* and β-actin expression. RNAs were separated in 1.0% agarose gel containing 2% formaldehyde, transferred to nitrocellulose, and hybridized to a nick-translated ^{32}P-labeled *Eco*RI–*Dra* I fragment (0.9 kbp) of s-*myc* and *Eco*RI–*Hpa* II fragment (1.3 kbp) of the rat β-actin gene (25). Total RNA was prepared by the guanidinium thiocyanate/cesium chloride method (18), and poly(A)$^+$ RNA was selected by $(dT)_{15-20}$-cellulose column chromatography. Lanes 1–7: total RNA from rat liver (lane 1), kidney (lane 2), thymus (lane 3), whole body of rat embryo (lane 4), rat 3Y1 fibroblasts (lane 5), GH3 tumor cells (lane 6), and RT4-AC tumor cells (lane 7). Lanes 8, 9, 10, and 12: total RNA from A2, A18, A44, and Y17 cells (s-*myc* transfectants derived from RT4-AC). Lanes 11 and 13: poly(A)$^+$ RNA from A44 and Y17 cells. Position of 28S rRNA is indicated.

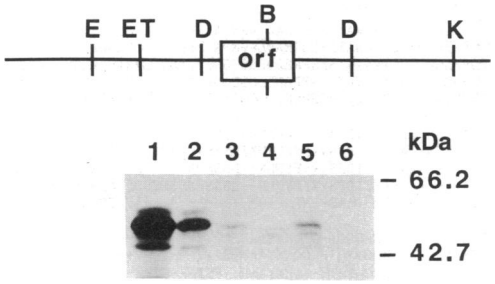

FIG. 3. *In vitro* translation of s-*myc* RNA. DNA fragments containing the s-*myc* open reading frame (orf) were inserted into vector pTZ18R. Insertion of *Dra* I–*Dra* I (DD), *Eco*T22I–*Dra* I (ETD), *Eco*RI–*Dra* I (ED), and *Eco*RI–*Kpn* I (EK) fragments yielded plasmids pTDD, pTETD, pTED, and pTEK, respectively. Capped RNAs synthesized *in vitro* by T7 RNA polymerase were translated *in vitro* with a rabbit reticulocyte lysate in the presence of [^{35}S]methionine. The ^{35}S-labeled translation products were analyzed by SDS/polyacrylamide gel electrophoresis. Lanes 1–5: translation products of RNAs from pTDD, pTETD, pTED, pTEK, and pTEK, respectively. In lane 4, the template DNA from pTEK for RNA synthesis was digested with *Bam*HI (B), which cleaves the orf. Lane 6: translation products without template RNAs.

plasmid DNA with pSV2neo DNA into NIH 3T3 cells or RT4-AC cells. A number of G418-resistant colonies were isolated and examined for their morphological and growth characteristics. Moreover, the copy number of the transfected gene was analyzed by Southern hybridization with the 1.2-kbp *Pvu* II fragment of pNM3 as a probe. None of the NIH 3T3 mouse cell lines transfected with the s-*myc* gene exhibited significant changes in morphology or growth rate. By contrast, a majority of the transfected RT4-AC cell lines exhibited distinct morphological changes. Four lines (A2, A18, A44, and Y17) were selected for further studies to analyze their tumorigenicity. The transfected cell lines A2, A18, and A44 contained approximately 3, 15, and 40 copies of pSVNM3, respectively, and the transfected cell line Y17 had approximately 20 copies of pNM3. B3 and BY were RT4-AC cell lines transfected with pSV2neo only. Under the culture conditions employed, a slight decrease in growth rate, dependent upon the expression level of the transfected gene, was observed except for B3 and BY cells. B3 and BY cells, which were similar morphologically to the original RT4-AC tumor cells, proliferated with almost the same doubling time as RT4-AC tumor cells (data not shown). Both Y17 and A44 cells, transfected with pNM3 and pSVNM3, respectively, produced significant amounts of a polyadenylylated 4.7-kb RNA corresponding to the s-*myc* gene (Fig. 2). Moreover, the level of s-*myc* expression in transfected cells was closely correlated with the copy number of the transfected s-*myc* gene (Fig. 2). The tumorigenicity of RT4-AC cells transfected with the s-*myc* gene was examined. About 1.5×10^6 cells of each transfected line were transplanted into each nude mouse and the sizes of the tumors produced were measured 35 days after the transplantation. The tumor-forming activity of transfected RT4-AC cells was greatly suppressed, proportional to the increased copy number of the s-*myc* gene present in the transfected cells (Table 1).

Table 1. Growth of rat RT4-AC cells transfected with the s-*myc* gene in nude mice

Transplanted cell line	DNA transfected	No. of s-*myc* copies transfected	Tumor volume, mm^3
RT4-AC	None	0	3380
B3	pSV2neo	0	3150
BY	pSV2neo	0	3038
Y17	pNM3	20	117
A2	pSVNM3	3	2160
A18	pSVNM3	15	270
A44	pSVNM3	40	6

Cells ($1.5–2 \times 10^6$) suspended in 0.5 ml of phosphate-buffered saline were transplanted into both the left and the right shoulder of nude mice. The growth of transplanted cells in each shoulder of nude mice was determined by measuring the length (l) and width (w) of the tumors 35 days after transplantation. The tumor volume (v) was estimated as $v = 0.5\, lw^2$. Each value is the average of the volume of tumors grown in four individual nude mice, except for B3 and BY transplantation. For B3 and BY cells, two nude mice per cell line were used.

DISCUSSION

We have determined the nucleotide sequence of a *myc*-related gene isolated from a rat genomic library. This gene, named s-*myc* due to its suppressive effect on tumorigenicity, contains an open reading frame of 1287 bp that could encode a polypeptide of ≈47 kDa. There are two other ATG codons close together in the same frame immediately downstream of the ATG codon at position 4134 (Fig. 1). It is more likely that the second ATG codon at position 4158 is actually used as the translation initiation codon, because it is surrounded by the consensus sequence, CCRCCATG(G), that is preferred for translation initiation of mammalian mRNA (19). The translation initiation sites of mouse and human N-*myc* mRNA also show similar structural features (1, 17).

In vitro transcription–translation analysis of the s-*myc* gene showed that the open reading frame spanning positions 4134–5421 is linearly translated, resulting in the formation of a protein of 50 kDa (Fig. 3). Moreover, the DNA sequence of the s-*myc* gene revealed no consensus splice-donor or splice-acceptor sequences as are found at the exon/intron boundaries of the *myc*-family genes (Fig. 1). Since the *myc* genes reported so far, except for the v-*myc* and L-*myc* pseudogenes, always consist of three exons separated by two intervening sequences, the intronless s-*myc* gene is quite different from other *myc* genes in this respect (1–5).

Comparison of the protein predicted from the DNA sequence of the s-*myc* gene with those predicted from the mouse c-, N-, and L-*myc* and rat c-*myc* sequences revealed that this protein contains six of the seven highly conserved amino acid sequences found in mouse and rat Myc-family proteins (Fig. 4). In the homologous amino-terminal region of the s-Myc protein, there is a tyrosine residue preceded by an acidic amino acid-rich sequence that is known to be a consensus sequence for substrates of protein-tyrosine kinase (20).

Amino acid residues 106–143, 320–328, and 364–412 of the human c-Myc protein are known to be important for the transport of the protein to the nucleus and its retention in the nucleus (21, 22). These domains, except for residues 320–328, are well conserved in s-Myc (Fig. 4). In addition, the predicted s-Myc protein also contains a sequence rich in basic amino acids followed by a potential α-helical leucine-zipper structure in the C-terminal region (7, 23). These features suggest that s-Myc is a nuclear DNA-binding protein.

In contrast to the sequence homologies in the C- and N-terminal regions, the fourth conserved region, which contains a stretch of acidic amino acids, is not present in s-Myc (amino acid residues 233–248 in Fig. 4). This region is also missing in the L-Myc protein, but L-Myc contains a stretch of glutamic residues encoded from further downstream within exon 3 of the L-*myc* gene (the region boxed with a dotted line in Fig. 4) (3). A similar amino acid sequence was also found in domain 2 of the adenovirus E1A protein (7, 24).

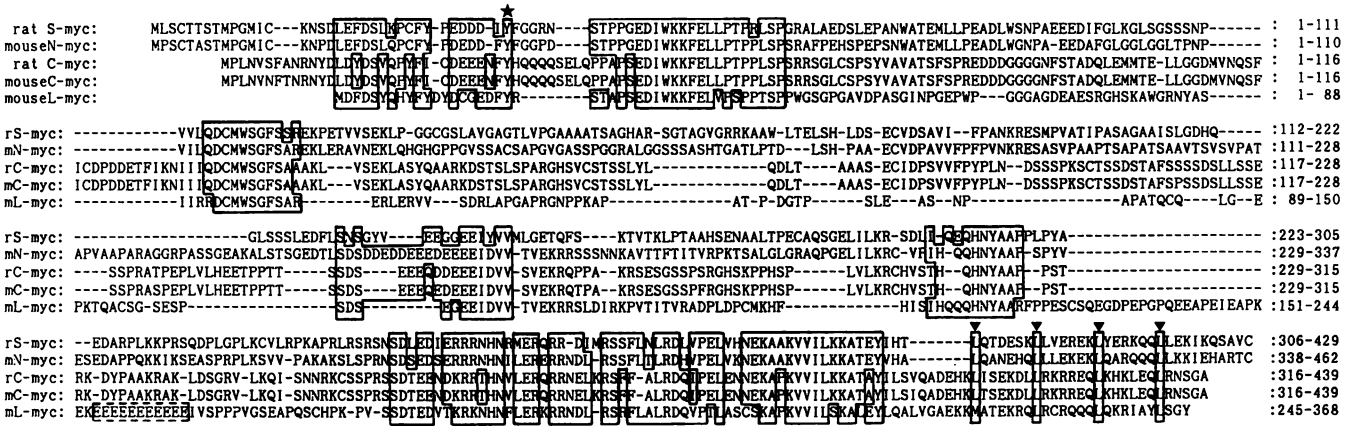

FIG. 4. Alignment of homologous amino acid sequences of mouse and rat Myc-family proteins and rat s-Myc protein. Amino acid sequences predicted from the DNA sequences reported so far (1–5) were aligned to achieve maximum homology. Boxes show conserved amino acid sequences in the proteins. The star indicates the tyrosine residue thought to be the target for phosphorylation by protein-tyrosine kinase.

We have demonstrated that the s-*myc* gene product suppressed the transforming activity as well as tumorigenicity of RT4-AC tumor cells. However, it is likely that the suppressive effect of the s-*myc* gene product on the transforming activity and tumorigenicity of RT4-AC tumor cells is not very strong, since complete inhibition was observed only when many copies of s-*myc* were inserted into the cells and expressed in sufficient quantity (Table 1; Fig. 2).

In naturally occurring cells, s-*myc* gene expression was low even though the β-actin gene was actively expressed (Fig. 2). This implies that under certain conditions s-*myc* transcription is repressed. Therefore, studies to determine whether a specific factor(s) affects transcription of the s-*myc* gene will be important in order to understand the physiological function (e.g., tumor suppression) of the s-*myc* gene product. In any event, the discovery of s-*myc*, a *myc*-related gene of unique character, opens an alternative way to elucidate biological functions of the *myc*-family genes, as well as to regulate their transforming activity.

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