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Biological activities of v-*myc* and rearranged c-*myc* oncogenes in rat fibroblast cells in culture

(immortalization/transformation/serum dependence)

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ABSTRACT **Two distinct forms of the *myc* oncogene were assayed for their ability to induce, in cultured rat fibroblast cells, the alterations of cellular growth controls observed upon transfer of the gene of polyoma virus encoding only the large T protein (*plt*). Both of these rearranged *myc* genes and the *plt* gene had been previously shown to cooperate with *ras* oncogenes for transformation of rat embryo fibroblasts (REF) and were thought to induce the same early step ("immortalization") of the tumoral transformation pathway. We now report that these two different oncogenes elicite the same response in the following biological assays: (*i*) reduction of the requirements in serum factors for growth in culture of cells of the established FR3T3 line; (*ii*) expression of transformed properties in low serum medium after transfer into FR3T3 cells expressing only the middle T protein of polyoma virus (MTT lines); (*iii*) conferring on REF cells the ability to grow as clonal colonies after seeding at low cell density; (*iv*) conferring on REF cells the ability to grow continuously in cell culture. These congruent phenotypes suggest that the activities of the large T and myc proteins result in the induction of the same molecular events. These results also provide simple biological assays and selective systems for oncogenes of the *myc* class.**

Two steps could be genetically defined in the transformation by polyoma virus of primary rat embryo fibroblasts (REF) (see ref. 1 for review). A first step is mediated by modified viral genomes (2), which express either the complete large T protein (105 kDa) (3) (*plt* gene) or only its amino-terminal region (4, 5). REF cells expressing these proteins were shown to acquire an unlimited potential for growth in culture ("immortalization") and the ability to grow in the presence of low concentrations of serum factors. Unlike the original REF cells, the permanent lines established after transfer of *plt* could be subsequently transformed to a tumorigenic state with the gene encoding the viral middle T protein. A similar two-step mechanism was demonstrated in adenovirus-induced transformation. The *Ela* transcription unit is required for complete transformation by the *Elb* genes and, by itself, is able to immortalize primary culture cells (6). These multigenic models of cell transformation were recently extended by Land *et al.* (7) and by Ruley (8) to cellular oncogenes: neither *ras* genes cloned from human tumors nor *myc* genes cloned from the genome of MC29 virus or from tumoral mouse cells are able to transform primary rat fibroblasts, but combinations of *myc* and *ras*, of *plt* and *ras*, and of *Ela* and *ras* were efficient in inducing a tumorigenic state.

These results may suggest that the proteins encoded by the *myc*, *Ela*, and *plt* genes act on common cellular target(s) to induce the same first step in oncogenic transformation. This assumption appears plausible, as these three genes encode nuclear proteins (at least two of them with DNA-binding properties) (9–17). Alternatively, considering that an extended growth potential is definitely required in experiments based on the selection in culture of tumorigenic derivatives from REF cells, we cannot exclude the fact that this property could be acquired through distinct, possibly unrelated, mechanisms. Cooperation of *myc* and *plt* with the *ras* oncogenes might in this case be purely coincidental: cells expressing *myc* and *plt* would be different in other properties than immortality and would not correspond to a unique step in tumorigenesis. Such a situation was already suggested by the comparative study of the spontaneously established 3T3 lines (18) and of the *plt*-immortalized lines, as they consistently differ in a series of critical properties, such as their dependence on serum factors (refs. 4 and 5; this report).

A more complete comparison of the growth properties of cells expressing either the *plt* or a *myc* oncogene, and of 3T3 cells, would therefore be informative. It might, in addition, provide biological assays, and possibly selective conditions, for genes of the *myc* class. We compared the phenotypes induced in fibroblast cultures by the polyoma *plt* gene and by two different forms of the *myc* gene, the *gag–myc* fused gene from MC29 virus (v-*myc*) and rearranged c-*myc* sequences from a mouse plasmacytoma line (7). Immortalization after transfer of the various genes was monitored by two different assays: plating efficiency of REF cells at low input density and long-term growth in mass culture. Serum independence was assayed by colony formation in medium containing 0.5% newborn calf serum by cells of the highly serum-dependent FR3T3 line (19). An independent assay for serum effects was provided by the observation that transfer of the *plt* gene into cells that express only the middle T protein of polyoma virus (MTT cells) leads to focus formation and growth in suspension in low serum medium (4). In all these assays, congruent phenotypes, clearly distinct from that of FR3T3 cells, were observed for the *plt* and for the rearranged *myc* genes.

MATERIALS AND METHODS

Cell Cultures. Cell lines, preparation of rat embryo cultures, and culture conditions have been described (4, 5).

Cloned Oncogenes. (*i*) *Polyoma virus genes.* Plasmid pPyLT1 carries the complete form of the *plt* gene (deletion of the large T intron) encoding the full-sized (105-kDa) polyoma virus large T protein, and plasmid pLT214 only encodes its truncated form (amino-terminal 40 kDa expressed from the *Bam*HI/EcoRI fragment of the gene) (3, 4).

(*ii*) *Cellular oncogenes.* Plasmid pSVc-*myc*-1 carries a rearranged form of the mouse c-*myc* gene cloned from a plasmacytoma cell line and pEJ6.6, the human Ha-*ras* gene; pSVv-*myc* carries the viral *gag–myc* fused gene; pSVv-*myc*-del is a deleted form of the latter lacking the *myc* coding sequence (7): these plasmids were kindly provided by R. A.

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Abbreviation: REF, rat embryo fibroblasts.

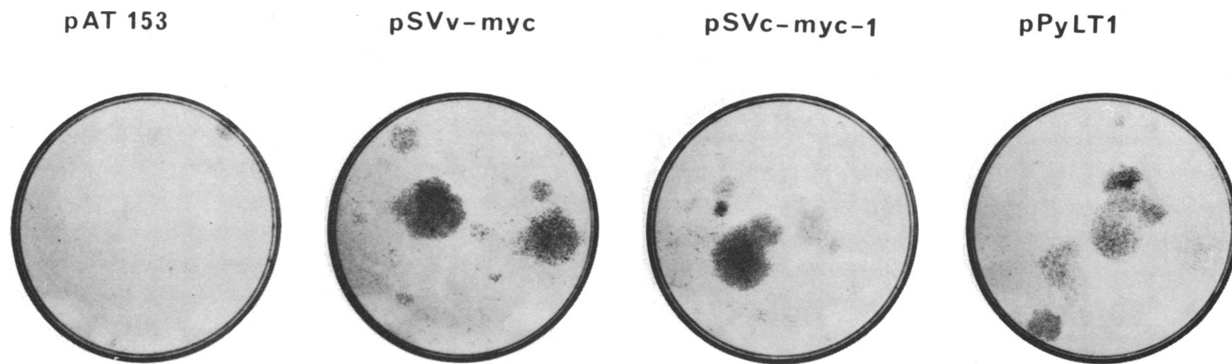

FIG. 1. Plating efficiency of REF cells after transfer of the *plt* and *myc* oncogenes. The indicated plasmid DNA was transferred into primary REF cells by the Ca^{2+} transfection technique (5 μg of DNA per plate); 24 hr later, cells were trypsinized and seeded (1000 cells per 60-mm plate) in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum (GIBCO). Plates were stained with Giemsa after 5 weeks.

Weinberg. Vectors are pBR322 for the *myc* and *ras* genes and a deleted form of pBR322, pAT153 (2), for the polyoma virus genes.

Gene Transfer Techniques. Plasmid DNA was introduced into cells in culture by using either the protoplast fusion technique (20) or the calcium phosphate transfection procedure (21). For the latter, 5 μg of plasmid DNA was used per 2–5 $\times 10^5$ cells in a 6-cm Petri plate, and the DNA precipitate was left for 24 hr in contact with the cell layer. The cells were then washed and fresh medium was added. The next day, cells were trypsinized and seeded at the required densities.

RESULTS

Immortalization Potential of the v-*myc* and Rearranged c-*myc* Genes. (*i*) *Colony formation by REF cells*. Cells of established lines, unlike primary culture cells, can produce colonies after seeding at low cell density (18). We used this assay previously to show that the *plt* gene is able to confer on REF cells an indefinite growth potential in culture (5). The same result was observed after transfer of the v-*myc* and rearranged c-*myc* oncogenes (Fig. 1 and Table 1). The efficiency of colony formation after seeding at cell densities in the range of 1000 cells per 6-cm Petri plate was the same as with the polyoma *plt* gene.

(*ii*) *Long-term growth in mass culture*. After transfer of the *myc* and *plt* genes under the same conditions as in the experiment described in Fig. 1, cells were initially seeded at a density of 5×10^3–10^4 cells per 6-cm Petri plate and further propagated by serial transfers at a 1:4 dilution when the cultures reached confluency. As expected, control cultures that had received either no DNA or plasmid pAT153 could only grow for 4–5 additional generations. By contrast, cultures treated with the *myc* genes could be successfully maintained for at least 20 generations and are still actively growing. The same ability for long-term growth was observed for cells picked from the colonies grown at low cell densities after treatment with the *myc* or *plt* genes, but not, as previously observed (5), for the few colonies observed for untreated cells or cells transfected with vector DNA.

Assay for Serum-Independent Growth of FR3T3 Cells. We reported previously that clones able to grow in low serum medium (0.5%–1% newborn calf serum) could be derived from cells of the highly serum-dependent FR3T3 line after transfer of the full-sized or truncated *plt* genes (4). The same experiment was carried out using the two *myc* oncogenes. Although a background plating efficiency in the range of 0.5–1 $\times 10^{-3}$ was observed for the control FR3T3 cells, this value was increased about 3-fold after transfer of either *plt* or *myc* genes, colonies being in this case morphologically different from the controls and of a larger size (Fig. 2 and Table 1). Cells derived from such large colonies after transfer of *myc* genes could be successfully maintained in culture in the presence of 0.5% newborn calf serum.

Complementation of MTT Cells at Low Serum Concentration. MTT lines were derived from FR3T3 cells by transformation in medium with high serum concentration with the polyoma virus middle T only gene. They exhibit the typical growth properties of transformed cells in medium with high serum concentration, but they revert to an apparently normal growth control in the presence of only 0.5% serum. Transfer of the genes encoding the full-sized or the truncated form of the large T protein led to the appearance of foci or agar colonies in medium with low serum concentration (4). The same complementation experiment was carried out by transferring the v-*myc* and c-*myc* genes into FR3T3-MTT4

Table 1. Plating efficiencies of REF cells in medium with high serum concentration and of FR3T3 cells in medium with low serum concentration after transfer of the *plt* and *myc* oncogenes

Transfer of plasmid*	Immortalization assay: cloning efficiency of REF cells (10% calf serum)	Cloning efficiency of FR3T3 cells (0.5% calf serum)	
		Colonies, total	Large colonies†
None	0, 1‡	17	0, 1
pAT153	0, 0, 0, 0, 1, 1‡	15, 15, 17, 20, 21	0, 0, 1, 3
pPyLT1	1, 2, 3, 3, 3	35, 35, 40, 40, 45	15, 17, 19, 20
pSVv-*myc*	2, 2, 3, 3, 3	44, 45, 48, 48, 50	17, 20, 21, 22
pSVc-*myc*-1	2, 2, 3, 3, 3	35, 40, 45, 52	20, 25, 34

*The indicated plasmid DNA was transferred by protoplast fusion (20); 24 hr later, cells were trypsinized and seeded at a density of 1000 cells per 60-mm plate. The number of colonies on each plate, counted after Giemsa staining 5 weeks later, is indicated.

†See text and Fig. 2.

‡Cells picked from these colonies could not be further propagated in culture (see text).

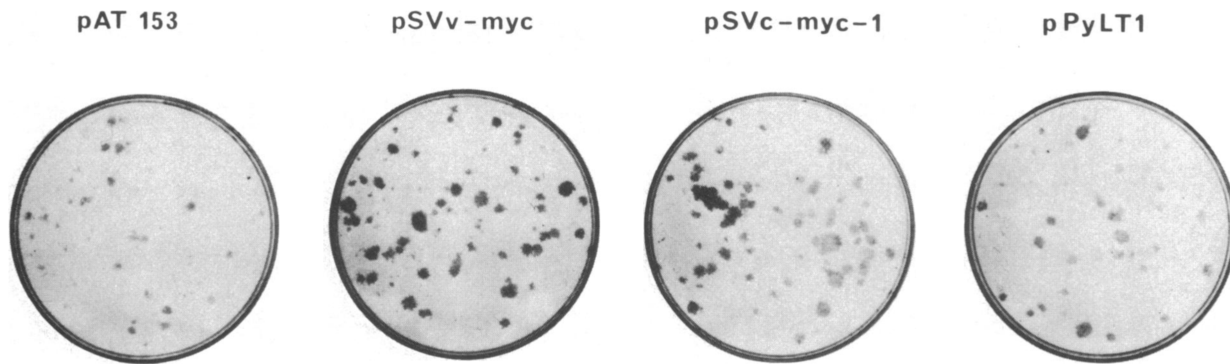

FIG. 2. Colony formation in medium with low serum concentration by FR3T3 cells after transfer of the *plt* and *myc* oncogenes. FR3T3 cells were transfected with the indicated plasmid DNA and further treated as described in the legend of Fig. 1. In this case, cells were plated in medium supplemented with 0.5% newborn calf serum.

cells and selecting for colonies in agarose medium supplemented with 0.5% newborn calf serum. As shown in Table 2, the same efficiency of colony formation ($0.5–1 \times 10^{-3}$) was observed with the plasmids encoding the myc and the large T proteins, with background values in the range of 10^{-5} for cells that received either no DNA or vector DNA. A series of colonies resulting from complementation with *myc* genes were picked and further grown in medium with low serum concentration. As MTT cells complemented with *plt*, they exhibited the morphological changes and growth at high density characteristic of fully transformed cells.

Cooperation of the *myc* and *plt* Genes and of the *ras* Gene for Transformation of REF Cells. It was shown by Land et al. (7) that the truncated form of the *plt* gene encoding only the 40-kDa amino-terminal part of the protein (4) cooperates with *ras* genes for transformation of secondary REF cells. These authors could not study, in this respect, the activity of the full-sized (105-kDa) polyoma protein because of a toxic effect after gene transfer at high multiplicity by the Ca^{2+} transfection method. Although this toxicity of high intracellular concentrations of large T could be observed in many instances (unpublished results), it was not apparent when the complete *plt* gene was transferred by the protoplast fusion method (4, 5). We therefore carried out similar transformation experiments on tertiary REF cultures by polyethylene glycol-induced fusion with mixtures of protoplasts carrying the pEJ6.6 plasmid (Ha-*ras*) and of protoplasts carrying either pPyLT1 or pLT214 (full-sized or truncated large T) or the *myc* plasmids. Transfer into tertiary culture cells presents the additional advantage, as compared with primary or secondary cultures, of having the control cultures show a lower background of cells growing at high density, thus allowing a direct assay by focus formation without selection for a cotransferred resistance gene. As exemplified in Fig. 3, results demonstrated that the genes encoding the 105-kDa large T protein and the 40-kDa truncated forms exhibited comparable efficiencies of induction of foci when cotransferred with the *ras* gene and that this efficiency was again comparable to that of the two *myc* genes in the same assay (5–20 foci per 10^5 cells).

Table 2. Complementation of FR3T3-MTT4 cells for colony formation in agarose medium at low serum concentration

Fusion of FR3T3-MTT4 cells with protoplasts carrying plasmid	Oncogene	Colonies per 10^5 cells	
		0.5% serum	10% serum
pBR322	None	0–1	$>5 \times 10^4$
pPyLT1	Polyoma *plt*	55–70	NT
pSVv-*myc*	MC29 *gag–myc* (v-*myc*)	85–95	NT
pSVc-*myc*-1	Rearranged c-*myc*	65–80	NT
pSVv-*myc*-del	Deleted MC29 v-*myc*	0–1	NT
Untreated MTT4 cells	—	0–1	$>5 \times 10^4$

The indicated plasmid DNA was transferred into FR3T3-MTT4 cells (4) by the protoplast fusion technique (20); 24 hr later, the cells were trypsinized and seeded (10^5 cells per 60-mm Petri plate) in agarose medium (4) supplemented with the indicated concentration of newborn calf serum (GIBCO). Colonies were counted after 5 weeks. Extreme values observed (3–5 identical plates) are indicated. NT, not tested.

DISCUSSION

We established previously that transfer of the *plt* gene of polyoma virus into rodent embryo fibroblasts results in the expression of a series of phenotypic characters. Assays were developed for three groups of properties (4, 5): (*i*) immortalization in cell culture, (*ii*) growth in medium with low serum concentration of normal cells and anchorage-independent growth of cells transformed with the polyoma *pmt* gene, and (*iii*) cooperation with the *pmt* gene for the transformation of primary embryonic cells. A coordinated expression of these various characters was always observed in both mouse and rat cells (unpublished results), and by using either the complete *plt* gene (105-kDa protein) or the truncated form encoding only the amino-terminal 40% of large T. It was independently reported that both the polyoma *plt* and the *myc* genes cooperate with an activated *ras* oncogene for transformation of primary rat fibroblasts, and thus suggested that expression of the *myc* protein might confer immortality on these cells (7).

We asked whether all the changes observed in rat fibroblast cells after transfer of the polyoma *plt* gene were also expressed after transfer into these cells of the same two *myc*-related genes that were shown to complement Ha-*ras* for cell transformation, the MC29 *gag–myc* fusion gene, and rearranged c-*myc* sequences from a mouse plasmacytoma line (7). We could confirm, using a different assay (focus formation without selection for a cotransferred resistance gene), that the full-sized *plt* gene, as its amino-terminal truncated form, and the *myc* genes allowed efficient transformation of primary cells by the *ras* oncogene. Both the *myc* and *plt* genes conferred on REF cells an unlimited potential for growth in culture, and decreased serum requirements were evidenced in the two assays previously used, colony formation by the highly serum-dependent FR3T3 cells in the presence of 0.5% calf serum and complementation for growth in suspension at the same low serum concentration of the partially transformed MTT cells.

As done by others under similar circumstances (7), we assumed that in all cases, the observed effects are due to the activity of the proteins encoded by the transferred genes. Experiments to check directly for the expression of the

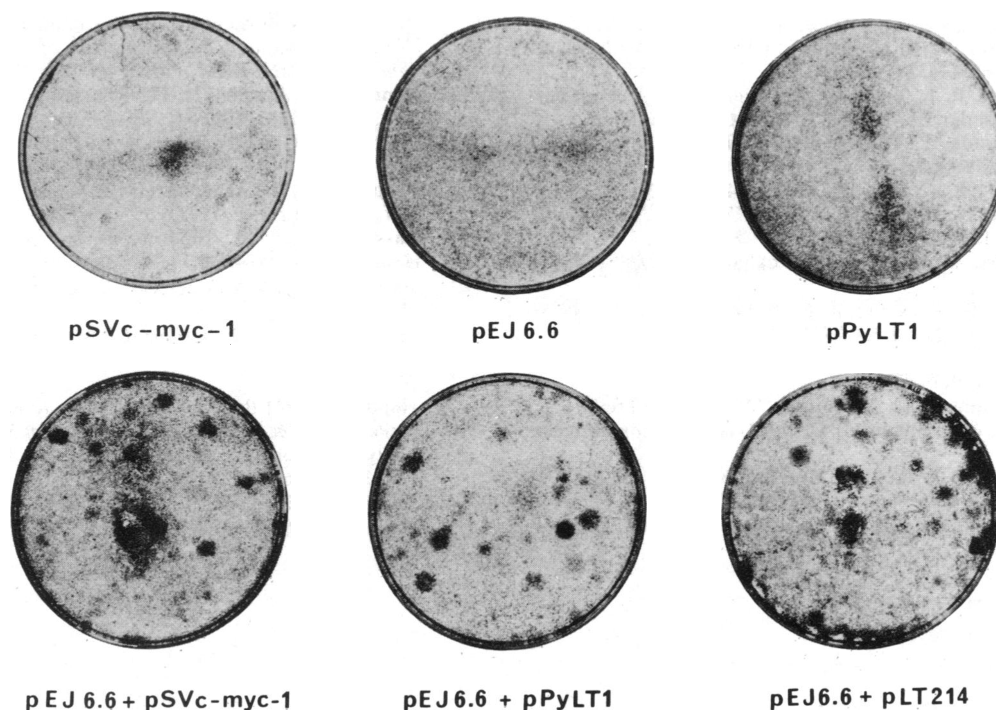

FIG. 3. Focus formation by REF cells after transfer of the full-sized and truncated *plt* genes and of *myc* genes in combination with Ha-*ras*. Secondary REF cells were trypsinized and plated at a density of 2×10^5 cells per 35-mm plate; 24 hr later, the indicated plasmid DNAs were transferred into cells of these tertiary cultures by the protoplast fusion technique (20), using either 2×10^9 protoplasts per plate for single plasmid transfer or a mixture of 1×10^9 protoplasts of each type for combinations of two plasmids. Cells were transferred the next day at a density of 1.5×10^5 cells per 60-mm plate in medium containing 10% newborn calf serum. Plates were stained with Giemsa after 4 weeks.

transferred *myc* genes are made technically difficult because of the known low levels of expression of these genes and by the fact that expression of the transferred genes has to be distinguished from that of the endogenous c-*myc* gene. In the case of the polyoma large T protein, where there is no problem of endogenous synthesis of a similar product, with biochemical assays and *ts* mutants readily available, we demonstrated previously that the ability to grow in medium with low serum concentration as well as continuous growth in culture requires the continuous expression of the viral gene (4, 5).

Changes induced by oncogenes of this group in cells in culture are not likely to be limited to these properties. It was, for instance, recently observed (C. Cerni, personal communication) that the increased frequency in sister chromatid exchange characteristic of polyoma virus-transformed cells depends on the expression of the large T protein and that transfer of the *plt* gene makes normal cells sensitive to the transforming effect of tumor promoters (G. Connan, personal communication).

In addition to a more complete description of the changes in cell growth regulation induced by oncogenes of the *myc*–*plt* family, these results provide quantitative assays for their biological function(s). It will, for instance, be possible to compare in these assays the efficiency of nonrearranged c-*myc* sequences, of the various rearranged forms observed in tumor cells (for review, see ref. 22), and of mutant and recombinant genes prepared *in vitro*. Some of these assays, such as complementation of MTT cells in medium with low serum concentration, may also provide selective procedures for the search of new oncogenes acting at the same step in tumorigenesis. The background level of the MTT complementation assay is low enough for the detection of rare events. More studies are required, however, before using such selective systems in transfection experiments with genomic tumor DNA, because the efficiency of DNA uptake by cells of the presently available lines is limited as compared with that of the currently used NIH3T3 cells (unpublished results).

The tight correlation observed between a series of phenotypic characteristics after transfer of apparently unrelated genes strongly suggests that expression of these genes results in the same primary molecular event(s). Both *myc* and *plt* gene products are nuclear proteins that bind DNA with high affinity (9–17). The polyoma large T protein, as the homologous simian virus 40 protein, is a *trans*-acting regulatory protein that modulates the expression of viral genes (for review, see ref. 23; ref. 24). One possible group of hypotheses would be that the myc and large T proteins regulate either the same subset or overlapping groups of cellular genes. Another type of model may be deduced from recent observations (25, 26), indicating that the expression of the c-*myc* gene in normal cells is correlated with specific events in the cell cycle. Effects observed upon artificial addition of exogenous c-*myc* sequences by transfection might be the results of their constitutive expression, changes in the neighboring nucleotide sequences leading to escape from the physiological controls. This result might also be achieved by the translocations observed in many tumors (for review, see ref. 22) and by a specific regulatory effect exerted by the large T protein on the expression of the c-*myc* gene.

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