Fibroblast Transformation Parameters Induced by the Avian *v-mil* Oncogene

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An avian retrovirus containing only the *v-mil* oncogene (PA200-MH2) was analyzed for its ability to induce a transformed phenotype in chicken embryo fibroblasts. Infected cultures exhibited an altered morphology, disarranged actin cable filaments, and a decrease in the amount of cell surface fibronectin. In addition, these cells showed a high level of plasminogen activator protease activity and were also capable of growth in low serum concentrations. In contrast, PA200-MH2 was very inefficient at inducing foci under agar and colonies in semisolid medium relative to the Mill Hill 2 and Rous sarcoma viruses. This inefficiency was further reflected in vivo by the total inability of PA200-MH2 to induce wing tumors in young birds. However, 40% of the birds inoculated in the wing web with PA200-MH2-infected cells did develop slow-growing tumors at the site of injection, with no evidence of hematopoietic involvement. Our results indicate that the *v-mil* oncogene is transforming both in vitro and in vivo and that each of the oncogenes in the Mill Hill 2 virus, *v-mil* and *v-myc*, can independently transform fibroblasts. These data suggest that *v-mil* is functionally related to its homologous murine counterpart, *v-raf*, which also transforms fibroblasts.

The *v-mil* oncogene is one of two unlinked cellular genes in the Mill Hill 2 (MH2) retrovirus (5, 13, 14); the other transduced sequence contained in MH2, *v-myc*, is the major transforming gene (12, 17, 29) and is also found in three other independent virus isolates (MC29, OK10, and CMII), all of which lack additional cellular inserts (8). These four viruses transform macrophages and fibroblasts in cultures and induce leukemias, carcinomas, and sarcomas in vivo (2, 4, 20, 22, 25, 27). However, the presence of *v-mil* (also termed *v-mht*) in MH2 seems to be responsible for an increased oncogenic potential in birds relative to MC29 (9, 16). Likewise, the *v-mil* gene appears to play an ancillary role in MH2-mediated transformation by enhancing the macrophage-transforming ability of *v-myc* in vitro; this effect is manifested by growth independent of exogenous growth factors (1, 9; unpublished results). The protein encoded by *v-mil* contains an associated serine-threonine kinase activity and is related to the *src* family of tyrosine kinases (19). Recently, a spontaneous *myc* deletion mutant of MH2, PA200-MH2, was isolated and found to contain helper virus envelope sequences substituted for most of the *myc* sequences (3). In addition, infected cells no longer express an *myc*-containing subgenomic mRNA. With the availability of this mutant, studies assessing the transforming capacity of the *v-mil* gene alone were made possible. Indeed, it has already been demonstrated that neuroretinal cells exhibiting a seemingly normal morphology could be induced to proliferate by PA200-MH2 (3). Since the *v-mil* nucleotide sequence is highly homologous with that of the fibrosarcoma-inducing murine *v-raf* gene of mouse sarcoma virus 3611 (11, 15, 23, 26), we were particularly interested in determining whether *v-mil* could alter the growth pattern of chicken fibroblasts. In the present study, we have characterized the PA200-MH2 retrovirus for eight parameters of fibroblast

transformation and determined that the *v-mil* oncogene is transforming both in vitro and in vivo.

A morphologically normal neuroretinal cell line producing molecularly cloned PA200-MH2 and Rous-associated virus-1 (RAV-1) and described by Bechade et al. (3) was provided by Simon Saule (Institut Pasteur, Lille, France). Tertiary chicken embryo fibroblasts (CEF) were prepared from 9-day-old embryos obtained from a group-specific antigen-negative chicken flock maintained in Hamilton. Cells infected for 5 to 7 days with filtered supernatants from the neuroretinal cell culture exhibited an elongated morphology and grew in a criss-cross, disorganized pattern (Fig. 1A) relative to RAV-1-infected control cells (Fig. 1C). The *v-mil* gene-infected cultures also metabolized growth medium at a high rate and tended to detach from the substrate in sheets if not trypsinized daily. Tertiary quail embryo fibroblasts infected for the same number of days with PA200-MH2 appeared very filamentous (Fig. 1B) as compared with RAV-1-infected cells (Fig. 1D), and upon prolonged passage, they became totally suspended. They could not be grown in sufficient numbers to be characterized further. It should be noted that after repeated attempts, the PA200-MH2 virus was found to possess no transforming activity on hematopoietic cells (unpublished results).

The disarrangement of actin cable microfilaments and a scarcity in the amount of fibronectin are known properties of transformed fibroblasts (10). To determine the proportion of cells containing actin filaments and the relative abundance of cell surface fibronectin, we used a double fluorescence staining procedure (21, 22). This technique permitted both parameters to be visualized simultaneously and involved the use of antifibronectin antiserum (the gift of Richard O. Hynes, Massachusetts Institute of Technology, Cambridge, Mass.) (18) and a fluorescently labeled phalloidine preparation specific for filamentous actin (provided by T. Wieland, Max Planck Institute for Medical Research, Heidelberg, Federal Republic of Germany) (28). Only 9% of the *v-mil*-infected cells expressed actin cables (Fig. 2A), whereas 91%

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FIG. 1. Phase-contrast micrographs showing morphological changes induced by PA200-MH2. CEF (A and C) and quail embryo fibroblast (B and D) mass cultures were infected 7 days earlier with either PA200-MH2 (RAV-1) (A and B) or RAV-1 alone (C and D). Cell cultures were prepared in accordance with published procedures (21) and were grown in Dulbecco modified Eagle medium containing 8% fetal calf serum, 2% heat-inactivated chicken serum, and 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer (pH 7.3) (growth medium). Magnification, $\times 300$.

of the RAV-1-infected cells were actin cable positive (Fig. 2B). Likewise, the fibronectin network, which normally forms a thick extracellular matrix (Fig. 2D), was very weakly expressed and disorganized for the *v-mil* virus-infected culture (Fig. 2C). Thus, the *v-mil* oncogene induced morphological and structural alterations similar to those induced by most other acutely transforming avian retroviruses (10, 22, 25).

Virus-transformed fibroblasts have been previously shown both to secrete and to contain a trypsinlike enzyme called plasminogen activator protease (24). This protease activity is correlated with the ability of transformed cells to lyse casein contained in an agar overlay (6). A test assessing caseinolytic activity was performed for cells infected with PA200-MH2 (Table 1). Infection with the PA200-MH2 virus induced a high level of caseinolysis comparable to the activity found in Rous sarcoma virus (RSV)- and MH2-transformed CEF. In agreement with previous results, fibroblasts transformed by MC29 showed only a slightly elevated protease activity (25). Since other viruses containing only the *v-myc* gene also have been reported to induce relatively low caseinolysis (22, 25), it appears likely that the high activity seen with MH2 is due to the presence of *v-mil*.

Avian fibroblasts transformed by all four *v-myc*-containing

virus isolates have an average population doubling time of 10 to 13 h (22, 25). However, PA200-MH2-infected cells displayed no such characteristic when assessed for a similar induction of rapid growth rate (data not shown). These cells were further tested for their ability to grow in medium containing 1% serum. PA200-MH2-infected cells steadily increased in numbers, their growth rate closely resembling that seen with RSV-transformed fibroblasts (Fig. 3). After sustaining an initial increase during the first 48 h, MH2-transformed CEF greatly decreased in numbers during the final 3 days of the test and were nearly all dead after 5 days, as were cells in the RAV-1-infected control culture. MC29-transformed CEF also have been shown to lack the capacity to proliferate when cultured in medium containing a low serum concentration (25). Taken together, these results indicate that the *v-mil* gene can lower the growth factor requirements of fibroblasts in the absence of *v-myc*. However, *v-mil* cannot reduce the serum requirements of rapidly dividing *myc*-transformed cells; apparently, *myc*-transformed CEF are highly susceptible to death in suboptimal culture conditions, and this susceptibility cannot be lowered by the *v-mil* gene.

The data presented thus far have focused strictly upon the characteristics exhibited by previously infected CEF mass

FIG. 2. Double fluorescence staining of CEF cultures infected with PA200-MH2 (RAV-1) (A and C) or RAV-1 alone (B and D). The same cell fields were stained for both actin (A and B) and fibronectin (C and D). The details of this procedure are described elsewhere (21). Cells containing more than three extended actin microfilaments were scored as positive, and at least 100 cells per sample were counted. Magnification, $\times 720$.

cultures in which infectivity and initial changes induced by the respective viruses were not considered. To observe these effects, we performed focus formation and colony formation assays. Low numbers of foci and colonies were obtained with PA200-MH2 relative to the other transforming viruses tested (Table 2). In addition, the foci induced by PA200-MH2 contained a more diffuse cell arrangement and were smaller than either RSV- or MH2-induced foci. The fibroblast colonies formed in the presence of PA200-MH2 were also small as compared with those formed in the presence of RSV; MH2 induced intermediate-sized colonies. Similar data for both tests were obtained when PA200-MH2 was harvested from either neuroretinal cells or infected fibroblast cultures, indicating that the virus replicated equally well in both cell types. Despite this finding, the apparent inability of PA200-MH2 to replicate to a high titer

is one possible explanation for low numbers of foci and colonies. However, since PA200-MH2 does not appear to be replication defective (3 and this report), the possibility that it is highly inefficient in inducing fibroblast foci and colonies must also be strongly considered. If this is the case, primary neuroretinal cells may be a more suitable cell type for determining virus concentration, since they appear to be very sensitive to infection by PA200-MH2 (3).

The final parameter studied was sarcomagenicity. Young birds were injected in the wing web either with fibroblast populations infected *in vitro* or with virus only and examined biweekly for the appearance of tumors. Both types of inocula were used because attempts to increase the relatively low concentration of PA200-MH2 above 100 to 200 focus-forming units per ml were unsuccessful for unknown reasons. Table 3 shows that 40% of the birds inoculated with PA200-MH2-infected cells developed tumors at the site of injection and died as a result. These tumors were palpable 5 to 7 days after infection and were slow growing, as judged by a mean survival time of 54 days. In only one case was a secondary tumor observed at the apex of the heart, suggesting a low frequency of metastasis. It could not be determined whether this secondary tumor was caused by the original injected cells or by circulating virus. However, the latter possibility appears unlikely, since no tumors were observed in any of the birds inoculated with only PA200-MH2 virus. The same lack of tumorigenicity was found by Graf et al. (9) when birds were injected intravenously with PA200-MH2.

Blood smears prepared weekly from both groups of PA200-MH2-infected animals were also negative for the presence of leukemic cells. Although small wing nodules

TABLE 1. Plasminogen activator protease activity as measured by caseinolysis^a

Infecting virus	Oncogene	Caseinolytic activity after 3 days ^b
PA200-MH2 (RAV-1)	<i>mil</i>	+++
MH2 (RAV-1)	<i>mil</i> and <i>myc</i>	+++
MC29 (RAV-1)	<i>myc</i>	+
RSV (Schmidt-Ruppin A)	<i>src</i>	+++
RAV-1	None	—

^a The preparation of the agar overlay and other details were previously reported (6, 21).

^b —, No lysis; +, light clearing of casein overlay; + + +, total clearing of casein overlay.

TABLE 2. Focus-forming and colony-forming abilities of avian retrovirus strains

Virus used	Focus-forming units/ml ^{a,b}	Colony-forming units/ml ^{a,c}
PA200-MH2 (RAV-1)	1.0×10^2	8.0×10^1
MH2 (RAV-1)	1.5×10^4	1.1×10^3
RSV (Schmidt-Ruppin A)	1.0×10^5	8.3×10^3
RAV-1	0	0

^a Scored after 10 to 14 days. Values were averaged from duplicate dishes in two independent experiments.

^b The focus formation assay was performed as previously described (7).

^c Cultures were infected for 18 h, trypsinized, and seeded in a methylcellulose-containing medium (21).

were seen in some of the surviving birds injected with PA200-MH2-infected CEF, these tumors either remained small or disappeared during the course of the experiment. Birds injected with RSV-transformed CEF or RSV had extremely short mean survival times, with a rapid development of large wing tumors. MH2-infected birds also died relatively early, but in these birds multiple neoplasms were found (Table 3). Leukemic bone marrow cells taken from several birds infected with MH2 grew rapidly in cultures and displayed a morphology indistinguishable from that of in vitro MH2-transformed macrophages (data not shown). This experiment indicates that the efficiency of disease induction by PA200-MH2, as well as by the other transforming viruses tested, was significantly amplified when infected cell cultures were used.

This study was undertaken to define, with several assays of fibroblast transformation, the characteristics of cells infected with the *v-mil* gene-containing virus PA200-MH2. The approach used was to compare the properties of PA200-MH2 with those of known oncogene-containing virus isolates to determine if *v-mil* could induce a similar transformed phenotype. During the course of this work, separate isolates of PA200-MH2 were tested for the presence of MH2-like revertant viruses by in vitro bone marrow transformation assays and bird inoculations. At no time were any revertants observed.

The data indicate that PA200-MH2 exhibited a moderate to strong transforming ability for all parameters tested with passaged virus-infected fibroblasts. These included cellular morphology, integrity of actin cables, content of fibronectin,

FIG. 3. Growth kinetics of fibroblasts grown in medium containing a low serum concentration. Fibroblast cultures were infected 10 days earlier with the viruses shown (CONTROL = RAV-1-infected cells) and seeded at a density of 3×10^5 cells per 35-mm tissue culture dish. At 2 h later, the growth medium was changed to Dulbecco modified Eagle medium containing 1% fetal calf serum (25). Each point represents the ratio of the total cell number found at that time divided by the original cell number seeded. The numbers of both surviving adherent and suspended cells were determined by using a hemacytometer. Duplicate dishes per sample were averaged.

activity of plasminogen activator protease, growth in low serum concentrations, and sarcomagenicity. In contrast, PA200-MH2 appeared to be very inefficient at inducing foci or colonies of fibroblasts in vitro and was completely deficient at inducing tumors in vivo. Possible explanations for these results are (i) a partial defect in replication, (ii) a low infectivity, or (iii) an unusually long period of time required to establish transformation-specific effects. Since PA200-

TABLE 3. Comparison of oncogenic properties of inoculated CEF cultures and viruses in chickens

Transforming agent	No. of birds infected ^a	No. of deaths (% incidence)	Mean survival time (days) ^b	No. with neoplasms			
				Leukemia	Wing sarcoma	Heart	Liver or kidney
CEF infected with ^c :							
PA200-MH2 (RAV-1)	25	10 (40)	54	0	10 ^d	1	0
MH2 (RAV-1)	16	15 (94)	24	10	4	0	6
RSV (Schmidt-Ruppin A)	10	10 (100)	13	0	10	0	0
RAV-1	5	0 (0)		0	0	0	0
Virus ^e :							
PA200-MH2 (RAV-1)	10	0 (0)		0	0	0	0
MH2 (RAV-1)	10	5 (50)	35	4	1	0	2
RSV (Schmidt-Ruppin A)	10	8 (80)	19	0	8	0	0
RAV-1	2	0 (0)		0	0	0	0

^a One- to four-day-old birds were used.

^b Calculated for infected birds with nonregressing tumors. Surviving birds were kept for at least 100 days.

^c Cells (2×10^6) in 0.1 ml of growth medium minus serum were inoculated in the wing web of each bird.

^d Tumors from two birds were mixtures of fibroblasts and relatively pure populations of erythrocytes that were incapable of independent growth.

^e Virus (10 to 20 focus-forming units or PFU) in 0.1 ml of growth medium minus serum were inoculated in the wing web of each bird.

MH2 has already been shown to infect avian cells and replicate in a manner similar to that of other retroviruses (3), we believe the last possibility to be most likely. This interpretation is in agreement with the findings of Zhou et al. (29), who reported that an independently isolated *v-mil*-containing virus was completely negative for focus formation in quail embryo fibroblasts. Similarly, Bechade et al. (3), in their original communication describing PA200-MH2, observed no transforming activity, nor did Graf et al. (9), who subsequently found the virus to be negative for tumor induction. Whereas these groups based their conclusion that *v-mil* possessed no capacity to transform exclusively on focus formation and tumor induction by virus inoculation, we have used the additional cellular criteria outlined in this report to conclude that *v-mil* can transform CEF. Genetic differences in each of the cell systems used and culture conditions may be secondary reasons why the results reported above were not seen before, although a "weak" transforming effect for the *v-mil* gene was previously reported by von Weizsäcker et al. (27). Since the murine *v-raf* gene has been shown to possess a high degree of homology with *v-mil* (26), our results suggest that a functional relationship exists between these two oncogenes as well. It will be interesting to determine if the protein encoded by the *v-mil* gene or an induced growth factor similar to cMGF (1) is responsible for the transforming properties of PA200-MH2.

We thank Kathryn Radke and Bruce Chesebro for comments on the manuscript before submission. We also thank Gary Hettrich and Robert Evans for photographic assistance and Sandra Mulkey and Helen Blahnik for preparing the manuscript.

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