MOLECULAR AND CELLULAR BIOLOGY, Oct. 1988, p. 4529–4532
0270-7306/88/104529-04$02.00/0

Vol. 8, No. 10

Subnuclear Localization and Antitransforming Activity of N-*myc*:β-Galactosidase Fusion Proteins

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Received 9 May 1988/Accepted 24 June 1988

N-*myc* expression is under stage- and tissue-specific regulation in mammalian development, but its function is totally unknown. We sought agents to block N-*myc* activity in order to infer from the effect the possible function of N-*myc* in the apparently complex processes. As candidates for such agents, we tested fusion genes encoding N-*myc*:β-galactosidase fusion proteins for their effects on the formation of transformed foci of rat embryo primary fibroblasts as the result of transfection with N-*myc* and activated H-*ras*. One of the gene constructs very efficiently antagonized N-*myc* activity, as assessed by its effect on focus formation, but did not appreciably affect cell viability. The product of this gene was not only targeted to the nucleus but also accumulated in subnuclear loci which may represent the sites where normal N-*myc* proteins reside. The occurrence of antagonistic effect at a low stoichiometric ratio suggested that the fusion protein gene competed with the N-*myc* gene in a fashion analogous to a dominant negative mutation.

The N-*myc* gene, first discovered as a result of its amplification in some of the neuroblastomas (9, 14), is a member of the *myc* gene family; it shares homology to c-*myc* (1, 4, 10, 19, 22) and encodes a nuclear protein (7, 13, 17). In contrast to c-*myc* expression, which is ubiquitous and nearly constant in various tissues, N-*myc* expression is extremely high in the early embryonic period and declines drastically in later periods, except in nervous and other limited kinds of tissues (8, 26). Presumably, this stage- and tissue-specific regulation of N-*myc* expression is highly significant for early developmental processes, but the real function of N-*myc* has not yet been elucidated.

Analysis of the function of a putative regulatory gene is greatly facilitated by experimental conditions in which the activity of the gene is artificially blocked. Thus, we attempted to create defective genes whose products antagonize the function of the normal N-*myc* gene. As candidates, we examined genes which encode fusion proteins of mouse N-*myc* and bacterial β-galactosidase (N-*myc*:β-gal).

Three different lengths of the N-*myc* base sequences coding for 75, 278, and 444 N-terminal amino acids were fused in phase to the base sequence coding for the β-galactosidase moiety. N-*myc* genomic DNA fragments starting at the *Nar*I site (at position −161 relative to the cap site [8a]) and ending at the *Nsp*(7524)V site in exon 2 (position +1664: the coordinate is based on the nucleotide sequence of Katoh et al. [8a] and Taya et al. [22]), the *Bst*EII site in exon 3 (position +4278), and a *Pst*I site in exon 3 (position +4780) were excised from a genomic clone carrying the 7.5-kilobase *Eco*RI fragment (Fig. 1), and the termini were made blunt with the aid of T4 DNA polymerase. Since the N-*myc* promoter located downstream of the *Nar*I site is not very active in fibroblasts (our unpublished result), we added Moloney murine leukemia virus enhancer-promoter complex (from −1077 to +16 relative to the cap site [16] as the *Eco*RI-*Sma*I fragment of pδKp-Mov [5] to assist expression in rat embryo primary fibroblasts (REF). Fusion to the coding sequence of β-galactosidase was done by blunt end ligation by using one of the restriction sites in the polylinker of pMC1871 (15), *Sma*I [versus *Nsp*(7524)V] for construct 1, *Eco*RI (versus *Bst*EII) for construct 2, or *Xma*I (versus *Pst*I) for construct 3. The transcriptional units were made as a complete structure by attaching tandemly arranged poly(A) signals of herpes simplex virus thymidine kinase (from positions 1415 to 1797 [11]) and simian virus 40 (the *Sma*I-*Eco*RI fragment of pSV2neo [18]) and put on the plasmid vector pUC19.

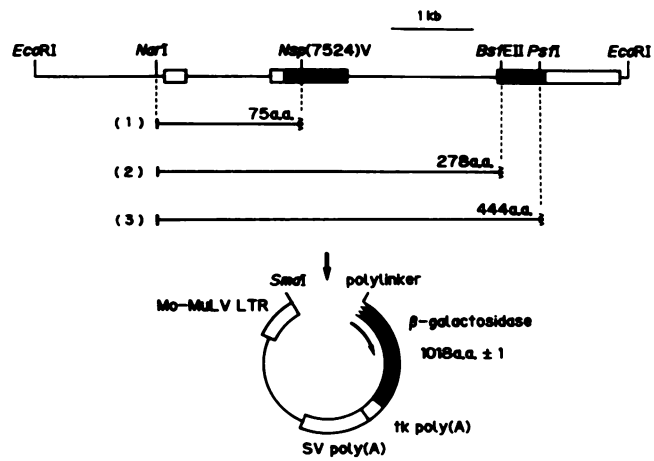

FIG. 1. Structure of the mouse N-*myc* gene (4, 8a, 22) and construction of the N-*myc*:β-gal fusion genes. On the N-*myc* map, exons are indicated by boxes; coding regions are filled-in areas. The segments of the N-*myc* gene with the *Nar*I site at the 5′ end and a restriction site at the 3′ end were cloned on the plasmid vector shown on the lower part of the figure so that the N-*myc* coding sequence continues in phase, through the polylinker sequence of pMC1871, to the β-galactosidase sequence. The numbers of amino acid residues (a.a.) derived from N-*myc* are also indicated. The vector is derived from pUC19 and contains the enhancer-promoter complex of Moloney murine leukemia virus long terminal repeats (Mo-MuLV LTR), herpes simplex virus thymidine kinase (tk), and simian virus 40 (SV) poly(A) addition signals. The map of the vector is not drawn to scale.

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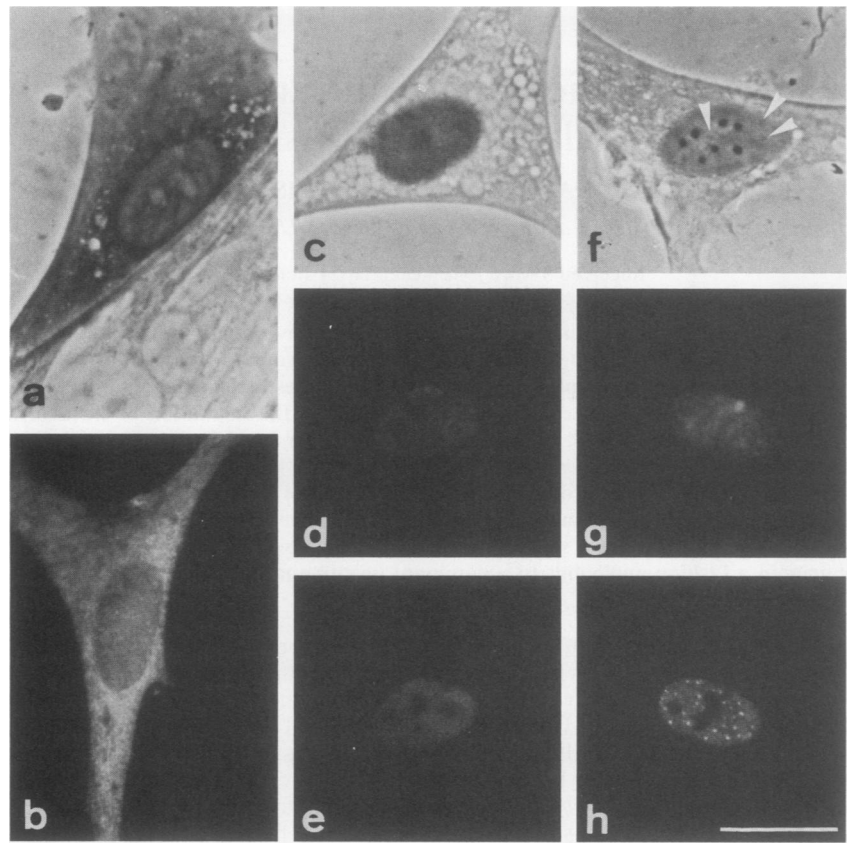

FIG. 2. Expression and subcellular localization of N-*myc*:β-gal fusion proteins after transfection of REF with construct 1 (a and b), construct 2 (c and e), and construct 3 (f and h). Panels a, c, and f were stained with X-Gal for β-galactosidase activity (23), and panels b, e, and h show rhodamine immunofluorescence of β-galactosidase. Panels d and g are the same fields as panels e and h but are fluorescence stained with 4′,6′-diamidino-2-phenylindole dihydrochloride to localize DNA on the nuclei. In panel f, a number of weakly stained spots (some are indicated by white arrowheads) were observed in addition to the prominent spots. Not all the spots are reproduced in the figure. Bar, 20 μm.

We transfected REF with the gene constructs, and transiently expressed gene products were at first analyzed by histochemical means to detect β-galactosidase activity (Fig. 2). If the fusion genes have biological activity relevant to N-*myc*, the products should be localized in the nucleus. When the fusion was done at amino acid codon 75 in the second exon of N-*myc* (N-*myc*:β-gal#1), β-galactosidase activity was detected only in the cytoplasm (Fig. 2a), but when fused at the third exon (N-*myc*:β-gal#2 and #3), the activity was found mainly in the nucleus (Fig. 2c and f). Thus, the N-terminal 278 amino acids contain signals which can target N-*myc* protein to the nucleus.

Judging from the β-galactosidase activity, hybrid protein N-*myc*:β-gal#2 was evenly distributed in the nucleus (Fig. 2c), but N-*myc*:β-gal#3 was concentrated to several to multiples of ten spots in the majority of the nuclei found to be positive (Fig. 2f). This difference in subcellular and subnuclear localization of the hybrid proteins was confirmed by immunofluorescence using anti–β-galactosidase antibodies (Fig. 2b, e, and h). Immunofluorescence of N-*myc*:β-gal#3, which detected the β-galactosidase moiety of the fusion protein, showed a number of specks, but that of N-*myc*:β-gal#2 revealed much greater homogeneity in the nucleus. In both cases, the fusion proteins appeared to be excluded from the nucleoli. It may be noted that the distribution of N-*myc*:β-gal#3 protein did not coincide with staining with 4′,6′-diamidino-2-phenylindoledihydrochloride, which detected DNA (Fig. 2g). Antibodies specific to N-*myc* have been reported (7, 13, 17); however, histochemical detection of the N-*myc* protein with these antibodies was possible only in cell lines and tumors expressing an extreme excess of N-*myc* (7, 17; our unpublished result), and no clear subnuclear localization of N-*myc* was indicated. The localization of N-*myc*:β-gal#3 protein may demonstrate for the first time the actual sites at which N-*myc* proteins carry out their functions. It is interesting to note that Sullivan et al. (20) observed a "speckled pattern" of c-*myc* and v-*myc* protein distribution in a nucleus by using specific antibodies.

The region of exon 3 bracketed by the N-*myc* break points of constructs 2 and 3 must contain a domain essential for the activity of the N-*myc* protein after its entrance into the nucleus. The amino acid sequence of the N-*myc* protein is the best conserved in this region among human, mouse, and chicken (4, 10, 19, 22; our unpublished results). Western blotting (immunoblotting) using anti–β-galactosidase antibodies demonstrated synthesis of a fusion protein with an apparent molecular weight of 180,000 in REF transfected with the gene for N-*myc*:β-gal#3 (data not shown).

REF do not express significant levels of endogenous N-*myc*. However, the same subnuclear localization of the fusion protein encoded by construct 3 was observed in transfected neuroblastoma line NBA2 (26) and in primitive endodermal line PSA5-E (8a), which highly express endogenous N-*myc*. Therefore, localization of the fusion protein

TABLE 1. Effect of N-*myc*:β-gal fusion genes on N-*myc*-, "activated" *ras*-dependent focus formation of REF

Transfected gene(s)[a]	Foci/10^6 transfected REF[b] (no. of foci with heavy β-galactosidase staining) in expt:			
	1	2	3	4
T24*ras*	0	0	0	0
T24*ras*, N-*myc*	91	128	52	90
T24*ras*, N-*myc*, N-*myc*:β-gal#1			49 (36)	60 (48)
T24*ras*, N-*myc*, N-*myc*:β-gal#2			64 (53)	105 (89)
T24*ras*, N-*myc*, N-*myc*:β-gal#3	18	21	9 (0)	8 (0)

[a] DNAs were prepared in the form of pUC19-based plasmids: pUCT24, the 6.4-kilobase *Bam*HI fragment of T24*ras* (21) cloned on pUC19; pN-*myc*, the clone of the *Nar*I-*Eco*RI fragment of N-*myc* on the vector shown in Fig. 1, from which the β-galactosidase and herpes simplex virus thymidine kinase poly(A) portions were removed. Plasmids for the fusion protein genes are shown in Fig. 1.

[b] Each 9-cm dish inoculated with 10^6 REF (25) was transfected with 10.5 μg of DNA, which was a mixture of 0.5 μg of pUCT24, 5.0 μg of pN-*myc*, and 5.0 μg of one of the pN-*myc*:β-gal constructs, with the remainder being pUC19. After 24 h, the REF in one dish were distributed into four dishes, and after 9 more days the cultures were fixed with 1% glutaraldehyde and stained with Giemsa. In experiments 3 and 4, the dishes were also stained for β-galactosidase (23).

was not affected, regardless of whether the cells were in the condition for endogenous N-*myc* expression or whether the sites for N-*myc* protein were already occupied by normal N-*myc* protein.

We examined the focus-forming and focus-suppressing activities of the fusion genes by transfection of REF in combination with activated H-*ras* (T24*ras*) (21) on the plasmid pUCT24. Although long terminal repeat-driven N-*myc* on plasmid pN-*myc* produced foci, as reported by Yancopoulos et al. (25), none of the fusion genes expressed activity to support focus formation. However, the fusion gene for N-*myc*:β-gal#3 exhibited a very strong suppressing effect on N-*myc*-, T24*ras*-dependent focus formation, while no appreciable effect was observed with either N-*myc*:β-gal#1 or #2 (Table 1). The significance of this difference between the fusion gene constructs was strengthened by staining the transformed foci for β-galactosidase activity. When the plasmid pN-*myc*:β-gal#2 was cotransfected with pN-*myc*, cells in the majority of the foci were heavily stained at the nucleus, indicating that the fusion gene was in fact cotransfected and expressed but that it did not interfere with N-*myc*. In contrast, in the small number of foci which appeared in dishes transfected with pN-*myc*:β-gal#3, either the cells were not stained at all or only a very small fraction of the cells was weakly stained, indicating that the foci represent cell clones which failed to incorporate or to express efficiently the fusion protein gene. The expression of N-*myc*:β-gal#3 per se is not toxic to the cells, because the frequencies of β-galactosidase–expressing cells after transfection of REF with either construct but without pUCT24 were similar between the fusion genes when measured after 24 h (ca. 3%) as well as after 10 days (ca. 0.5%).

Various amounts of the DNA of pN-*myc*:β-gal#3 were mixed with a constant amount of pN-*myc* DNA, and the frequencies of the transformed focus were scored (Fig. 3). Suppression of foci was noticeable even at the molar ratio of 0.2; the foci progressively decreased in number with increases in the fusion gene DNA, and suppression was as great as 80% at the molar ratio of 0.6. This antagonizing effect is expected, for instance, if N-*myc* and the fusion protein compete for the same site of interaction with nuclear components, presumably on DNA (13), and if the interaction of the function-defective fusion protein is stronger or more stable than that of normal ones. Alternatively, if N-*myc* proteins function as multimers, interaction of the fusion proteins may interfere with functional multimeric forms due to either the bulkiness of the β-galactosidase moiety or the tetrameric nature of the β-galactosidase itself.

To determine whether focus suppression by N-*myc*:β-gal#3 is specific to N-*myc*, we examined the effect on the c-*myc*–dependent foci. As shown in Fig. 3, no significant effect on foci was observed by cotransfection of pN-*myc*:β-gal#3 with pc-*myc* under the conditions in which the number of N-*myc*–dependent foci was very much decreased. Thus, focus suppression by the fusion gene for N-*myc*:β-gal#3 is specific to N-*myc*.

Thus, expression of N-*myc*:β-gal fusion proteins in REF demonstrated two different aspects of utilizing the gene fusion which will be extended to the analysis of a variety of other regulatory genes. First, the β-galactosidase moiety provided an excellent molecular tag, enzymatically as well as antigenically, which allowed us to very precisely track down where the gene product was localized. We could allocate regions required for nuclear targeting and functional subnuclear localization on the N-*myc* polypeptide, comparing various lengths of the N-*myc* portion fused to the same β-galactosidase moiety. The subnuclear loci where the N-*myc*:β-gal#3 fusion protein accumulates presumably represent the sites for activity of normal N-*myc* proteins, which have not been known previously owing to the lack of proper protein probes. An analogous, successful case was the

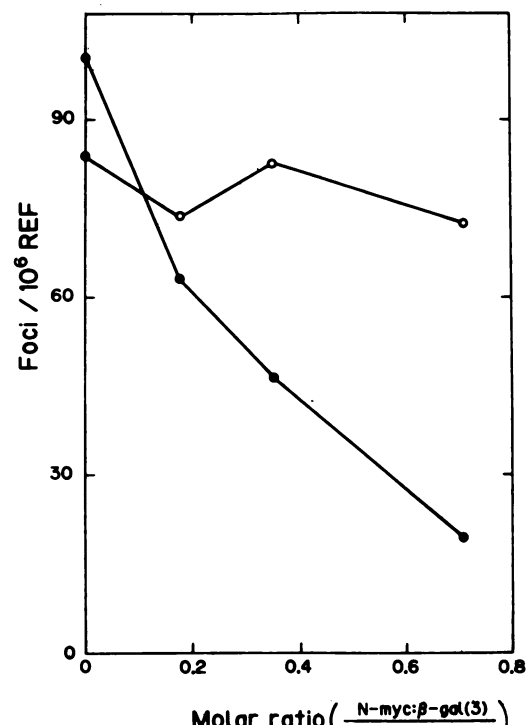

FIG. 3. Effect of N-*myc*-β-gal#3 on N-*myc* (●)- or c-*myc* (○)-dependent focus formation. A total of 10^6 REF were transfected with DNAs: 0.5 μg of pUCT24, 5 μg of pN-*myc*, or 3 μg of pc-*myc* plus various amounts of pN-*myc*:β-gal#3 and pUC19 DNA so that the total amount of DNAs came to 10.5 μg. pc-*myc* is the clone of the 7.5-kilobase *Sma*I-*Bam*HI fragment of the mouse c-*myc* gene (2) on the same vector as pN-*myc*. Cultures were processed as described in footnote *b* Table 1 and scored for foci after 10 days.

analysis of nucleus-targeting signals of glucocorticoid receptor protein by Picard and Yamamoto (12). Second, one of the N-*myc*:β-gal fusion proteins was used as an antagonizing agent to block N-*myc* function. This can be regarded as an example of "functional inactivation of genes by dominant negative mutations," which has been prospectively reviewed by Herskowitz (6). N-*myc*:β-gal#3 fusion protein exerted a strong antagonistic effect on N-*myc*–dependent transformation of REF. How the fusion protein suppresses transformed foci is not clearly understood but must be highly relevant to the action of N-*myc* protein. We are extending the application of N-*myc*:β-gal#3 to early mouse embryos to examine the function of N-*myc* expression during that period. Recent discoveries that some of the genes involved in cell differentiation share homologies with the *myc* gene family (3, 24) emphasize the versatility of the approach we have undertaken.

K. Ueno was on leave from the Department of Biology, Tokyo Institute of Technology. We thank Y. Hiramoto for encouragement; Kenji Shimizu and Kenji Watanabe for providing pUCT24 and antibodies, respectively; and Shoji Sawai for assistance.

This work was supported by grants from the Ministry of Education, Science and Culture of Japan.

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