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Quail embryo fibroblasts transformed by four *v-myc*-containing virus isolates show enhanced proliferation but are non tumorigenic

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Quail embryo fibroblasts infected with any of the four natural avian *myc* gene-containing virus strains (MC29, CMII, OK10 and MH2) or with the *myb,ets*-containing E26 acute leukemia virus, were examined for their expression of several transformation-associated parameters. All *myc*-containing viruses, but not E26 or Rous sarcoma virus (used as a control) induced a dramatic stimulation of cell proliferation. In addition, the *myc* virus-transformed cells exhibited prominent nucleoli, possibly as a consequence of their increased proliferation. Cells transformed by MC29, OK10, MH2 and E26 were capable of growing in semi-solid medium and showed a loss of actin cables and, in most cases, of an ordered fibronectin distribution. All of the *myc* virus-transformed fibroblasts, as well as the E26-transformed cells, were unable to form tumors in nude mice, indicating that the *myc* gene (and the *myb/ets* genes) are not sufficient for the induction of a fully malignant phenotype in avian fibroblasts.
Key words: myc gene-containing virus/transformation/tumorigenicity

Introduction

Myc is the oncogene contained in the avian myelocytomatosis virus strain MC29, as well as in three other natural retrovirus isolates (for review, see Graf and Stèhelin, 1982). Most of these viruses induce a variety of neoplasms (myelocytomatosis, kidney and liver carcinomas, endotheliomas and probably B-cell tumors) in chickens and quails and can transform both fibroblasts and macrophages *in vitro* (reviewed by Graf and Beug, 1978; Moscovici and Gazzolo, 1982). Several deletion mutants in the *myc* gene have been isolated (Ramsay *et al.*, 1980). These mutants show a reduced macrophage-transforming activity and an altered spectrum of tumors (Ramsay *et al.*, 1980; Enrietto *et al.*, 1983) indicating that the *v-myc* gene itself encodes the viral transforming capacity.

However, it is not clear whether *myc* is sufficient to fully transform cells or whether it activates one or more cellular oncogenes which then act as the ultimate transforming agents. The recent observation that cloned *v-myc* DNA does not appear to induce transformed foci in primary rat cells, but does so only in combination with DNA from another oncogene such as the *ras* gene (which also cannot fully transform primary rat cells by itself) (Land *et al.*, 1983) tends to support the latter possibility. In addition, earlier studies with chicken cells showed that, although MC29 is capable of inducing transformed foci in primary chick embryo fibroblasts (Langlois *et al.*, 1967; Graf, 1973), the transformed cells were neither tumorigenic in syngeneic animals nor immortalized in culture (Royer-Pokora *et al.*, 1978). However, the MC29-transformed chicken fibroblasts showed an enhanced rate of proliferation (Bolognesi *et al.*, 1968; Royer-Pokora *et al.*, 1978).

The present work was undertaken to determine whether these properties are unique to the MC29 strain or whether they are characteristic of all *myc*-containing viruses. We therefore examined quail embryo fibroblasts (chosen because of their longer lifespan *in vitro* relative to chicken cells) infected with the *myc*-containing viruses MC29, OK10, MH2 and CMII for their expression of several transformation-associated parameters. In addition, we analyzed cells transformed by the E26 virus (which contains the *myb* and *ets* oncogenes; Leprince *et al.*, 1983) and, as a positive control, by Rous sarcoma virus (RSV) (which contains the *src* oncogene).

Results

Rate of proliferation of virus-transformed quail embryo fibroblasts

As shown in Figure 1, cells transformed by all four of the *myc*-containing viruses showed a significantly increased rate of growth relative to helper virus (RAV-1)-infected cells and to E26- or RSV-transformed cells. They had average popula-

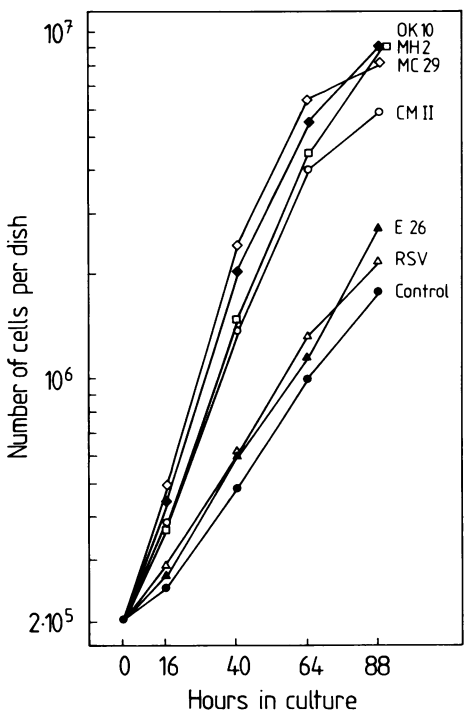

Fig. 1. Growth curves of virus-infected quail embryo fibroblasts. Each point represents the total number of cells per culture at the times indicated. Duplicate dishes per sample were averaged.

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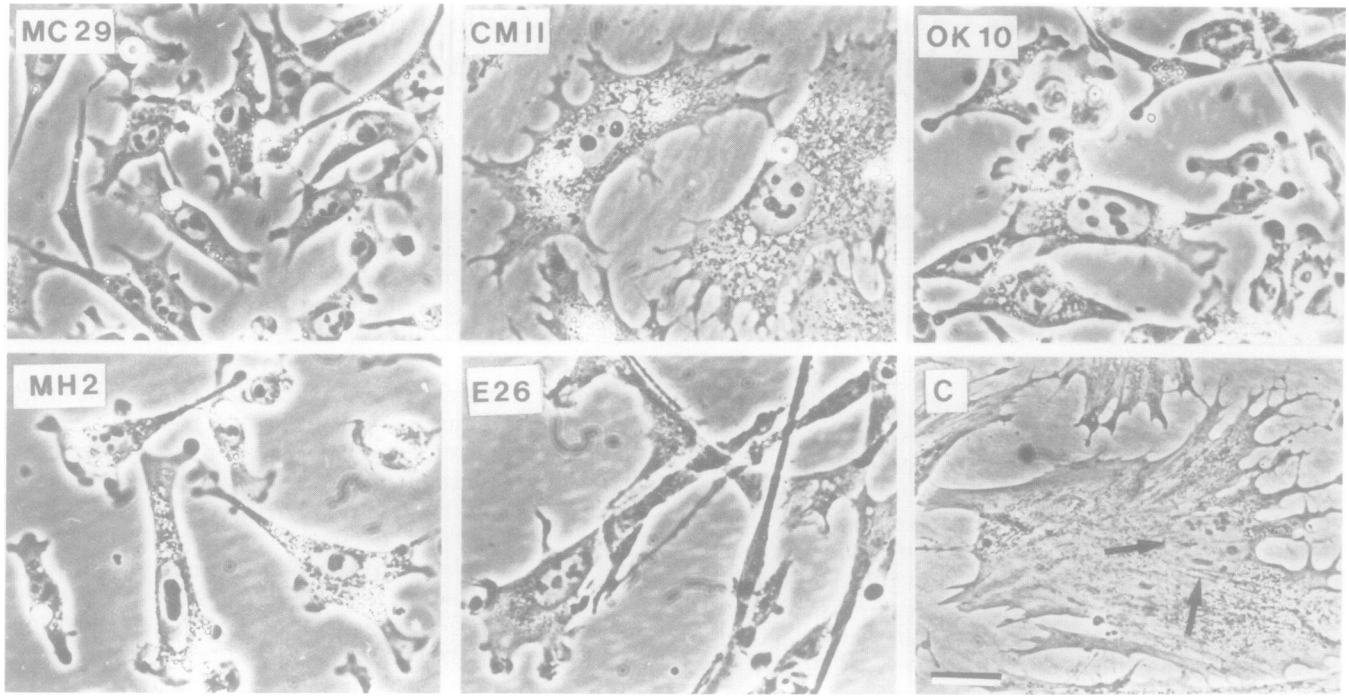

Fig. 2. Morphology of virus-infected quail embryo fibroblasts as shown by phase contrast micrographs of living cells. C = control cells infected with RAV-1. The arrows in C indicate the position of the nucleus. Bar = 10 μm.

tion doubling times between ~10 h (MC29) and 13 h (CMII), compared with ~24 h for all other types of cells. In addition, all of the *myc* virus-transformed cultures shed high numbers of cells into the medium after reaching confluence. This effect was less pronounced in the CMII-transformed cells and was not seen in the cultures infected by RSV, E26 or RAV-1.

Virus-induced morphological changes

To determine whether the *myc* gene induces specific morphological changes, quail embryo fibroblasts were infected and passaged 3–5 times, until all cells appeared to be morphologically transformed. As shown in Figure 2, cells transformed by each of the four *myc*-containing viruses had prominent nucleoli, a property which was not seen in the RSV-, E26- or RAV-1-infected cells. MC29-, OK10- and MH2-transformed cells showed additional changes: they were stellate in shape, much smaller and less adherent than normal cells, and had smaller nuclei. An exception was the CMII-transformed cells. These cells, although exhibiting large nucleoli, resembled normal cells in their overall morphology. E26-transformed fibroblasts also looked different: they were thin and elongated in appearance and tended to grow on top of one another.

Distribution of actin cables and fibronectin

The loss of thick, highly ordered actin-containing microfilament bundles and a reduction in the amount and the organization of the cell surface glycoprotein fibronectin are among the most common alterations observed in virus-transformed fibroblasts (Hynes, 1973; Edelman and Yahara, 1976). To determine whether such changes also occur in *myc* virus-transformed cells, actin and fibronectin were stained in fixed cell preparations using a double immunofluorescence technique. As shown in Figure 3 and in Table I, cultures transformed with MC29, OK10 or MH2 viruses contained very low numbers of actin cables, and those few which were still present usually did not extend across the full length of the cells. Furthermore, these cultures completely lacked an organized fibronectin network (photographs of OK10- and MH2-infected cells not shown). Thus, they resembled RSV-transformed cells with respect to the distribution of both actin and fibronectin. In contrast, cells transformed by CMII contained essentially normal actin cables but still showed an abnormal distribution of fibronectin. E26-transformed cells had the reciprocal phenotype: most cells lacked organized actin microfilaments but exhibited an almost normal pattern of fibronectin.

Focus formation under agar and induction of caseinolytic activity

All four *myc*-containing viruses, as well as E26 were capable of inducing foci under an agar overlay, thereby confirming previous observations obtained with these viruses (Graf and Beug, 1978; Graf *et al.*, 1979). The foci induced by MC29, OK10 and MH2 in quail embryo fibroblasts were small but distinct while CMII-induced foci were larger and more diffuse. Foci induced by E26 were very small and diffuse and could not be counted with a high degree of accuracy. The titers obtained from fully transformed cultures varied significantly. As shown in Table II, MC29 induced the highest number of foci (similar to that obtained with RSV), followed by OK10 (~10-fold less) and then by CMII, MH2 and E26 (~500- to 1000-fold less). These differences in replication ability might reflect differences in the efficiency with which the long terminal repeats (LTRs) of the defective transforming viruses promote viral RNA transcription.

We also tested whether the virus-transformed cells showed an increased production of plasminogen activator, a proteolytic enzyme which is synthesized at high levels by most virus-transformed fibroblasts (Unkeless *et al.*, 1973). The assay

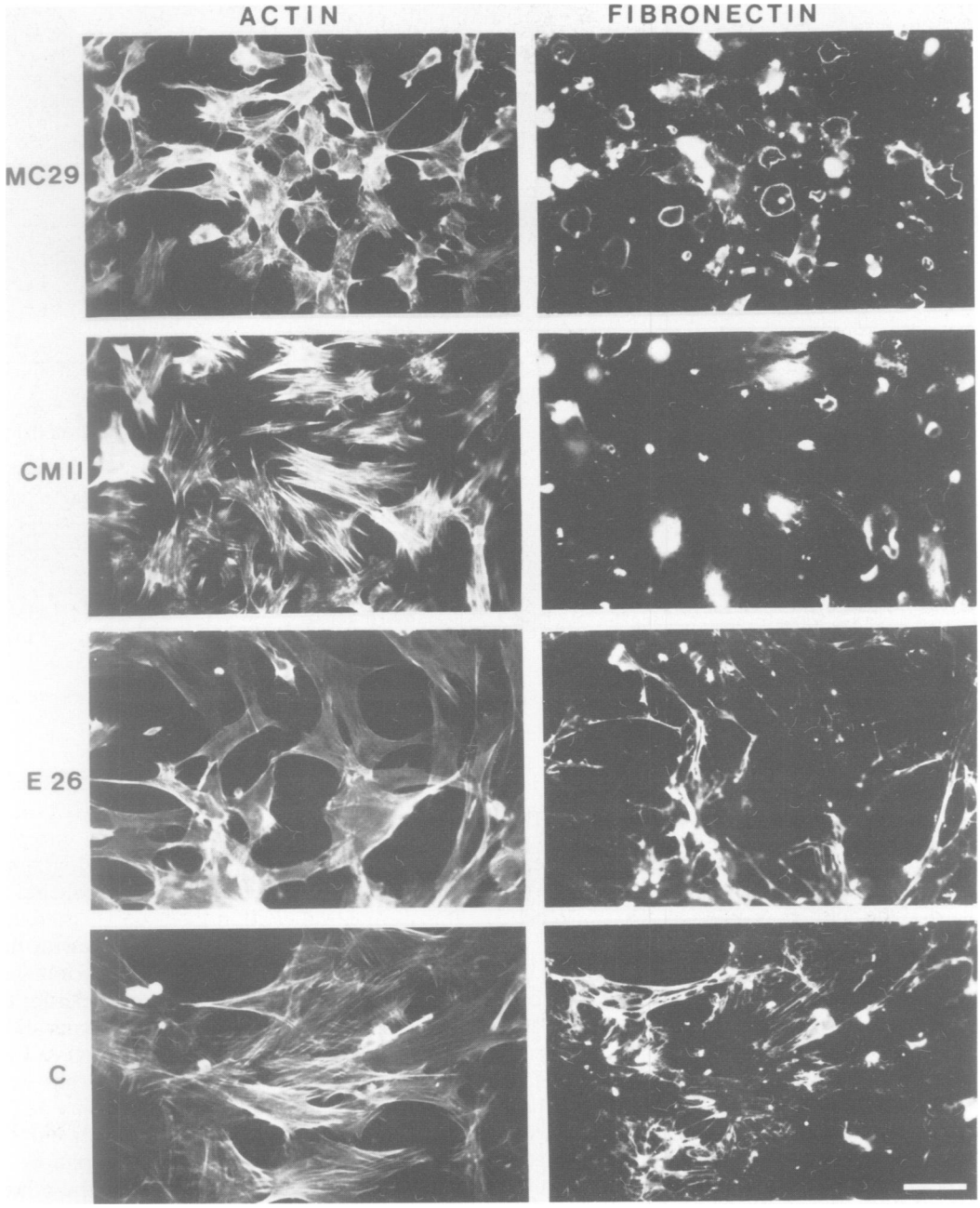

Fig. 3. Actin cable and fibronectin patterns in infected quail embryo fibroblasts as shown by double immunofluorescence. For the actin staining, rhodamine-labelled phalloidin was used; for the fibronectin staining, FITC-labelled rabbit antibodies were employed. C = RAV-1 infected cells. Bar = 50 μm.

which we used is based on the fact that plasminogen activator secreted into the medium is able to lyse casein (added to the culture in an agar overlay), thereby creating a clear area over the cells (Goldberg, 1974). As summarized in Table II, MH2 was the only virus which induced levels of caseinolytic activity similar to that seen for RSV-transformed cells.

Colony formation in semi-solid medium and tumorigenicity in nude mice

Fibroblasts transformed with oncogenic viruses usually acquire the ability to proliferate in semi-solid medium (reviewed by Freedman and Shin, 1978). To test the capacity of the *myc*-containing viruses to induce this change, we seeded various numbers of virus-transformed cells into growth medium containing 1% methylcellulose and then counted the number of colonies 10 days later. As shown in Table III, three of four *myc* virus-transformed cells (all except those transformed by CMII), as well as the E26-transformed cells, were able to form colonies in methylcellulose (and in agar; results not shown). However, the colonies were smaller and more compact and the plating efficiencies were 3- to 10-fold lower than with RSV-transformed cells.

Lastly, we tested the tumorigenic potential of the virus-transformed quail fibroblasts in nude mice. Quails were not used as recipients in these experiments because we wished to avoid the possibility that host cells might be transformed by the virus which the injected cells produce. Since the avian viruses which we have used are not able to infect mammalian cells, any tumors obtained in nude mice must therefore reflect the inherent neoplastic growth potential of the injected cells.

Table I. Organization of actin cables and fibronectin in virus-transformed quail fibroblasts

Infecting virus	Percent of cells with actin cables	Fibronectin network
MC29 (RAV-1)	6[a]	–
CMII (RAV-1)	54	–
OK10 (RAV-1)	1	–
MH2 (RAV-1)	1	–
E26 (RAV-1)	13	+
RSV	0	–
RAV-1	87	+

[a]Cells with three or more microfilament cables extending across the entire cell were scored as positive.

Table II. Focus-forming and casein-plaque-forming activity of various virus strains

	Foci/ml	Lysis of casein
MC29 (RAV-1)	1.5×10^5	+
CMII (RAV-1)	3.3×10^2	+
OK10 (RAV-1)	3.3×10^4	+
MH2 (RAV-1)	2.5×10^2	+ + +
E26 (RAV-1)	2.0×10^2	(+)
RSV	1.0×10^5	+ + +
RAV-1	0	–

(+), very light clearing of the casein overlay after 3 days; +, moderate clearing after 3 days; + + +, total clearing after 1 day; –, no clearing.

The results of this experiment are shown in Table III. Although the RSV-transformed cells formed tumors in most of the injected animals, neither the E26-transformed cells nor any of the *myc* virus-transformed cells were tumorigenic, even at inocula of 10×10^6 cells.

Discussion

The phenotypic changes induced in quail embryo fibroblasts transformed with the four *myc*-containing viruses as well as with the *myb,ets*-containing virus E26 are summarized in Table IV. Two of the alterations examined, an increase in the rate of cell proliferation and increased prominence of the nucleoli, were common to all of the *myc* virus-transformed cells but were not detected in RSV- or E26-transformed cultures. Furthermore, they have not been consistently observed in cells transformed by other oncogenic viruses. This result is in agreement with the observation that macrophages transformed by *myc*-containing viruses also appear to differ from their normal counterparts mainly in their increased proliferative capacity (Beug *et al.*, 1979; unpublished observations) and by the presence of large nucleoli (Langlois *et al.*, 1967; Beug *et al.*, 1979).

Since the nucleolus is the site of ribosome assembly (Alberts *et al.*, 1983) the large nucleoli in *myc* virus-transformed cells might be a consequence of increased protein synthesis which is probably needed to support an increased proliferation rate. The unusual proliferation-inducing capacity of the *v-myc* gene could be related to the fact that the *myc*-encoded protein, in contrast to most other oncogene products, is localized in the nucleus (Donner *et al.*, 1982; Abrams *et al.*, 1982), where it might stimulate cell division through an interaction either with DNA or with the nuclear matrix. Studies on *myc* proteins have revealed that they are able to bind DNA in a cell-free system (Donner *et al.*, 1982), but that

Table III. Colony and tumor-forming ability of *in vitro* transformed quail fibroblasts

Infecting virus	Colony formation in Methocel (Cols./10^5 cells)	Incidence of tumors in nude mice
MC29 (RAV-1)	4.6×10^3	0/6
CMII (RAV-1)	0	0/6
OK10 (RAV-1)	5.2×10^3	0/6
MH2 (RAV-1)	4.7×10^3	0/6
E26 (RAV-1)	1.2×10^3	0/6
RSV	1.6×10^4	4/6
RAV-1	0	N.T.

Table IV. Summary of transformation-related changes induced in quail fibroblasts

	Changes induced in cells infected by:					
	MC29	CMII	OK10	MH2	E26	RSV
Induction of cell proliferation	+	+	+	+	–	–
Decrease in cell size; increased prominence of nucleoli	+	(+)	+	+	–	–
Colony formation in semi-solid medium	+	–	+	+	+	+
Loss of fibronectin network	+	+	+	+	–	+
Loss of organized actin cables	+	–	+	+	+	+
Focus formation under agar	+	+	+	+	(+)	+
Induction of plasminogen activator synthesis	(+)	(+)	(+)	+	–	+
Tumorigenicity in nude mice	–	–	–	–	–	+

in the cell they are localized mostly with the nuclear matrix (R.Eisenman, personal communication).

Among the remaining *in vitro* properties which we examined, a reduction in the level and organization of cell surface fibronectin was found to be common to the *myc* virus- and RSV-transformed cells. All of the cells except those transformed by CMII acquired the ability to form colonies in semi-solid medium. This agrees with our previous observation that CMII-transformed macrophages are more normal than macrophages transformed by the other *myc*-containing viruses (Graf *et al.*, 1977). It is unlikely that this is due to different modes of expression, since the *myc*-encoded protein from CMII appears to be synthesized at similar levels as the MC29 protein and is also translated from genomic RNA as a *gag-myc* polyprotein (Bister *et al.*, 1979; Hayman *et al.*, 1979). Lastly, fibroblasts transformed by the various *myc*-containing viruses differed from one another with respect to their levels of plasminogen activator: although all *myc* virus-transformed cells were positive, only MH2 cells showed activities which were similar to those seen in RSV-transformed cells. Whether or not this is related to the presence of the second (putative) oncogene in MH2 (D.Stéhelin and K.Bister, personal communication) remains to be determined.

E26-transformed fibroblasts differed from *myc* virus-transformed cells with respect to several phenotypes: they were not induced to proliferate at a faster rate, nor did they exhibit prominent nucleoli. In addition, the foci induced by E26 virus were extremely diffuse and the cells had a nearly normal fibronectin distribution. However, the E26- and the

myc virus-transformed cells (not including CMII) were similar with respect to certain other transformation-associated properties: they were able to form colonies in semi-solid medium, showed a loss of organized actin cables, a transformed morphology, and secreted low levels of plasminogen activator. Recently, Leprince *et al.* (1983) found that the E26 genome contains two putative oncogenes: *myb*, which is also present in the avian myeloblastosis virus (AMV), and *ets*, which had not previously been detected. It is not yet known which of these two genes is responsible for the fibroblast transforming capacity of E26, although the fact that AMV cannot transform fibroblasts suggests that the *ets* gene encodes this function.

We also examined the ability of the different virus-transformed fibroblasts to form tumors in nude mice. Fibroblasts transformed by the *myc*-containing viruses and by E26 all proved to be non tumorigenic. This result is consistent with our previous observation that chick embryo fibroblasts transformed by MC29 were non-sarcomagenic in syngeneic animals (Royer-Pokora *et al.*, 1978) and with recent studies by Enrietto *et al.* (1983), who were unable to induce sarcomas in chickens injected with MC29 or with various mutants exhibiting a high fibroblast transforming potential. Furthermore, it is known from previous work that the latency of tumors induced by *myc*-containing viruses usually exceeds 2 months. This is significantly longer than the latency of acute leukemia viruses such as AEV, AMV or E26, or that of avian sarcoma viruses (Graf and Beug, 1978). Taken together, these results suggest that the *myc* gene alone causes some changes characteristic of *in vitro* transformed cells and that additional changes are necessary to generate neoplastic cells.

As mentioned in the Introduction, experiments performed in rodent cells have led to similar conclusions. However, unlike in avian cells, cloned *myc* DNA does not appear to induce any morphological or proliferative changes in 3T3 cells or in primary rat embryo fibroblasts. Instead, it was proposed that the *myc* gene promotes the process of cell line establishment in rodents (Land *et al.*, 1983). In contrast to these results, certain pseudotypes of MC29 have been reported to induce foci in Rat-1 cells, an established line of rat embryo fibroblasts (Quade, 1979). Further experiments will be necessary to clarify the influence of the host species, the type of cells used (primary cultures *versus* established cell lines) and the mode of introduction of the *myc* gene into cells, on the biological effects of this oncogene.

Materials and methods

Cells and viruses

Japanese quail embryo fibroblasts were prepared from 11-day-old embryos from our Heidelberg flock. Cells were grown in Dulbecco's medium containing 10^{-2} M HEPES pH 7.3, 8% fetal calf serum, and 2% heat-inactivated chicken serum (Gibco). The origin of the viruses MC29 (Graf, 1973), CMII (Graf *et al.*, 1977), MH2 (Hu *et al.*, 1978), OK10, and E26 (Graf *et al.*, 1979) have all been described previously. The Schmidt Ruppin-1 strain of Rous sarcoma virus (RSV) and RAV-1 were originally provided by P.Vogt.

To obtain pseudotypes of MC29, CMII, OK10 and MH2 viruses, non-producer chick embryo fibroblasts transformed by each of the virus strains were superinfected with RAV-1. [For the E26 (RAV-1) pseudotype, non-producer chicken myeloblasts were used instead of fibroblasts.] Viral stocks were obtained by collecting supernatants from these cultures 5 – 7 days after superinfection.

Preparation of virus-transformed mass cultures

Primary quail embryo fibroblast cultures were infected with the viral stocks described above, passaged until they appeared completely morphologically transformed (3 – 5 times over the next 10 days), and then examined for the expression of transformation-associated properties. RAV-1-infected control fibroblasts were treated in the same way. To assess their morphology, fully transformed cultures were trypsinized, seeded at 2 x 10^5 cells per 35 mm dish and then photographed the next day after removing the medium and placing a coverslip over the cells.

Assay for transforming parameters

The following assays were carried out according to previously described procedures: determination of cell growth rate (Royer-Pokora *et al.*, 1978); distribution of fibronectin and actin cables, caseinolysis assay, colony formation in methylcellulose (Palmieri *et al.*, 1982), and focus formation under agar (Graf, 1973).

Tumorigenicity in nude mice

The use of nude mice to determine the tumorigenic potential of virus-transformed avian cells has been described previously (Kahn *et al.*, 1982). Briefly, transformed cells were thawed from liquid nitrogen and grown in culture for several days. The cells were then trypsinized and 5 – 10 x 10^6 cells suspended in phosphate buffered saline were injected s.c. into 4 – 6-week-old BALB/c nude mice. Animals were monitored for tumor formation for 4 months following the injections.

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