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Cooperation between the Polyomavirus Middle-T-Antigen Gene and the Human c-*myc* Oncogene in a Rat Thyroid Epithelial Differentiated Cell Line: Model of In Vitro Progression

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Two rat thyroid epithelial differentiated cell lines, PC Cl 3 and PC *myc*, were infected with the polyoma murine leukemia virus (PyMLV) carrying the Middle-T-antigen gene of polyomavirus. After infection, both cell lines acquired the typical markers of neoplastic transformation; however, the PC *myc* cells showed a greater malignant phenotype. Furthermore, the thyroid differentiated functions were completely suppressed in PC *myc* cells transformed by PyMLV, whereas they were, at least partially, retained in PC Cl 3 cells transformed by PyMLV, and in particular, thyroglobulin synthesis and secretion were not affected at all. Since no differences in the expression of the middle-T-antigen gene were observed in the two PyMLV-transformed cell lines, the different properties shown by these two infected cell lines must be ascribed to the expression of the c-*myc* oncogene.

Studies of chemical carcinogenesis, as well as epidemiological analysis of malignancies in humans, strongly suggest that neoplastic transformation is a multistage process (2, 3, 5, 6, 13, 14). Furthermore, the observation that two different oncogenes are required in concert for malignant conversion of nonestablished rat cells (23, 25, 31) confirms this point of view. However, most of the studies concerning cooperation between oncogenes have so far been performed with primary cultures of undifferentiated fibroblasts. A permanent epithelial differentiated cell system could provide new insights about the role and modalities of cooperation of these genes in transformation and differentiation. In this study, we used two permanent epithelial thyroid cell lines, PC Cl 3 and PC *myc*. The PC Cl 3 cell line (15) derives from Fischer rat thyroid and retains in vitro the typical markers of thyroid differentiation (i.e., thyroglobulin [TG] synthesis and secretion, ability to trap iodide from the culture medium, and dependence of growth on six growth factors, including thyrotropin, the physiological thyroid stimulator). The PC *myc* cell line (15) was obtained by transfection of the PC Cl 3 cells with the plasmid pMCGM1 (32), which carries the human *myc* oncogene (34). Both thyroid epithelial cell lines were infected with the polyoma murine leukemia virus (PyMLV), which carries the polyomavirus middle-T-antigen gene (11).

The normal and the infected cell lines were cultured in modified F12 medium supplemented with 5% calf serum (Flow Laboratories, Inc., McLean, Va.) and six growth factors (thyrotropic hormone, hydrocortisone, insulin, transferrin, somatostatin, and glycyl-histidyl-lysine [Sigma Chemical Co., St. Louis, Mo.]). The epithelial infected cell lines used in this work were designated as follows: PC PyMLV, PC Cl 3 cells transformed by PyMLV; PC *myc* PyMLV, PC Cl 3 *myc* cells transformed by PyMLV; PC Py-T, a cell line established in vitro from a PC PyMLV-induced tumor; Pc *myc* Py-T, a cell line established in vitro from a PC *myc* PyMLV-induced tumor; PC Homer 6 PyMLV, PC Cl 3 cells transfected with Homer 6 (35) and then infected with PyMLV. FRT-Fibro cells are Fischer rat thyroid fibroblasts, and FRT-Fibro Py cells are FRT-Fibro cells transformed by PyMLV. Transfections were performed by described procedures (20).

PC Cl 3 and PC Cl 3 *myc* cell lines were infected with PyMLV essentially as described previously (8, 16–18). The PyMLV preparation contained 2×10^6 focus-forming units per ml when titrated on NIH 3T3 cells.

RNAs purified from cultured cells by a modification of the guanidine hydrochloride extraction method as described elsewhere (1) were analyzed by dot-blot and Northern (RNA) blot hybridizations performed as described previously (27). Nick translations of cloned cDNA TG fragments (9), of plasmid pMT-1 (35) containing the middle T antigen gene, and of plasmid pMCGM1 were performed by using the Amersham nick translation kit as described previously (27).

Immunoprecipitation analysis of the polyomavirus middle T protein synthesized in PC PyMLV and PC *myc* PyMLV cells was performed by labeling the cell lines, grown until nearly confluent, for 4 h with 200 μCi of [^{35}S]methionine per ml (600 Ci/mmol; 1 Ci = 3.7 × 10 Bq) (Amersham Corp.). Extracts were prepared and analyzed after immunoprecipitation with anti-polyomavirus middle-T-antigen antibodies (10) as described previously (8).

Infection of the PC Cl 3 (Fig. 1A) and PC *myc* (Fig. 1C) cell lines with PyMLV was followed after 4 to 5 days by readily detectable morphological changes that were different in these two cell lines. In fact, for the PC Cl 3 cells, the morphological changes were not dramatic and folliclelike structures appeared, that is, the cells still kept an epithelial morphology and grew as islets (Fig. 1B). In the infected PC *myc* cells, the changes were dramatic. The cells lost their

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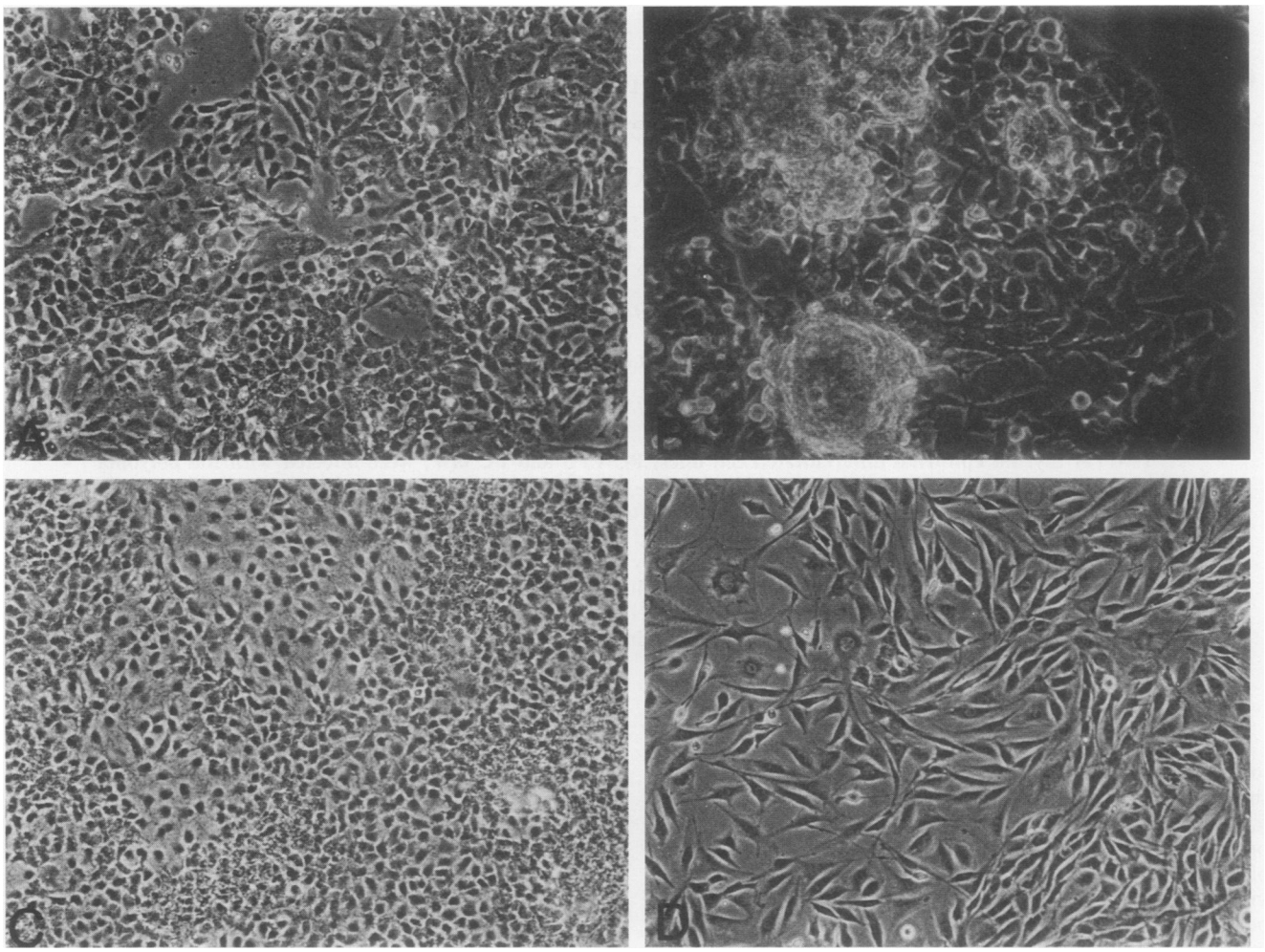

FIG. 1. Transformation of PC Cl 3 and PC Cl 3 *myc* with PyMLV. (A) Uninfected PC Cl 3 cells (magnification, ×150). (C) Uninfected PC Cl 3 *myc* cells (magnification, ×150). (B) PC Cl 3 cells infected by PyMLV (magnification, ×150). (D) PC Cl 3 *myc* cells infected by PyMLV (magnification, ×150).

typical epithelial morphology and became spindle shaped or round and grew in a disordered way (Fig. 1D).

After infection, three independent cell clones from each infected cell line were isolated by morphological criteria and analyzed for the expression of the differentiated and transformed phenotypes. The data, shown below, represent the average of the results obtained with different clones, even though no significant differences were observed among homologous clones. Moreover, the total infected cell population, selected for the capability to grow without the addition of the six growth factors, did not show any significant differences from the analyzed cell clones.

After infection, a dramatic change in the growth factor requirement was observed. The growth of the PC PyMLV cell line was still at least partially dependent on the six growth factors for optimal growth, but the cells also showed

TABLE 1. Analysis of the transformation markers of PC Cl 3 AND PC Cl 3 *myc* infected by PyMLV

Cell type[a]	Colony-forming efficiency (%)	Tumor incidence[b]	Latency period	Histological type of tumor
PC Cl 3	0	0/4		
PC Cl 3 *myc*	0	0/4		
PC PyMLV	1–2	8/8	3–4 weeks	Differentiated carcinoma
PC Homer 6 PyMLV	1–2	3/3	3–4 weeks	Differentiated carcinoma
PC *myc* PyMLV	30	15/15	5–7 days	Undifferentiated carcinoma
PC PyMLV *myc*[c]	1–2	3/3	3–4 weeks	Differentiated carcinoma
FRT-Fibro	0	0/5		
FRT-Fibro PyMLV	35	4/4	2–3 weeks	Fibrosarcoma

[a] See the text for explanation of cell designations.
[b] Assayed by injecting 2×10^6 cells into athymic mice (4 to 6 weeks old). Number with tumor/number injected.
[c] A clone of PyMLV-infected PC Cl 3 cells was transfected with the plasmid pMCGM1.

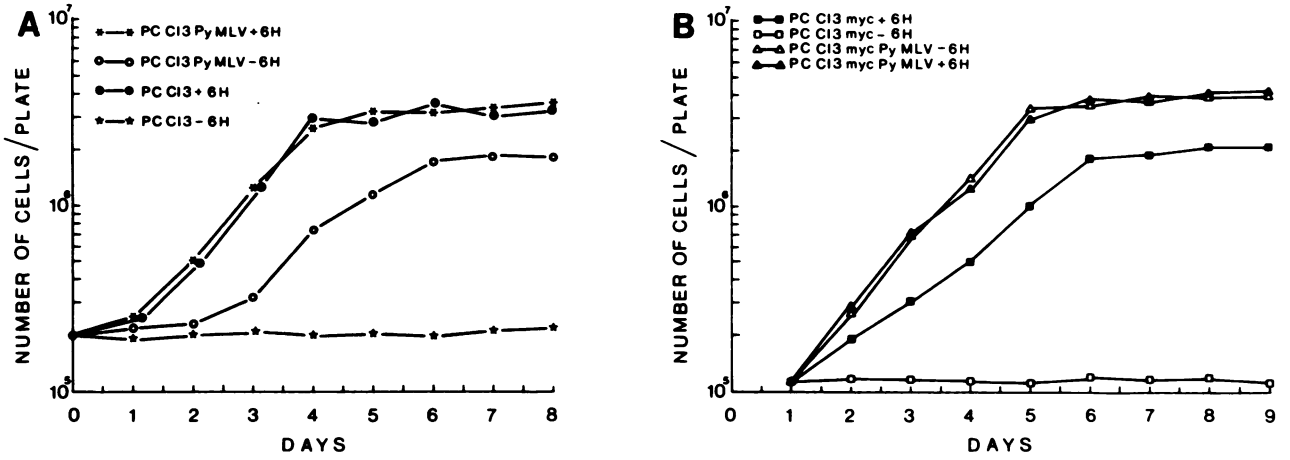

FIG. 2. Growth curves of uninfected PC Cl 3 and PC Cl 3 *myc* cell lines and of the same cells infected with PyMLV and grown in the presence or absence of the six growth factors (6H). (A) PC Cl 3 and PC PyMLV cells grown in the presence or absence of the six growth factors. (B) PC Cl 3 *myc* and PC *myc* PyMLV cells grown in the presence or absence of the six growth factors.

the capability to grow without the factors (Fig. 2). In contrast, the growth of uninfected cells was completely arrested without the addition of the six growth factors to the culture medium. No significant differences in the doubling time and the saturation density were observed between PC PyMLV and PC Cl 3 cells. In contrast, the PC *myc* PyMLV-infected cell lines were no longer dependent on the six growth factors for their growth. These cells also showed a decreased doubling time and an increased saturation density. The acquisition of anchorage independence by the infected cell lines was tested by the assay of growth in semisolid medium performed by the technique described previously (26). The data (Table 1) demonstrate that both infected cell lines were capable of forming colonies in a semisolid medium, but the colony-forming efficiency was higher for the PC *myc* PyMLV cells. The tumorigenicity of PyMLV-infected cell lines was tested by injecting 2×10^6 cells into athymic mice. The mice were palpated at the inoculation site twice a week. Tumor excision, fixation, and staining were performed as described previously (16). Tumors appeared at the inoculation site after injection of both infected cell lines, but the latency period was much shorter for the PC *myc* PyMLV cell line (7 to 10 days) than for the PC PyMLV cells (4 weeks). Moreover, the tumors induced by PC PyMLV were quite stable, not exceeding 1 cm, whereas those induced by PC *myc* PyMLV were very invasive, reaching very large dimensions (4 to 5 cm in diameter) and leading to the death of the animals.

Histological analysis of the excised tumors induced by PC PyMLV cells showed typical epithelial cells with the basal membrane also forming some glandlike structures. These tumors may be considered well-differentiated carcinomas. In contrast, those induced by PC *myc* PyMLV (Fig. 3) did not show any glandlike formation; even though typical epithelial cells could be observed, the basal membrane was absent. There were no evidences of morphological differentiation, and a high number of mitoses was observable. Because of these features, these tumors may be classified as undifferentiated carcinomas. Cell lines established from the tumors are morphologically identical to the injected cells, and the PC Py-T cells (established from the tumors induced by the PC PyMLV cells) still synthesized and secreted TG, as demonstrated by dot-blot hybridization (data not shown) and radioimmunoassay (Table 2).

The transformed cells were assayed for the ability to produce and secrete TG by a radioimmunoassay and by measuring TG mRNA levels by dot-blot hybridization with a probe specific for TG mRNA. The results (Table 2) indicated that the PC *myc* PyMLV cell line lost the capability to secrete TG, whereas this differentiated property remained completely unaltered in the PC Cl 3 cells transformed by PyMLV. Dot-blot RNA hybridization (Fig. 4) demonstrated the absence of TG-specific mRNA in PC *myc* PyMLV cells, whereas no significant reduction was present in the PyMLV-infected PC Cl 3 cells, as confirmed by densitometric analysis. The same filters were hybridized with an actin probe that showed that the amounts of RNA spotted were almost identical (data not shown). These results are therefore consistent with the interpretation that TG production is blocked in PC *myc* PyMLV at the mRNA synthesis level.

We also tested the ability of PyMLV-infected cell lines to concentrate radioactive iodide (a property unique to thyroid cells in vivo and in vitro). The results (Table 2) demonstrated that this property is completely lost in the PyMLV-infected PC *myc* cells, whereas a certain capability to trap iodide (about 15%) is still maintained by the PC PyMLV cells.

TABLE 2. Differentiated thyroid functions in PC Cl 3 and PC Cl 3 *myc* infected by PyMLV

Cell type	Iodide uptake[a]	TG production[b]
PC Cl 3	16.5	600
PC Cl 3 *myc*	4.2	570
PC PyMLV	1.8	650
PC Homer 6 PyMLV	1.9	700
PC *myc* PyMLV	0.3	ND[c]
PC PyMLV *myc*[d]	1.2	570
Rat-2	0.4	ND

[a] Counts per minute of ^{125}I incorporated $\times 10^6$ cells, expressed as percentage of the total ^{125}I added to the medium.

[b] Expressed as nanograms of TG produced per milliliter $\times 10^6$ cells as determined by radioimmunoassay.

[c] ND, Not detectable.

[d] See Table 1, footnote *c*.

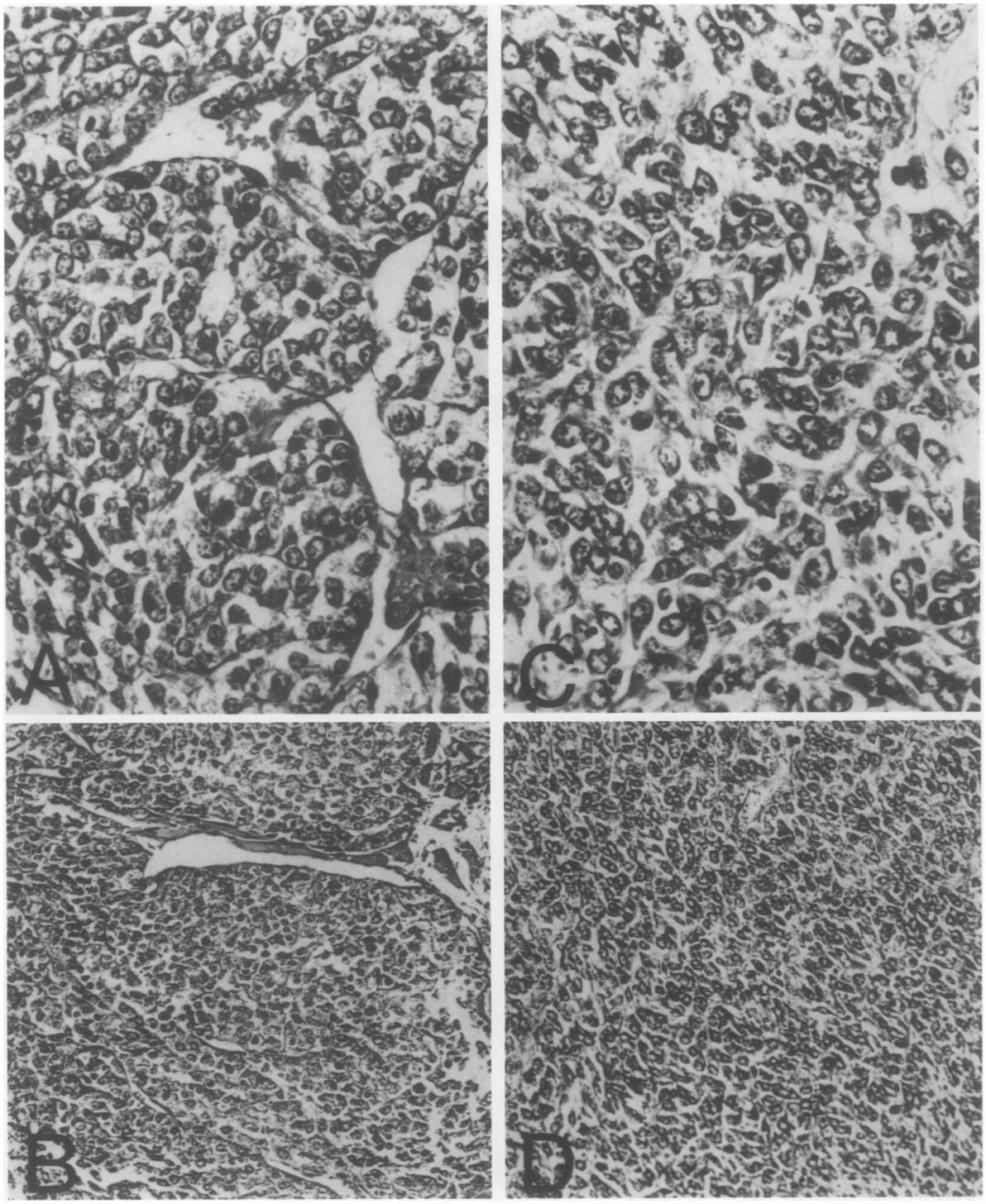

FIG. 3. (A) Histological appearance of tumors induced by PC Cl 3 cells transformed by PyMLV (magnification, ×450). (B) Same as panel A (magnification, ×240). (C) Histological appearance of tumors induced by PC Cl 3 *myc* cells transformed by PyMLV (magnification, ×450). (D) Same as panel C (magnification, ×240).

The hypothesis that differences between PC PyMLV and PC *myc* PyMLV in the expression of the differentiated and transformed phenotypes could be explained by a different quantitative expression of the middle-T-antigen gene was evaluated by dot-blot RNA and Northern hybridizations with a probe specific for the middle-T-antigen gene (35) and by immunoprecipitation with antibodies against the p56 middle T protein (10). The results demonstrated that specific mRNA for the middle-T-antigen gene is expressed at comparable levels in both PyMLV-infected cell lines (data not shown). Also, no striking differences between the two infected cell lines in the expression of the viral transforming

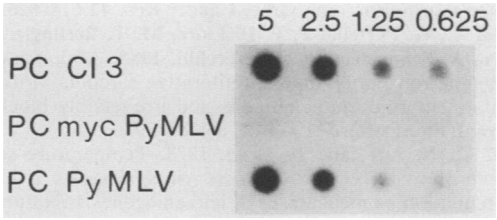

FIG. 4. Dot-blot hybridization of total RNA from transformed and normal thyroid cell lines with the 57.5 cDNA TG probe. RNA was spotted at the indicated quantities (micrograms) onto nitrocellulose filters and hybridized with 4×10^6 cpm of in vitro nick-translated TG cDNA. PC Cl 3, uninfected PC Cl 3 cells; PC PyMLV, PC Cl 3 cells infected by PyMLV; PC *myc* PyMLV, PC Cl 3 *myc* cells infected by PyMLV.

protein were observed after immunoprecipitation (Fig. 5), confirming the dot-blot RNA hybridization data.

Then we investigated the levels of expression of the human c-*myc* gene in the normal and transformed cell lines by Northern blot hybridization using a human *myc* probe (29) that is also able to detect the rat *myc* gene because of the high homology between human and rat *myc* sequences. The results shown in Fig. 6 indicate that the *myc* oncogene is expressed in the PC Cl 3 *myc* cell line and that a high increase of this expression results in PC *myc* PyMLV cells, whereas *myc* expression is almost undetectable in PC Cl 3 and PC PyMLV cells. Northern blotting, however, did not enable us to discriminate between the rat endogenous and the human exogenous c-*myc* gene expression in the PC *myc* PyMLV cells, because the mRNAs for human and rat *myc* oncogenes have the same size. Therefore, an S1 nuclease analysis of the human c-*myc* transcript was done. No expression of the human *myc* gene was detectable in PC Cl 3 cells, whereas it was clearly expressed in PC *myc* and in PC *myc* PyMLV cell lines. However, no observable differences between these two cell lines were found (data not shown), thus suggesting that the expression of the endogenous rat *myc* gene is increased in PC *myc* PyMLV cells.

To investigate whether the functions of the human c-*myc* gene and the middle-T-antigen gene of polyomavirus need to be sequentially expressed to induce the previously described effects, we transfected the PC PyMLV cell line with the plasmid pMCGM1 and the transfected cells were selected in a G418-containing medium. The cell lines so obtained showed a reduced capability to trap iodide, but they did not

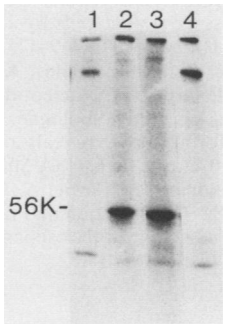

FIG. 5. The cells were labeled with [^{35}S]methionine and lysed, and samples of lysate were immunoprecipitated with antibodies versus the middle T antigen and analyzed on sodium dodecyl sulfate-polyacrylamide gels. Lanes: 1, PC Cl 3; 2, PC *myc* PyMLV; 3, PC PymLV; 4, PC *myc*. K, 10^3.

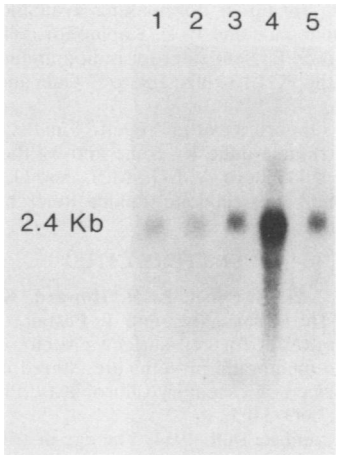

FIG. 6. Expression of human *myc* sequences in normal and PyMLV-infected cells. Autoradiograph of a Northern blot hybridized with the 1.3-kilobase (Kb) *Pst*I-*Pst*I fragment of the plasmid pRyc7.4 specific for the human *myc* gene. A 10-μg sample of total RNA was run for each cell line. Sources of RNA were as follows: lane 1, PC Cl 3 cells; lane 2, PC PyMLV; lane 3, PC Cl 3 *myc*; lane 4, PC *myc* PyMLV; lane 5, PC PyMLV plus *myc* (PC PyMLV cells transfected with the human oncogene).

acquire the same malignant properties and block of TG expression as the PC *myc* PyMLV cells did (Tables 1 and 2).

Retroviral transformation is generally associated with a block in the expression of the differentiated functions (4, 8, 12, 15, 17–19, 22). In contrast, in this study we demonstrated that transformation of rat thyroid cells by a virus carrying the middle-T-antigen gene of polyomavirus is associated with the maintenance of some differentiated functions; for instance, synthesis and secretion of TG are not affected at all in the PC PyMLV cell line, even though this cell line behaves as a transformed one. This phenomenon is not restricted to the thyroid system. It has also been described with rat adipocytes, which, after transformation with the middle-T-antigen gene of polyomavirus, were able to differentiate in vitro and to give rise to adipose tumors in vivo (24). Moreover, the PC Cl 3 cells transformed by PyMLV alone show for the first time a dissociation of the thyroid differentiation properties. In fact, while TG synthesis is not affected at all, the dependence on thyrotropin for growth is partially abolished and the capability to trap iodide is significantly reduced.

The expression of the human *myc* oncogene enhances enormously the malignancy induced by PyMLV with the complete suppression of the thyroid-differentiated functions. The effect of c-*myc* on rat thyroid cells is somewhat comparable to that of the v-*myc* gene, rearranged c-*myc* genes, and large-T-antigen gene of polyomavirus on fibroblast cells and on adipocytes (7, 28, 30). It was very interesting to observe that the tumors induced by PC PyMLV and PC *myc* PyMLV showed a very high analogy with neoplastic thyroid pathology that includes benign very well differentiated and highly malignant undifferentiated carcinomas.

This cell system may therefore represent an interesting model to study the multistep process of carcinogenesis in vitro and its relationship with the expression of the differentiated state.

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