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Neoplastic transformation and tumorigenesis by the human protooncogene *MYC*

(primary cells/retroviral vector/leukemia/cellular phenotype)

GARY M. RAMSAY*, GIOVANNELLA MOSCOVICI†, CARLO MOSCOVICI†, AND J. MICHAEL BISHOP*

*Department of Microbiology and Immunology and The G. W. Hooper Research Foundation, University of California, San Francisco, CA 94143; and †Veterans Administration Medical Center and Department of Pathology, University of Florida, Gainesville, FL 32610

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ABSTRACT **Damage to the protooncogene *MYC* has been implicated in the genesis of diverse human tumors, but the tumorigenic potential of the isolated gene has been disputed. Here we report the use of a retroviral vector to test the potency of human *MYC* for neoplastic transformation in avian cells. We found that sustained and abundant expression of *MYC* can transform both embryonic fibroblasts and hematopoietic cells and elicit granulocytic leukemias in chickens. Transformation by *MYC* is accompanied by changes in diverse aspects of cellular phenotype, including morphology, ability to grow in suspension, rate of proliferation, the structure of the cytoskeleton, and the composition of the extracellular matrix. Nevertheless, the biological potency of *MYC* is inherently constrained when compared to that of the retroviral oncogene v-*myc*. Our findings enlarge on previous descriptions of neoplastic transformation by *MYC* and sustain the view that ungoverned expression of the gene can contribute to the genesis of human tumors.**

The protooncogene *MYC* was first encountered as the progenitor for the retroviral oncogene v-*myc* (1, 2). Subsequently, *MYC* has been implicated in the governance of normal cellular proliferation and differentiation (3) and in the genesis of diverse human tumors (4). Two important questions arose from these findings. What is the tumorigenic potential of normal *MYC*, and how might damage to *MYC* augment that potential? The available answers to these questions remain clouded with ambiguity. On the one hand, it is generally agreed that damage to *MYC* can figure in the genesis of human tumors (4) and that abundant expression of the gene can transform a variety of cells in culture (5–8). But on the other hand, some observers attribute to the gene only a limited potency for neoplastic transformation, especially when tested in primary cultures of embryonic cells rather than in established cell lines (9, 10), and tumorigenicity by transgenes of *MYC* in mice typically seems to require additional factors (11–14). To test further the potency of *MYC* for neoplastic transformation, we used a retroviral vector that can infect and replicate in avian cells and that carries a cDNA representing human *MYC*. Our results document the ability of *MYC* to elicit neoplastic transformation in primary cultures of avian fibroblasts and hematopoietic cells, as described by others (15, 16, 31); demonstrate tumorigenicity of the gene in chickens; and reveal subtle constraints on the biological potency and specificity of *MYC* that may be relieved by mutations in v-*myc*.

MATERIALS AND METHODS

Cells and Viruses. The sources, propagation, and bioassays of MC29 and OK10 viruses have been reported (17, 18). Transfection of DNA into chicken embryo fibroblasts (CEFs) or an established line of quail fibroblasts, QT-6, was performed according to published protocols (19). The retroviral vector RCAS was derived from the genome of Rous sarcoma virus (RSV) and was provided by Stephen Hughes (20). A cDNA representing human *MYC* was inserted into the vector to give the strain RSV(*MYC*). A molecular clone that expresses the gene for resistance to neomycin under the control of the RSV long terminal repeat was kindly supplied by S. Gerondakis.

Stocks of RSV(*MYC*) were prepared in two ways. (*i*) DNA of RSV(*MYC*) was transfected into CEFs and the recipient cells were then propagated en masse until the virus reached high titers. (*ii*) DNA clones of both RSV(MYC) and the neomycin-resistance vector were cotransfected into QT-6 cells, which, 18 hr later, were suspended in Methocel (5×10^5 cells per 60-mm Petri dish) in the presence of G418 (250 μg/ml). Colonies of surviving cells were isolated after $\approx$16 days and propagated in the presence of G418 to quantities sufficient for harvesting of viral stocks.

The ability of the viral stocks to cause leukemia or hepatomas was tested by injecting standard amounts (200 focus-forming units) into the choroallantoic vein of 10-day-old embryos of SPAFAS (Norwich, CT) chickens. To determine sarcomagenic ability, 1-day-old chicks were injected in the wing web. Mortality from these procedures was negligible. Hatchlings were examined for leukemia or sarcomas at twice weekly intervals. Once evidence of leukemia or sarcomas appeared, the birds were killed and autopsied. Animals that failed to develop leukemia or sarcomas were killed and autopsied after 5 or 6 weeks. No evidence of macroscopic tumors was found.

Detection of Proteins by Immunoprecipitation, Western Blotting, and Immunofluorescence. Cells were labeled with [^{35}S]methionine and then analyzed by immunoprecipitation, using our antiserum CT1 and procedures reported previously (21). Western blotting was performed with the same antiserum according to standard protocols (22). For immunofluorescence, cells were propagated on glass coverslips and analyzed according to published procedures (23). Actin was detected with rhodamine-labeled phalloidin (Molecular Probes); fibronectin was detected with an antibody obtained from Collaborative Research and a second antibody labeled with fluorescein isothiocyanate.

RESULTS

Avian Retroviral Vector Carrying *MYC*. To assess the pathogenic potential of the protooncogene *MYC*, we used a retroviral vector derived from RSV (20) carrying a cDNA representing a normal allele of human *MYC* and designated RSV(*MYC*). The vector contained all of the viral functions required for replication and expressed the inserted *MYC* from a spliced mRNA.

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Abbreviations: CEF, chicken embryo fibroblast; RSV, Rous sarcoma virus.

Table 1. Ability of human *MYC* to transform primary cultures of chicken cells

	Foci	CEF colonies in methylcellulose	Macrophage colonies in Methocel
MC29	7×10^4	5×10^4	4×10^3
OK10	6×10^4	3×10^4	2×10^3
RSV(*MYC*)	2×10^4	8×10^3	7×10^2

We prepared infectious stocks of the vectors in two ways: (*i*) by transfection of CEFs with RSV(*MYC*), in the absence of a selectable marker, and (*ii*) by cotransfection of QT-6 cells with RSV(*MYC*) and a vector that expressed the gene for neomycin resistance. In the first of these approaches, virus stocks were harvested from cultures en masse; in the second approach, individual clones of cells were first selected with G418 in methylcellulose and then propagated for harvesting the virus. The virus prepared with CEFs arose from spread through the culture, was polyclonal in nature, and reached relatively high titers. By contrast, the virus harvested from clones of QT-6 cells presumably arose only from direct transfectants, not by spread through the culture, and had relatively low titers—as anticipated from previous experience with efforts to propagate RSV itself in QT-6 cells (unpublished results).

Transfection of Chicken Embryo Fibroblast Cells by *MYC*. Infection of CEFs with RSV(*MYC*) gave rise to foci of transformed cells in monolayer cultures and growth of colonies in methylcellulose (Table 1). Neither event occurred in the absence of infection. The titers of RSV(*MYC*) in both settings were akin to those of MC29 and OK10, two viruses that carry different alleles of the oncogene v-*myc* (18).

The potency of *MYC* in these assays differed from that of v-*myc*. First, foci induced by v-*myc* developed within 10 days; those induced by *MYC* developed within 16 days. Once developed, however, the two forms of foci were indistinguishable (data not shown). Second, the colonies elicited in methylcellulose by *MYC* grew to diameters only one-third those of colonies elicited by v-*myc* (Fig. 1 *A* and *B*). These differences prompted us to characterize further the phenotypes of CEFs transformed by *MYC* and v-*myc*.

Phenotypic Properties of Chicken Embryo Fibroblasts Transformed by *MYC* or v-*myc*. We first examined the microscopic morphology of CEFs transformed by *MYC* or v-*myc*. We documented complete infection of the cultures by using immunofluorescence to detect the protein products of the *MYC*/v-*myc* genes, which are not detectable by this means in uninfected CEFs (data not shown). The appearance of CEFs transformed by *MYC* was clearly different from that of normal cells (Fig. 1 *C* and *E*), but the morphologies of CEFs transformed by *MYC* and v-*myc* were also distinguishable (Fig. 1 *D* and *E*). In particular, cells transformed by v-*myc* displayed the characteristic features of increased refractility—rounding of shape and prominence of nuclei and nucleoli. The cells transformed by *MYC* displayed similar changes, but to a lesser extreme.

CEFs transformed by v-*myc* typically proliferate more rapidly than do their normal counterparts (24). The same proved to be true of cells transformed by *MYC*, although again, the change was less extreme than with v-*myc* (Fig. 2). When propagated under standardized conditions, CEFs

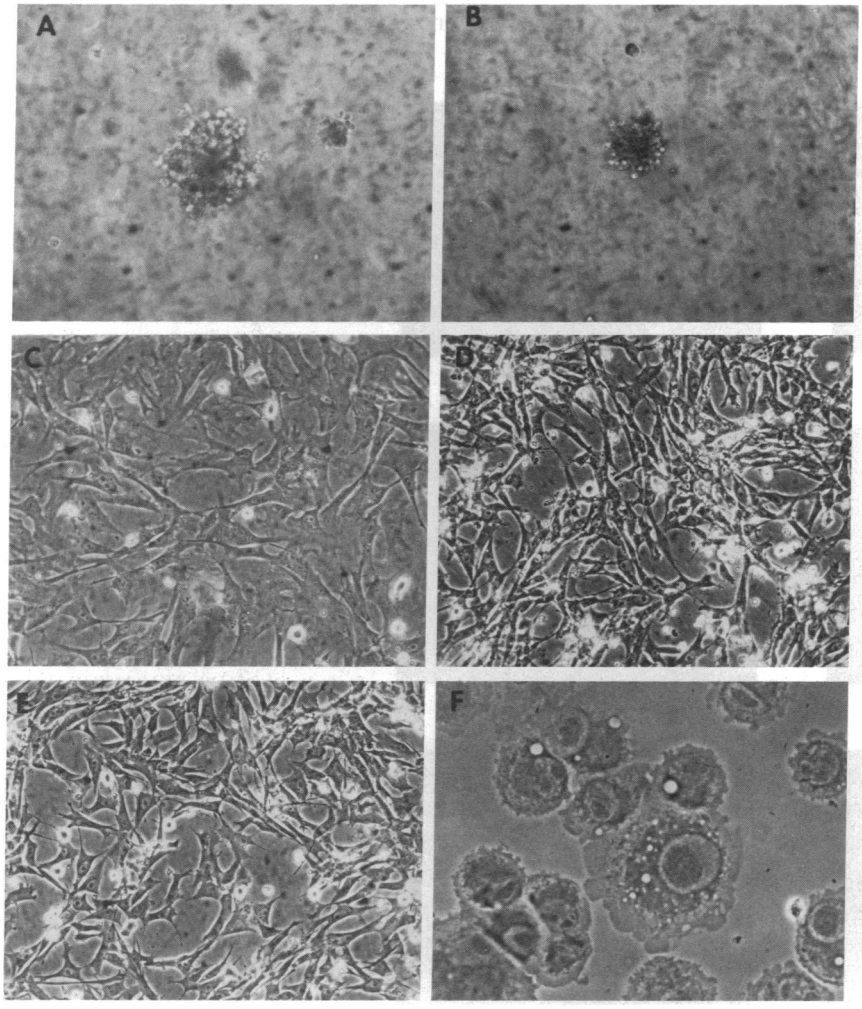

FIG. 1. The morphology of cells transformed by v-*myc* and *MYC*. CEFs were infected with MC29 virus or RSV(*MYC*) and either suspended in methylcellulose or propagated en masse until immunofluorescence documented uniform infection of the cultures. Bone marrow cells from 10-day-old chicken were infected and suspended in methylcellulose. (*A* and *B*) Colonies in methylcellulose of CEFs transformed by MC29 virus and RSV(*MYC*), respectively. (*C–E*) CEFs propagated in monolayer after mock infection (*C*), infection with MC29 virus (*D*), or infection with RSV(*MYC*) (*E*). (*F*) Bone marrow cells infected with RSV(*MYC*), propagated en masse, and stained with Wright's stain.

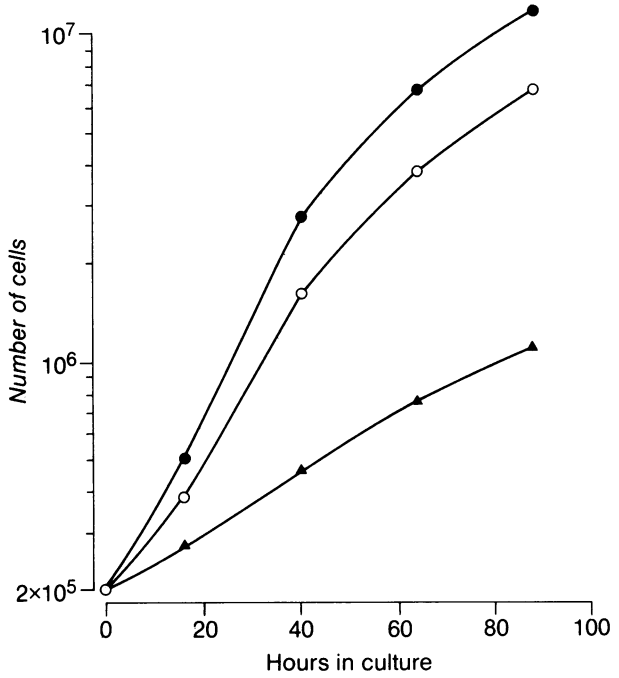

FIG. 2. Proliferation of CEFs transformed by v-*myc* or *MYC*. Cells (2×10^5) were plated on 90-mm Petri dishes in standard medium and then harvested for enumeration at 24-hr intervals. ▲, Uninfected CEFs; ●, CEFs transformed by OK10 virus; ○, CEFs transformed by RSV(*MYC*).

transformed by *MYC* and v-*myc* reached numbers that were, respectively, 6 times and 10 times greater than those of normal cells after 4 days of proliferation. The data appear to reflect differences in both rates of proliferation (as reflected in the relative slopes of the plots) and saturation densities for the cells. Although modest, the differences proved reproducible in repeated trials (data not shown).

Transformation by v-*myc* has characteristic effects on the cytoskeleton and extracellular matrix of CEFs, disrupting elements of the former and reducing the amount of fibronectin in the latter (24). We used both parameters to compare cells transformed by *MYC* and v-*myc*. Immunofluorescence revealed the expected abundance of well-organized actin cables within, and fibronectin on the surface of, normal CEFs (Fig. 3 *A* and *B*). By contrast, the actin cables were disrupted (Fig. 3 *C* and *E*) and the fibronectin was reduced (Fig. 3 *D* and *F*) to roughly equal extents in cells transformed by either *MYC* or v-*myc*.

Expression of *MYC* and v-*myc* in Infected Cells. The biological potencies of *MYC* and v-*myc* differed in several settings (see above). We used immunoprecipitation to explore the possibility that those differences might arise from the relative efficacy with which *MYC* and v-*myc* were expressed in the infected cells (Fig. 4 *A*). The results demonstrated that CEFs transformed by either *MYC* or the OK10 allele of v-*myc* contained roughly equivalent amounts of the pertinent gene products (62 and 58 kDa, respectively) and that cells transformed by the MC29 allele of v-*myc* contained a fewfold more of the 110-kDa oncogene product. Under the conditions used here, no MYC protein could be detected in uninfected CEFs. We conclude that constraints on biological potency may be inherent in the product of *MYC*.

Transformation of Hematopoietic Cells by *MYC* and v-*myc*. Diverse alleles of v-*myc* transform hematopoietic cells that resemble monocytes or macrophages in culture (18). We

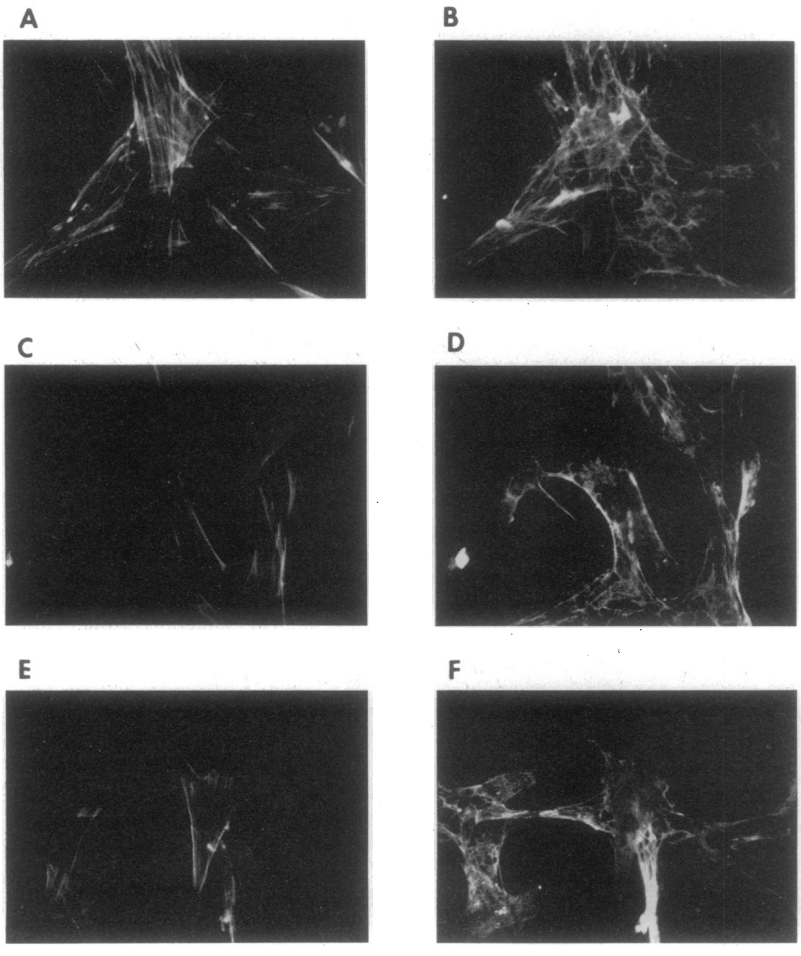

FIG. 3. Analysis of actin cables and fibronectin patterns by immunofluorescence. CEFs infected with MC29, RSV(*MYC*), or mock virus were subcultured and grown on glass coverslips for staining. (*A* and *B*) Mock-infected CEFs. (*C* and *D*) MC29-infected cells. (*E* and *F*) RSV(*MYC*)-infected cells. The CEFs in *A*, *C*, and *E* were stained with rhodamine-labeled phalloidin. The cells in *B*, *D*, and *F* have been stained with antibody against fibronectin followed by goat anti-rabbit antibody labeled with fluorescein isothiocyanate.

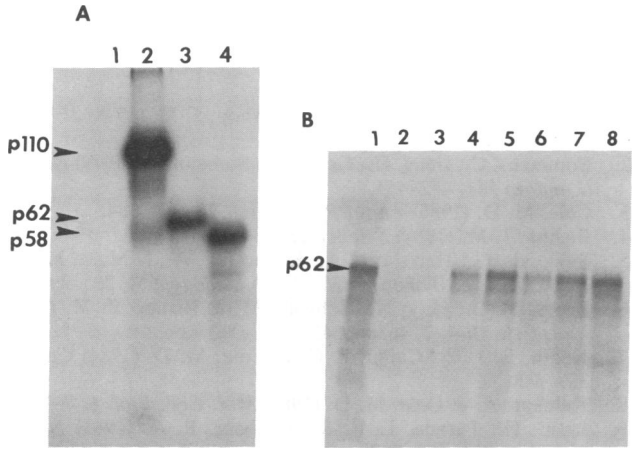

FIG. 4. Expression of *MYC* and v-*myc* in infected cells. (*A*) Analysis of cells in culture by immunoprecipitation. Cells infected with MC29, OK10, or RSV(*MYC*) were propagated en masse, labeled with [^{35}S]methionine, and analyzed as described. Approximately equivalent amounts of radioactivity were loaded in each lane. The cells analyzed by using anti-*myc* antiserum CT1 were as follows: lane 1, uninfected chicken cells; lane 2, MC29 virus-infected chicken cells; lane 3, RSV(*MYC*)-infected chicken cells; lane 4, OK10 virus-infected chicken cells. (*B*) Analysis of tumor cells by Western blotting. Tumor cells were analyzed and equal quantities of protein were loaded in each lane. The samples analyzed by using anti-*myc* antiserum were as follows: lane 1, RSV(*MYC*)-infected chicken cells; lane 2, spleen of uninfected chicken; lane 3, liver of uninfected chicken; lane 4, tumor cells from spleen of bird 1287; lane 5, tumor cells from liver of bird 1287; lane 6, tumor cells from spleen of bird 1293; lane 7, tumor cells from liver of bird 1293; lane 8, tumor cells from kidney of bird 1293.

found that RSV(MYC) elicited an analogous form of transformation when used to infect cultures of chicken bone marrow (Table 1). In contrast to the results with CEFs, the potencies of *MYC* and v-*myc* appeared similar in hematopoietic cells: MC29, OK10, and RSV(*MYC*)d elicited comparable numbers of transformed colonies, with comparable sizes and shapes, and over similar periods of time (Table 1; data not shown). Moreover, the microscopic morphology of cells transformed by *MYC* was characteristic of macrophages (Fig. 1*F*) and identical to that of bone marrow cells transformed by v-*myc* (data not shown). We conclude that the potency and specificity of *MYC* for transformation of bone marrow cells in culture may be equivalent to that of v-*myc*.

Tumorigenicity of *MYC* in Chickens. When chicken embryos were injected *in ovum* with MC29 virus, all of the subsequent hatchlings developed myeloid leukemia and hepatomas (Table 2). In the case of 1-day-old chicks injected in the wing web, 50% developed sarcomas. These were the results expected for a fully potent allele of v-*myc* (18, 25). By contrast, injection of a comparable amount of RSV(*MYC*)d gave rise only to leukemia, which developed in fewer than half of the hatchlings and after a relatively protracted incubation period (4–6 weeks, as opposed to 2–3 weeks with v-*myc*). Moreover, the leukemia involved a more mature cell (granulocyte) than that affected by v-*myc* (myelocyte). Abundant expression of *MYC* in the leukemia cells was documented by Western blotting (Fig. 4*B*). By contrast, the protein was undetectable by this means in uninfected cells (data not shown). We conclude that the sustained and abundant expression of *MYC* is leukemogenic, but there again appear to be constraints on the biological potency of the gene.

Table 2. Tumorigenic ability of human *MYC*

	Amount of virus injected, focus-forming units	Tumors obtained		
		Myeloid leukemia	Hepatoma	Sarcoma
MC29	2×10^2	8 (8)	8 (8)	6 (12)
RSV(*MYC*)	2×10^2	10 (24)	0 (24)	0 (12)

Tumorigenesis was tested as described in *Materials and Methods*. Total numbers of birds examined are given in parentheses.

Neoplastic Transformation by *MYC* Is Not Due to Genetic Variants. It was possible that the transformation by *MYC* reported here was due to mutations that arose in the gene during the preparation of viral stocks (26). In an effort to refute this possibility, we prepared multiple stocks of RSV(*MYC*) from clonal lines of QT-6 cells that had received the viral genome by transfection, but in which subsequent spread and replication of the virus should have been greatly constrained or prohibited (see *Materials and Methods* for details). The five clones that we examined gave rise to transforming virus with approximately equivalent titers, in the range of 5×10^2 focus-forming units/ml (data not shown). In view of these results, we believe it unlikely that mutations in *MYC* account for the transforming ability documented here (see below).

DISCUSSION

Neoplastic Transformation by Normal Allele(s) of *MYC*. The work reported here demonstrates that a normal allele of *MYC* can transform both CEFs and chicken macrophages in culture. In addition, we show that *MYC* can elicit granulocytic leukemia when introduced into the chicken embryo *in ovum*. Our findings are in accord with previous reports that *MYC* can transform avian fibroblasts (15, 16, 31), avian macrophages (16), and murine monocytes (5)—the latter in both culture and pristane-primed mice. The findings are particularly notable because they document two capabilities often denied for *MYC*: extensive and unilateral transformation of primary cultures and tumorigenicity in the absence of another oncogene.

CEFs transformed by either v-*myc* (24) or *MYC* (present data) reach higher population densities and proliferate more rapidly than do normal cells. The former is a ubiquitous property of cells transformed by diverse means, but the latter is much less common. Although we have yet to characterize the division cycle of cells transformed by *MYC*, we presume that the increased rate of proliferation is achieved by abbreviation of the G_1 period (27). Perhaps this change reflects some specific aspect of the mechanism by which *MYC* acts.

The leukemic cells elicited by *MYC* (granulocytes) do not correspond to the type of cell transformed by *MYC* in culture (macrophages). We have no explanation for this difference. We note, however, that a similar discrepancy applies to v-*myc*, which elicits leukemias of relatively undifferentiated myelocytes but transforms macrophages in culture (18).

The replication of retroviruses introduces mutations into the viral genome at exceptional frequencies (26), and this mutagenesis can give rise to transforming alleles of protooncogenes (28). We have several reasons to doubt that spontaneous mutations could account for the biological properties reported here for *MYC*. First, high titers of transforming virus arose promptly and reproducibly after transfection of RSV(*MYC*) into CEFs. By contrast, transforming alleles of c-*src* due to spontaneous mutation arose at a sluggish pace in a similar setting (28). Second, we have shown that transforming stocks of RSV(*MYC*) can be generated with great consistency without the spreading infection that facilitates mutagenesis of the retroviral genome. Third, other workers have used nucleotide sequencing to document the ability of normal *MYC* to elicit at least morphological transformation of avian fibroblasts (no other parameter of transformation was investigated) (15).

The Potency of *MYC* in Neoplastic Transformation. It appears from our data that the biological potency of *MYC* is constrained relative to that of v-*myc*: the phenotypic changes elicited in CEFs by *MYC* were less extreme than those elicited by v-*myc*, even though the genes were expressed in approximately equal abundance, and tumorigenicity by *MYC* required a longer incubation period than that by v-*myc* and was less frequent. The alleles of v-*myc* with which we worked contain several point mutations that cause substitutions of amino acids (29). Previous work has shown that these mutations enhance the biological potency of *MYC* in a combinatorial manner when tested in various murine or avian cells (refs. 5, 15 and 16; A. Bruskin and J.M.B., unpublished work). It seems likely that increased potency for transformation is a general property of v-*myc*, resulting from repeated selection for highly tumorigenic strains of virus carrying the gene.

The relatively protracted incubation period for tumorigenesis by *MYC* might represent the requirement for additional etiological events. A similar explanation has been offered for the delay in tumorigenesis elicited by transgenes of *MYC* in mice (11–13). Although we cannot refute this explanation of our findings, we note that tumorigenesis by some retroviral oncogenes is no more expeditious than that by *MYC* (30). Moreover, tumorigenesis in chickens that demonstrably requires several events usually takes place over the course of months rather than weeks (14).

The Spectrum of Transformation by *MYC*. In cell culture, *MYC* and v-*myc* both transform embryonic fibroblasts and monocytes–macrophages. By contrast, tumorigenicity by *MYC* may affect a narrower spectrum of tissues than does v-*myc*: in our hands, the former has elicited only leukemia, whereas the latter also causes sarcomas and highly malignant hepatomas (25). Moreover, the leukemic cells elicited by *MYC* (granulocytes) are more mature than those elicited by v-*myc* (myelocytes). There are at least two possible explanations for these discrepancies. First, the mutations of v-*myc* noted above may affect the specificity as well as the potency of its biological activity. Second, the RSV vector that we have used to deliver *MYC* to cells may have a different host range for entry or gene expression than that of MC29 or OK10. Both of these explanations are accessible to experimental test.

The Pathogenicity of *MYC* in Human Tumors. A variety of genetic damage afflicts *MYC* in human tumors, generally causing augmented and/or sustained expression of the gene (3, 4). Insertion of *MYC* into a retroviral vector has similar consequences and, thus, provides a physiological facsimile of the damage in human tumors. The tumorigenicity of this facsimile, demonstrated here, sustains the view that deregulation of an otherwise normal allele of *MYC* can contribute to tumorigenesis in natural settings. Similar conclusions have been reached by the use of mice bearing transgenic *MYC* (11–13).

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