JOURNAL OF VIROLOGY, Apr. 1988, p. 1211–1218
0022-538X/88/041211-08$02.00/0
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Vol. 62, No. 4

Transformed and Tumorigenic Phenotypes Induced by Avian Retroviruses Containing the v-*mil* Oncogene

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Received 8 September 1987/Accepted 4 January 1988

Avian retrovirus MH2 contains two oncogenes, v-*mil* and v-*myc*. We have previously shown that a spontaneous mutant of MH2 (PA200-MH2), expressing only the v-*mil* oncogene, is able to induce proliferation of quiescent neuroretina cells. In this study, we investigated the transforming and tumorigenic properties of v-*mil*. PA200 induced fibrosarcomas in about 60% of the injected chickens, whereas inoculation of MH2 resulted mainly in the appearance of kidney carcinomas. Analysis of several parameters of transformation showed that PA200, in contrast to MH2, induced only limited in vitro transformation of fibroblasts and neuroretina cells. These results suggest that v-*myc* is the major transforming and tumorigenic gene in MH2-infected cells. This low in vitro transforming capacity differentiates v-*mil* not only from other avian oncogenes, but also from the homologous murine v-*raf* gene.

The genome of avian retrovirus MH2 contains two oncogenes, v-*mil* and v-*myc*, transduced from independent loci in the chicken DNA (8, 21, 23, 37). The v-*mil* gene represents a truncated version of the chicken c-*mil* gene (19, 22) and is expressed as a 100-kilodalton polyprotein resulting from the fusion of *gag* and *mil* sequences (18). P100$^{gag\text{-}mil}$ is a cytoplasmic protein with associated serine/threonine kinase activity (7, 28) and is distantly related to the *src* family of tyrosine kinases (12). The v-*mil* gene is the avian homolog of the murine oncogene v-*raf* transduced in murine sarcoma virus 3611 (20, 24, 25, 40), which is highly transforming and tumorigenic (35). The second oncogene of MH2, v-*myc*, also present in three other independent virus isolates (MC29, CMII, and OK-10) (37), encodes a nuclear protein, p61/63^{myc}, that is translated from a spliced subgenomic mRNA (17, 29, 38).

Despite the presence of a second oncogene, the biological properties of MH2 are similar to those of MC29, CMII, and OK-10, which carry only the v-*myc* gene. All four viruses transform fibroblasts and macrophages in vitro and induce mainly carcinomas in vivo (4, 14). The role of v-*mil* in confering specific transformation properties to MH2 was assessed in two types of studies. First, MH2 was reported to be more tumorigenic in quails than MC29 (26). Second, it was shown that chicken macrophages transformed by MH2, but not by other v-*myc*-containing viruses, were able to grow in the absence of the hematopoietic growth factor cMGF (1). Based on these earlier reports, it was generally believed that v-*mil* played only an auxiliary role in the transforming properties of MH2.

The use of chicken neuroretina (NR) cells as a host system provided the first evidence for a direct effect of v-*mil* on cell growth regulation. We previously reported that a spontaneous variant of MH2, designated PA200, which carried a deletion in the v-*myc* gene, was able to induce sustained proliferation of these normally nondividing cells with the same efficiency as that of wild-type MH2 (3). However, in contrast to MH2-infected cells, NR cells infected with this mutant virus were not morphologically transformed. These differences in the cellular phenotypes induced by MH2 and PA200 led us to investigate the transforming and tumorigenic properties of v-*mil* and to examine the contribution of v-*mil* and v-*myc* to the oncogenic properties of MH2. We report that, in addition to its mitogenic effect in NR cells, PA200 induces fibrosarcomas in chickens. However, in contrast to other avian oncogenes and to the homologous murine gene v-*raf*, v-*mil* expresses only limited transforming capacity in cultured fibroblasts and NR cells.

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MATERIALS AND METHODS

Cell cultures and viruses. NR cell cultures were prepared from 7-day-old Brown Leghorn chick embryos (*gs*$^{+}$ *chf*$^{+}$) of the Edinburgh strain, as previously described (32). They were maintained and subcultured in Eagle basal medium supplemented with 5 to 10% fetal calf serum.

Chicken embryo fibroblasts (CEF) were prepared from 11-day-old embyros by standard procedures. Fibroblasts were grown in Dulbecco modified Eagle medium containing 3 to 5% newborn calf serum, 1% heat-inactivated chicken serum, and 10% tryptose phosphate broth.

Rous-associated virus type 1 (RAV-1) is a subgroup A lymphomatosis virus obtained by transfection of pRAV-1 DNA (a generous gift of J. M. Bishop) into CEF. Wild-type MH2 (RAV-1) was recovered from quail embryo fibroblasts cotransfected with pMH2-Hd (8) and helper pRAV-1 DNA. PA200-MH2 (RAV-1) is a spontaneous variant of MH2 (RAV-1) lacking the v-*myc* oncogene, which was previously characterized (3, 27). In the present studies, we used either the original isolate or virus obtained from NR cells cotransfected with a PA200 DNA clone and helper DNA pRAV-1. Both viral sources gave identical results.

MC29 (RAV-1) was obtained by superinfecting a nonproducer clone of transformed quail fibroblasts (6) with molecularly cloned RAV-1.

Assay of viral infectivity. NR cells were infected as previously described (32). The mitogenic activity of PA200 was quantitated by infecting NR cells with serial 10-fold dilutions of the virus. The mitogenic titer was defined as the reciprocal of the highest dilution inducing cell proliferation after one subcultivation and is expressed as mitogenic units per milli-

liter. Infectivity of wild-type MH2 (RAV-1) was measured by the standard focus assay on chicken or quail fibroblasts (43) and expressed as focus-forming units per milliliter.

Measurement of fibroblast growth. CEF were infected with undiluted viruses and subcultured twice. When cultures infected with MH2 became transformed, all virus-infected cells were seeded at low density in 60-mm dishes and counted at various intervals.

Anchorage-independent growth. NR cells or CEF, infected with virus and subcultured twice, were suspended in agar at concentrations ranging between 1×10^5 and 3×10^5 cells per 60-mm dish. Solid medium was minimal essential medium containing 0.70% agar, 10% tryptose phosphate broth, 2% chicken serum, and either 8% newborn calf serum (for CEF) or 8% fetal calf serum (for NR cells). Single-cell suspensions were prepared in the same media containing 0.35% agar.

Plasminogen activator production. Plasminogen activator production was monitored by the extent of casein hydrolysis in an agar overlay, in the presence or absence of plasminogen (Sigma), as described (13). Infected fibroblasts and NR cells were seeded at semiconfluency and overlaid with Eagle basal medium containing 0.4% agarose, 2% defatted dry milk, and 1% chicken serum. Cells were grown for 24 h in the absence or presence of 0.5 μg (for NR cells) or 4 μg (for CEF) of plasminogen per ml.

Fluorescence labeling of cells. Cells that had grown for at least 2 days on glass cover slips were fixed for 20 min with 3% formaldehyde in phosphate-buffered saline (pH 7.6) containing 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$. The cells were then permeabilized by treatment with 0.1% Triton X-100 for 4 min at room temperature and stained for F-actin by using 1 U of 7-nitrosobenzo-2-oxo-1,3-diazole (NBD)-phallacidin (Molecular Probes, Inc.) in phosphate-buffered saline (2). Fluorescence-labeled cytoskeletons were observed as previously described (33).

Tumorigenicity in chickens. One-day-old Brown Leghorn chicks were injected intraperitoneally or subcutaneously in each wing web with 0.1 ml of undiluted viruses. Control chicks received 0.1 ml of medium. The birds were examined for tumor development every 3 days over a period of 10 weeks. Autopsy was performed on dead animals and at the end of the experiments. Tumoral tissues were fixed and processed for histological examination as previously described (33).

DNA analysis. Frozen tumor tissues were fragmented in liquid nitrogen and Dounce homogenized in 10 mM Tris (pH 7.5)–0.1 M NaCl–1 mM EDTA. Sodium dodecyl sulfate was added to 0.5%, and DNA was prepared using proteinase K digestion and phenol-chloroform extraction (16). Digestion with the restriction enzyme *Eco*RI was carried out at 37°C for 4 h in 50-μl reaction mixtures containing 10 μg of DNA, buffer recommended by the supplier, and 40 U of restriction enzyme. Digested DNAs were electrophoresed in 1% agarose gels and transferred to nitrocellulose filters by the method of Southern (39). Blots were hybridized to v-*mil*-specific probe radioactively labeled by nick translation (36), as described by Wahl et al. (44). The v-*mil* probe used was obtained from the 1.1-kilobase *Bam*HI-*Hpa*I fragment of plasmid pMH2BS (8).

RESULTS

Characterization of an MH2 mutant deleted in the v-*myc* oncogene. PA200-MH2 (RAV-1) is a spontaneous variant isolated from a virus stock of MH2 (RAV-1) maintained in NR cells (3). It was selected on the basis of its ability to induce sustained proliferation of NR cells as efficiently as wild-type MH2. However, the morphology of PA200-infected NR cells was closer to that of uninfected cells, indicating that the mutant virus had different transformation properties.

Characterization of a biologically active DNA clone has shown that PA200 is a replication-defective virus, generated by recombination between the genomes of MH2 and RAV-1. Helper virus sequences (Δ*gag*-*pol*-Δ*env*) are substituted by Δ*gag*-*mil* and about 300 nucleotides of v-*myc*. The 3′ recombination junction occurred between v-*myc* and *env* sequences (27). Mutant virus-infected cells synthesize the $P100^{gag\text{-}mil}$ fusion protein (3). PA200 replicates with a much higher efficiency than does MH2, since virus titers were greater than 10^6 mitogenic units per ml, as compared to about 10^3 focus-forming units and 10^3 mitogenic units per ml for wild-type MH2 (RAV-1).

Effects of v-*mil*-containing viruses on morphology of CEF and NR cells. Striking morphological differences were observed among CEF and NR cells infected with MH2 or PA200. Infection of NR cells with MH2 resulted, within 7 to 10 days, in the appearance of refringent fusiform cells that actively multiplied. At later stages, these cells became rounded and tended to detach from the substrate (Fig. 1a). In contrast, NR cultures infected with PA200 contained only elongated cells that remained flat and adherent to the plates, even during further passages (Fig. 1b).

CEF infected with MH2 also became round or fusiform (Fig. 1d), whereas fibroblasts infected with PA200 retained an almost normal morphology (Fig. 1e) and did not form foci under agar. Occasional areas of fusiform cells were observed in chronically infected cells (data not shown). However, their number did not increase upon subsequent passages.

Cytoskeleton changes induced by v-*mil*-containing viruses. The arrangement of actin cables within fibroblasts and NR cells is greatly altered upon transformation by Rous sarcoma virus (9, 33). To determine whether such changes also take place in cells expressing the v-*mil* oncogene, we analyzed the cytoskeletons of both cell types infected with RAV-1, MH2, or PA200 by staining with the fluorescent probe NBD-phallacidin, wich specifically binds to polymerized actin (2).

RAV-1-infected CEF and NR cells exhibited an overall polygonal shape, with numerous and large actin cables (Fig. 2c and f). NR cells transformed by MH2 constituted a homogeneous population of aggregated round cells that completely lost their polymerized actin filaments. The actin staining was distributed either diffusely or in patches concentrated into a cap located at one pole of the cells (Fig. 2a). To observe the cytoskeleton of a homogeneous population of fibroblasts transformed by MH2, we selected a colony grown in soft agar. Transformed cells were three to five times smaller than RAV-1-infected fibroblasts and lacked a well-developed network. The actin staining was distributed diffusely, in thin filaments or in small patches all over the cell body (Fig. 2d).

NR cells infected with PA200 retained a polygonal shape and possessed a relatively well-developed actin skeleton. However, they were on average less well spread and had thinner actin cables than normal NR cells. Moreover, the majority of these cells exhibited a few abnormal fluorescent patches localized at the cell periphery, near the ends of actin bundles (Fig. 2b). Fibroblasts infected with the mutant virus also retained a well-developed actin skeleton. However, it was possible to distinguish two populations among these cells: one (15%) had the characteristics of RAV-1-infected

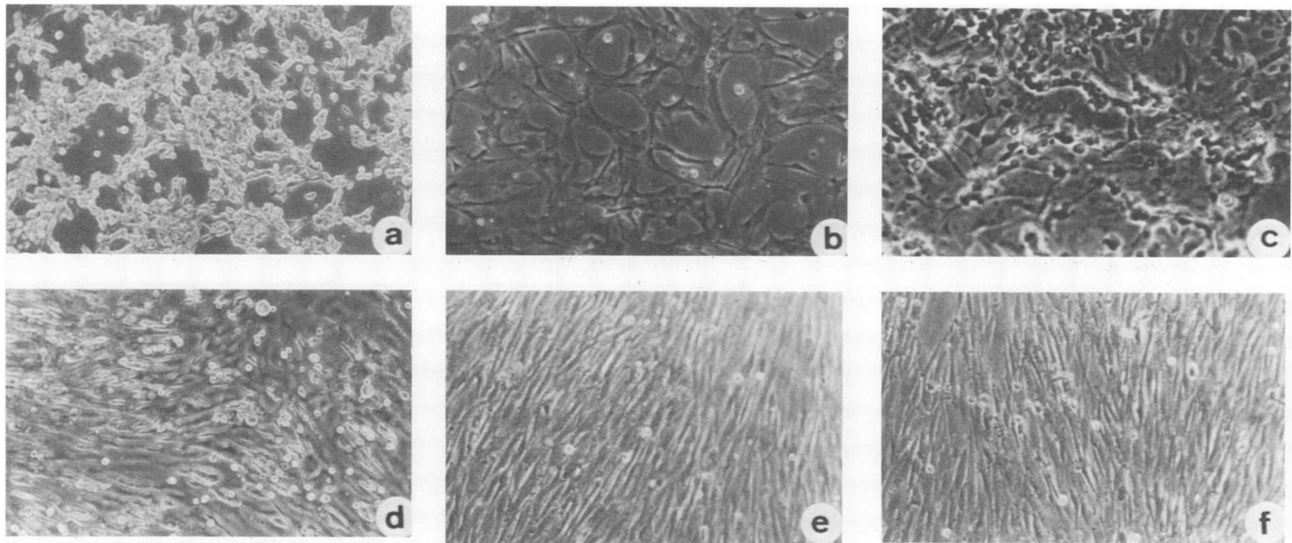

FIG. 1. Morphology of virus-infected cells. NR cells infected with MH2 (a), PA200 (b), or RAV-1 (c); CEF infected with MH2 (d), PA200 (e), or RAV-1 (f). Cultures were passaged twice. Magnification, ×250.

cells, and the other (85%) was composed of less well-spread cells with thinner actin cables. The latter population also exhibited numerous small patches of actin all over the cell body (Fig. 2e).

In conclusion, MH2 causes cytoskeleton alterations that are different from those induced by the v-*src* gene. In particular, both CEF and NR cells transformed by MH2 lack the characteristic patches (rosettes) located near the ventral surface of Rous sarcoma virus-transformed cells (9). In addition, PA200 induced characteristic changes in the cytoskeletons of both cell types that were less pronounced than those induced by wild-type virus.

Anchorage-independent growth. CEF and NR cells transformed by MH2 formed large and numerous colonies in soft-agar-containing medium (Fig. 3a and d). In contrast, NR cells induced to proliferate by PA200 gave rise to much smaller and fewer colonies (Fig. 3b). The mutant virus was also defective in promoting the growth of CEF in soft agar (Fig. 3e).

Growth properties of infected CEF in monolayer cultures. The v-*mil* oncogene induces sustained proliferation of nondividing NR cells (3) and autocrine growth of v-*myc*-transformed chicken macrophages (15). It was also reported that all v-*myc*-containing avian viruses stimulated proliferation of quail fibroblasts (30). We investigated the effects of the v-*mil* oncogene on the growth properties of CEF in medium containing 2% serum. Over a period of 4 days, the average doubling time of CEF chronically infected with either RAV-1

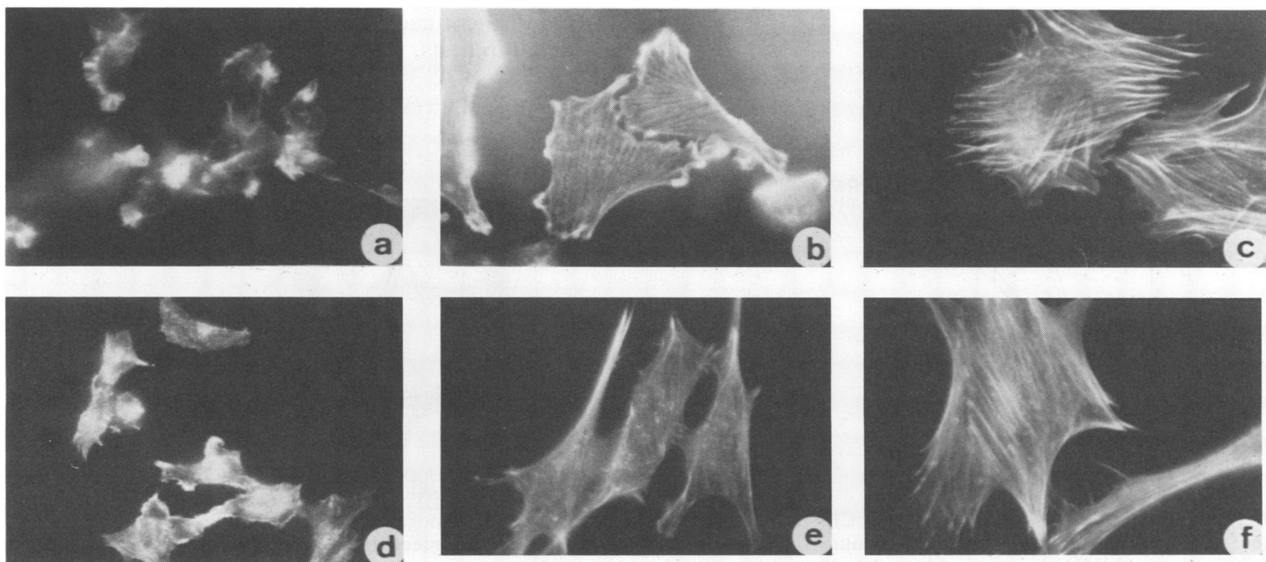

FIG. 2. Organization of polymerized actin in virus-infected cells. NR cells infected with MH2 (a), PA200 (b), or RAV-1 (c); CEF infected with MH2 (d), PA200 (e), or RAV-1 (f). Chronically infected cells were seeded in 35-mm dishes containing glass cover slips (3×10^4 cells per dish). Two days later, cells were fixed and incubated with NBD-phallacidin.

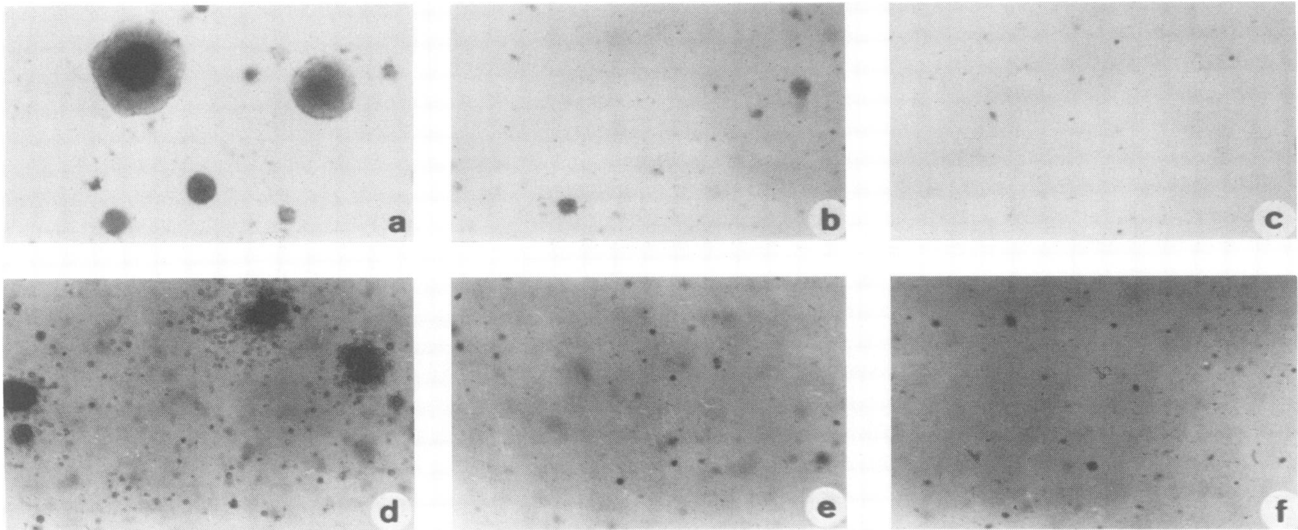

FIG. 3. Anchorage-independent growth of virus-infected cells. NR cells infected with MH2 (a), PA200 (b), or RAV-1 (c); CEF infected with MH2 (d), PA200 (e), or RAV-1 (f). Cultures were passaged twice and subsequently seeded in soft-agar-containing medium. Colonies were observed 10 to 14 days later.

or PA200 was 48 h. In contrast, the growth rate of MH2-transformed fibroblasts was markedly increased, with a doubling time of about 24 h (Fig. 4). Comparable results were obtained when the serum concentration was lowered to 1% (data not shown).

Plasminogen activator production. Rous sarcoma virus-transformed fibroblasts and NR cells produce increased levels of plasminogen activator (33, 41, 42). We compared the proteolytic activity in culture media from both cell types infected with RAV-1, MH2, and PA200. This assay was based on the lysis of casein present in the agar overlay (13). In contrast to the other parameters of transformation tested above, the levels of caseinolytic activity were comparable in MH2- or PA200-infected cells (Fig. 5).

Tumorigenicity of v-*mil*-containing viruses. Intraperitoneal and subcutaneous injection of MH2 (RAV-1), MC29 (RAV-1), and PA200-MH2 (RAV-1) into 1-day-old chickens led to the appearance of solid tumors (Table 1). In contrast to previous reports (4, 15), none of the viruses used in these experiments induced leukemias. Intraperitoneal and subcutaneous injection of MH2 resulted predominantly in the appearance of kidney tumors 7 to 9 weeks after inoculation. Occasional tumors were also detected in the liver, thymus, abdominal cavity, and comb. Renal tumors were often bilateral and, in most extreme cases, reached 8 to 10 cm in diameter. Microscopically, they exhibited the main features of carcinomas. Tumor cells were identified by their dense staining, increased nuclear/cytoplasm ratio, enlarged nuclei with prominent nucleoli, and high mitotic rate. They were clustered in nests, some of which resembled nonfunctional

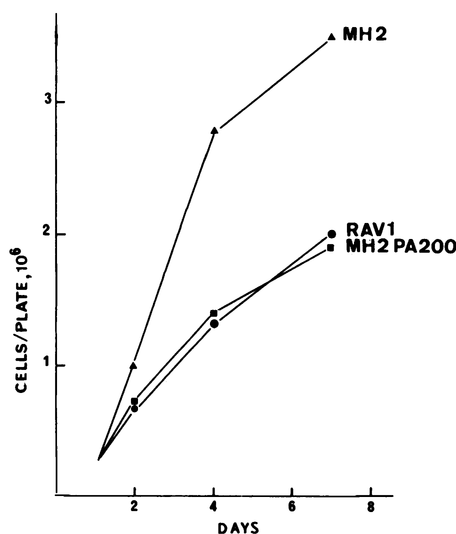

FIG. 4. Growth kinetics of virus-infected CEF. CEF were infected with the indicated viruses and subcultured twice. When transformation with MH2 became evident, 2×10^5 cells from each culture were seeded in 60-mm dishes and maintained in Dulbecco modified Eagle medium containing 2% newborn calf serum. Medium was renewed daily, and cells were counted on the days indicated.

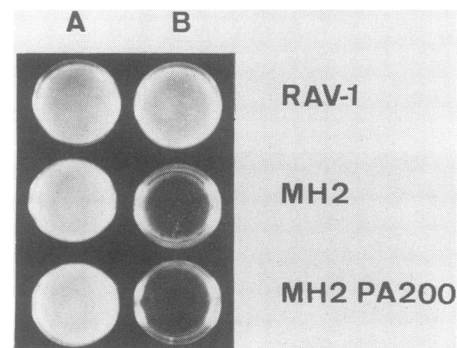

FIG. 5. Plasminogen activator production in virus-infected cells. CEF chronically infected with the indicated viruses were grown as monolayers under medium containing 0.4% agarose, 2% defatted dry milk, and 1% chicken serum. Cells were grown in the absence (A) or presence (B) of 4 μg of plasminogen per ml. Identical results were obtained with NR cells (data not shown).

TABLE 1. Tumor formation by MH2, MC29, and PA200[a]

Viruses	Route[b]	Incidence[c] (%)	Avg latency (wks)	Pathology[d]
MH2 (RAV-1)	i.p. s.c.	33/36 (92) 7/9 (78)	7	Kidney carcinomas (33/40); liver and thymus lymphomas (12/40); other tumors (13/40)
PA200-MH2 (RAV-1)	i.p. s.c.	9/14 (64) 13/22 (59)	4	Gizzard fibrosarcomas Subcutaneous fibrosarcomas
MC29 (RAV-1)	i.p. s.c.	8/8 (100) 5/6 (83)	7	Kidney carcinomas (13/13); liver carcinomas (2/13)

[a] Groups of 1-day-old chickens maintained in separate isolation rooms were inoculated with 0.1 ml of undiluted virus, as indicated.
[b] i.p., Intraperitoneal injection; s.c., subcutaneous injection.
[c] Number of birds developing tumors/total number of injected chickens in three experiments.
[d] Some birds developed more than one type of tumor.

tubules (Fig. 6a).Liver and thymus tumors were composed of lymphoid cells invading the liver parenchyma or replacing the normal structure of the thymus. Upon subcutaneous injection of MH2, small nodules 1 to 2 cm in diameter also developed at the site of inoculation and sometimes regressed. They were not processed for histological examination. MC29 also induced mainly kidney carcinomas, 6 to 8 weeks after intraperitoneal and subcutaneous injection (Fig. 6b).

Intraperitoneal inoculation of PA200 resulted in the growth of tumors emerging from the gizzard in about 60% of the birds. Upon subcutaneous injection into the wing web, tumors that reached a diameter of 5 to 8 cm were visible in 60% of the chickens after 4 to 6 weeks. Histological examination showed that PA200-induced tumors exhibited the microscopic features of sarcomas, with typical bundles of pleiomorphic and invasive tumor cells associated with proliferative fibromatosis. In addition, lymphocytic infiltrations were intense in the tumoral tissues, and clusters of lymphoid cells had the characteristics of anaplasia (Fig. 6c and d). Chickens injected with RAV-1 by either route did not develop any tumors (data not shown).

Four subcutaneous tumors induced by PA200 were also examined for the presence of virus. DNA was extracted from tumoral tissues, digested with *Eco*RI, and analyzed by Southern blotting. Hybridization to a v-*mil*-specific probe revealed the presence, in all four samples, of a 3.6-kilobase-pair PA200-specific DNA fragment, in addition to the 13-, 2.4-, and 1.2-kilobase-pair DNA fragments representing endogenous c-*mil* DNA (Fig. 7). This 3.6-kilobase-pair proviral DNA also hybridized to a long terminal repeat-specific probe (data not shown). Viruses were rescued from two

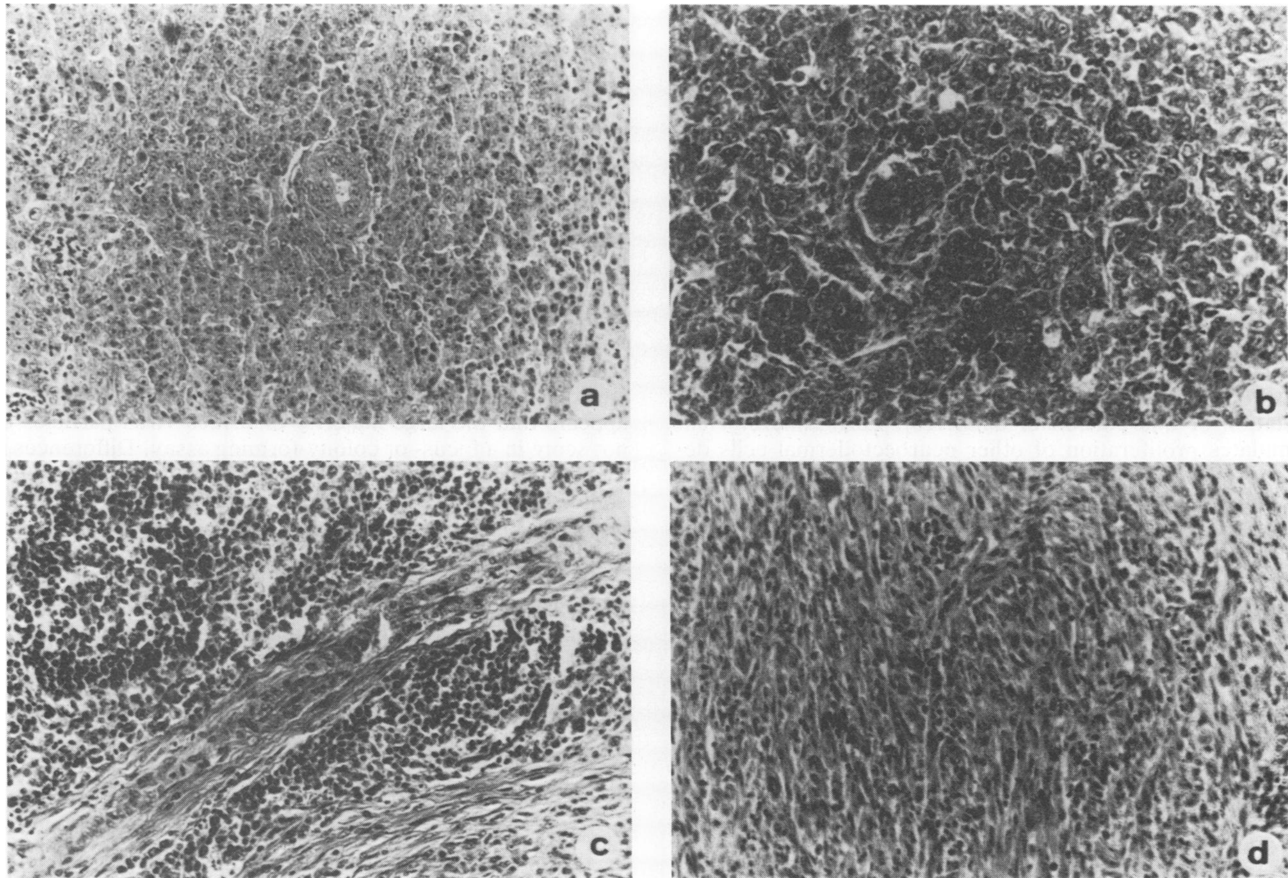

FIG. 6. Histological sections of virus-induced tumors. (a) Photomicrograph of MH2-induced kidney carcinoma, with a cluster of cells forming a pseudotube. (b) MC-29-induced kidney carcinoma. (c) PA200-induced tumor emerging from the gizzard, showing anaplasic lymphoid cells and proliferative fibromatosis. (d) Fibrosarcoma of the wing web induced by PA200. Magnification, ×80.

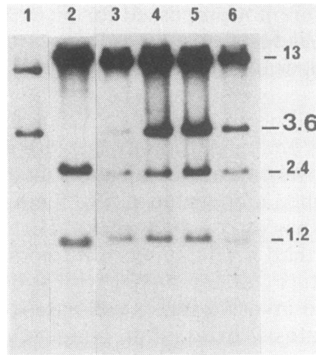

FIG. 7. Southern blot analysis of DNA from PA200-induced tumors. DNA was extracted from four subcutaneous tumors (lanes 3 through 6). Portions (10 μg) were digested with *Eco*RI and electrophoresed on 1% agarose gels. After being blotted onto nitrocellulose filters, DNA fragments were hybridized to a radioactive v-*mil* probe corresponding to the *Bam*HI-*Hpa*I fragment of plasmid pMH2BS. DNA extracted from quail NR cells infected with PA200 (lane 1) and from nontumoral tissues (lane 2) served as controls. λ DNA digested with *Hin*dIII was run as a size marker. The sizes of DNA fragments are expressed in kilobase pairs.

tumors. Their properties were not distinguishable from those of the original PA200: they displayed reduced transforming capacity in CEF and NR cells and lacked the ability to increase the growth rate of CEF in monolayer cultures (data not shown). These results established that PA200 itself was sarcomagenic.

DISCUSSION

v-*mil* expresses mitogenic activity in neural cells. PA200-MH2, which expresses only the v-*mil* oncogene, is able to initiate and to maintain proliferation of NR cells with an efficiency identical to that of wild-type MH2. Viruses similar to PA200 are generated, at a relatively high frequency, during propagation of MH2 in NR cells (C. Béchade and G. Calothy, unpublished results), suggesting that retention of v-*mil* sequences is essential for the growth of these cells. NR cells were also reported to proliferate upon transfection with a recombinant plasmid containing a long terminal repeat-activated carboxy-terminal coding region of c-*mil*, indicating that expression of the mitogenic activity does not require mutation of this portion of c-*mil* (10). Furthermore, v-*mil* stimulates proliferation of other neuroectodermal cells derived from the brain or the neural crest (S. Saule, personal communication). In contrast, v-*mil* is not able to induce multiplication of macrophages (15), nor is it capable of increasing the growth rate of CEF in monolayer cultures. Taken together, these results suggest a role for c-*mil* in the regulation of some essential function(s) in neural cells.

v-*mil* induces fibrosarcomas in chickens. We have shown that the v-*mil* oncogene is tumorigenic in 1-day-old chickens and that the two v-*mil*-containing viruses exhibit different target cell specificity in vivo. PA200 induces fibrosarcomas, mixed with invasive lymphoid cells, in about 60% of the birds, independently of the route of inoculation. Therefore, v-*mil* is functionally related to its murine counterpart v-*raf* by its sarcomagenic property. These results contrast with a previous report indicating that PA200 was not able to induce tumors in 8-day-old chickens (15). They also differ from those of Palmieri and Vogel (31), who showed that injection of PA200-infected cells resulted in the appearance of fibrosarcomas in about 40% of 1 to 4-day-old birds, whereas the mutant virus itself was not tumorigenic. These differences could be explained by genetic susceptibility or age of the chickens used in the various experiments. The sarcomagenic property of v-*mil* contrasts with its inability to alter the growth properties of CEF in vitro. Comparable results were obtained with a v-*src* mutant (33), suggesting that factors required for tumoral growth of fibroblasts could be supplied in vivo.

MH2, like MC29, induces mainly carcinomas, suggesting that v-*myc* is the dominant tumorigenic gene in this virus. Comparable shifts from sarcomas to other types of tumors were also reported with recombinant viruses expressing both v-*raf* and v-*myc* (34). While these results appear to question the role of v-*mil* in specifying the tumorigenic properties of MH2, they do not rule out the contribution of this gene to tumor progression, as suggested by previous reports (15, 26). This possibility is further supported by the finding that the v-*mil* gene, which is frequently lost in cultured fibroblasts, is preferentially retained in tumor cells (5).

v-*mil* exhibits low in vitro transforming capacity. PA200 was initially distinguished from the parental MH2 by its apparent inability to morphologically transform NR cells. Analysis of other transformation parameters confirmed that v-*mil*, in contrast to other avian oncogenes, causes limited in vitro transformation of CEF and NR cells. PA200 induces only minimal morphological changes in both cell types; it is largely defective in promoting anchorage-independent cell growth and lacks the capacity to stimulate the growth of fibroblasts. Moreover, cytoskeleton alterations of cells expressing only the v-*mil* oncogene are limited in comparison to those induced by transforming viruses, such as Rous sarcoma virus or MH2. Therefore, we conclude that v-*myc* is the major in vitro transforming gene of MH2.

Our results are in agreement with the previous report that in vitro-constructed mutants of MH2 expressing only the v-*mil* oncogene fail to induce focus formation and growth in soft agar of quail fibroblasts (45). In contrast, our results markedly differ from those of Palmieri and Vogel (31), who reported that a different viral stock of PA200 induced focus formation and colony formation by CEF and was able to stimulate proliferation of these cells in low serum concentration. However, the titer of the virus used in their experiments, expressed as focus-forming units or CFU, was several orders of magnitude lower than that of mitogenic particles normally present in our viral stocks. Therefore, it appears that the large majority of PA200 virus particles do not score in a focus- or colony-forming assay. Differences in the levels of expression of other transformation parameters could also be explained by variations in the response of the chicken cells used, although we obtained identical results in quail fibroblasts and NR cells (data not shown).

This low in vitro transforming capacity differentiates v-*mil* not only from the distantly related tyrosine kinase group of oncogenes but also from the homologous murine v-*raf* gene, which readily transforms mouse fibroblasts (35). The presence in v-*mil* of additional 5′ sequences derived from three exons of the c-*mil* chicken locus (11) could in part explain these differences. It may also be that avian and murine cells respond differently to the transformation functions of the v-*mil*/v-*raf* oncogene.

v-*mil* and v-*myc* are required for the transformation of NR cells infected with MH2. We have previously shown that, in the conditions used, viruses carrying only the v-*myc* gene fail to induce proliferation and transformation of chicken NR cells (3). However, the results reported here indicate that expression of v-*myc* is necessary to induce complete trans-

formation of MH2-infected NR cells. The mechanism(s) of the cooperation between the two oncogenes is, at present, unknown. It is possible that proliferation of NR cells constitutes a prerequisite to their transformation by v-*myc*. Alternatively, more specific interactions could take place between the two oncogenes. We are currently investigating whether other genes with mitogenic properties could also cooperate with v-*myc* for the transformation of NR cells.

ACKNOWLEDGMENTS

We thank R. Jove and B. Pessac for critical reading of the manuscript, M. Mesny-Moutaffian for interpretation of tumor histology, and F. Arnouilh for assistance in the preparation of the manuscript.

This work was supported by grants from the Institut National de la Santé et de la Recherche Médicale (CRE no. 852007), the Association pour la Recherche sur le Cancer, and the Fondation pour la Recherche Médicale. C.B. was a fellow of the Ligue Nationale Française Contre le Cancer.

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