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ARTICLES

Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes

Hartmut Land, Luis F. Parada & Robert A. Weinberg

Center for Cancer Research and Department of Biology, Massachusetts Institute of Technology, and Whitehead Institute for Biomedical Research, Cambridge, Massachusetts 02139, USA

Transfection of embryo fibroblasts by a human ras *oncogene does not convert them into tumour cells unless the fibroblasts are established and immortalized before transfection. The embryo fibroblasts become tumorigenic if a second oncogene such as a viral or cellular* myc *gene or the gene for the polyoma large-T antigen is introduced together with the* ras *gene.*

A LARGE and varied body of evidence indicates that carcinogenesis is a process involving multiple, independent steps. Epidemiological studies have suggested that cancer arises in proportion to a multiple power of elapsed lifetime. Pathological studies show that tumours progressively acquire new phenotypes by passing through a series of distinct stages such as anaplasia, metaplasia and neoplasia. Moreover, in many systems, experimental induction of a tumour requires at least two distinct types of stimulus, such as an initiator and a promoter (for example, see refs 1–5).

The evidence seems to be more equivocal at the cellular level. Although primary cultures of rodent cells can apparently become tumorigenic in a single step after infection *in vitro* by tumour viruses such as polyoma and adenovirus, this departure from multi-step carcinogenesis is more apparent than real. Recent studies have shown that each virus carries at least two genes encoding distinct functions, both of which must be expressed in order to realize the tumour cell phenotype[6–8]. Such work suggests that multi-step carcinogenesis might have an explanation at the genetic level: each step may require the activation of a distinct gene and the final phenotype may require the concomitant expression of many of the previously activated genes.

Other precedents support a model of multiple, cooperating, independently-activated genes. Induction of bursal lymphomas by avian leukosis virus seems to require the activation of two separate oncogenes during lymphomagenesis. The *myc* gene becomes activated by adjacent insertion of a provirus[9,10], while the B-*lym* gene acquires activity via a second, distinct mechanism whose nature is unclear[11]. This theme has been echoed in our own laboratory in a study of a promyelocytic leukaemia and an American Burkitt's lymphoma: in each case, the tumour cells carry altered versions of the *myc* gene, as well as activated versions of a second cellular oncogene, termed N-*ras*[12].

In apparent exception to this model of multiple genetic alteration, other studies have shown that a single oncogene can impart morphological alteration and tumorigenicity to NIH 3T3 mouse fibroblasts. The oncogene is usually introduced into the NIH 3T3 cells via calcium phosphate-mediated DNA transfection, which often results in establishment of multiple copies of the oncogene in the recipient fibroblasts[13] although a single copy of the gene is enough to produce full transformation (C. Tabin and R.A.W., unpublished results).

One possible explanation of this paradox is that NIH 3T3 cells, which were chosen because of their particular competence in taking up and expressing exogenous DNAs[14], behave abnormally in their response to oncogenes. These NIH 3T3 cells had been established (that is, adapted to grow indefinitely in monolayer culture) and then passaged extensively *in vitro*[15]. Thus, it seemed likely that they would deviate substantially

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Table 1 Transformation of Rat embryo fibroblasts and Rat-1 cells following transfection of the EJ c-Ha-*ras*-1 oncogene

Cells	Transfected DNA	Colonies or foci per 10^6 transfected cells: No of foci normal medium	*Ecogpt* selection medium monolayer: No. of colonies	*Ecogpt* selection medium monolayer: % Of colonies with morphologically transformed cells	Normal medium soft agar	Tumorigenicity in nude mice (no. of tumours/no. of injections)
3° REF	pSV2gpt	0	150	0	0	0/10
3° REF	pEJ6.6+pSV2gpt	0	200	80–90	200	0/11
Rat-1	pSV2gpt	0	1,200	0	0	0/5
Rat-1	pEJ6.6+pSV2gpt	2,400	1,200	40–50	2,400	6/6

Primary cultures of REFs were prepared as described elsewhere[60] from 12–14-day-old Fisher rat embryos; 3–4 days later the cells were passaged and 1.2×10^6 cells were seeded onto 100 mm Petri dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Hyclone)(normal medium). Parallel cultures of the Rat-1 cell line[29] were seeded at a density of 5×10^5 cells (normal medium) so that 18–24 h later both types of culture plated at similar cell densities (8×10^5–1.2×10^6 cells per dish). Transfections were carried out as described previously[26,61] using 75 μg REF carrier DNA, 10 μg pEJ6.6 DNA and 1 μg of pSV2gpt DNA per 2×10^6 cells (2 dishes). After 24 h, the transfected cells were pooled. The REFs and Rat-1 cells were split in a ratio of 1:3 and 1:10, respectively. One day later half of the cultures were subjected to mycophenolic acid selection[27]. Cultures were re-fed every 4 days. For plating in soft agar, 10^6 cells were seeded 36 h after transfection into normal medium containing 0.3% low-melting agarose (Sea Plaque). Foci or colonies were counted 14–16 days after transfection. To test for *in vivo* growth potential, the transfected cells were collected 16–18 days after transfection, washed with phosphate-buffered saline (without Ca^{2+}), and injected subcutaneously into 30–40-day-old nude mice. The mice had been irradiated (500 rad) 24 h before injection to eliminate natural killer cells. The cell content of one dish (5×10^6 cells) was used for a single injection. Cells which had been subjected to *Ecogpt* selection were adjusted to normal culture conditions 2–3 days before they were injected. This was done by re-feeding with *Ecogpt* selection medium[27] without mycophenolic acid and aminopterin. Finally, cells from one of these dishes were mixed with 5×10^6 untransfected 2° REFs and used for a single injection. The animals were observed for tumour formation on a weekly basis for 4 weeks. Tumours appeared between 7 and 10 days after injection.

from normal target cells for oncogenes *in vivo*. For example, the NIH 3T3 cells may, as a result of *in vitro* establishment, have acquired several alterations usually developed by a cell during its tumorigenic progression. These alterations may predispose the NIH 3T3 to tumorigenic conversion by a subsequent single-hit event. Thus, for the studies reported here we switched to rat embryo fibroblasts, reasoning that these cells more closely resembled normal targets of carcinogenic alteration.

Incomplete transformation by *ras* oncogene

The transforming gene used in our initial experiments was isolated from the human EJ bladder carcinoma cell line as a molecular clone termed pEJ6.6 (ref. 16). It represents a variant of the human c-Ha-*ras*1 proto-oncogene[17–19], and encodes a 21,000-molecular weight protein[17,20,21]. This EJ *ras* oncogene stands as a model for other human oncogenes as it is a member of the *ras* gene family and is thus closely related to the Ki-*ras* and N-*ras* oncogenes found to be active in several different tumour types[12,17–19,22–25].

Copies of the cloned oncogene were introduced into recipient cells using the calcium phosphate transfection procedure of Graham and van der Eb[26]. Mouse embryo fibroblasts were tested initially as recipients but were found to be unsatisfactory because of difficulties in detecting clones of stably transfected cells. Instead, we used secondary rat embryo fibroblasts (REFs) prepared from 12–14-day-old Fisher rat embryos. In the conditions of the focus assay (see Table 1 legend), no foci of morphologically altered cells were observed 14–21 days after transfection. These results were not due to an inability of the secondary (2°) REFs to take up and express exogenous DNA because when cultures of 10^6 2°REF cells were exposed to DNA of the Ecogpt clone[27] that serves as a dominant marker conferring resistance to growth inhibition by mycophenolic acid, 150 colonies were observable 14 days after transfection.

To examine further those cells in the culture that had taken up the EJ *ras* oncogene, but not yielded any obvious foci, we transfected the oncogene together with the *Ecogpt* marker and grew the cells in the presence of mycophenolic acid. (Cotransfected markers become incorporated together into competent cells in culture[28].) Of the resultant mycophenolic-acid-resistant colonies, 80–90% contained morphologically transformed cells (Fig. 1). Cells of the surrounding monolayer remained sparse and were unable to grow as they lacked resistance to the drug.

A second culture condition also permitted phenotypic expression of the introduced EJ *ras* oncogene. 2° REFs were exposed to DNA of the EJ *ras* oncogene, passaged, and introduced into soft agar suspension culture 36 h after transfection. The resultant 3° REF cultures formed 200 soft agar colonies per 10^6 initially transfected cells. In contrast, no discernible colonies (>8 cells per colony) were found after agar culture of untransfected 3° REFs.

We compared the behaviour of these 3° REFs with that of cells of an established line that might behave similarly to NIH 3T3 cells. We chose the Rat-1 cell line that originated from Fisher rat embryo fibroblasts[29]. When cultures of these cells were exposed to the EJ *ras* oncogene, large numbers of foci were seen (2,400 foci per 10^6 cells) whether or not we used *Ecogpt* DNA-cotransfection followed by selection with mycophenolic acid (Table 1). A control experiment demonstrated that the transfected Rat-1 cells could form foci in conditions closely resembling those which did not permit focus formation by the 3° REFs. 500 EJ *ras*-transformed Rat-1 cells were mised with 7.5×10^5 3° REFs and seeded in conditions identical to those of the earlier experiments; 20–30 foci were observed for every 100 transformed Rat-1 cells seeded into the REF monolayer. Thus, in virtually identical culture conditions, the transfected REFs were unable to form foci, while their Rat-1 counterparts did so quite efficiently (Table 2).

We found other contrasts between the behaviour of the transfected 3° REFs and Rat-1 cells. Oncogene-bearing cells could be recovered from *ras–Ecogpt*-cotransfected colonies of both types. The transformed Rat-1 cells yielded rapidly growing cell lines whereas colonies of the transformed 3° REFs entered cell crisis immediately on repassaging (they usually grew to a size of 500–5,000 cells, and then lost any further ability to divide).

Nude mice were inoculated with various transfected cultures and monitored for subsequent appearance of subcutaneous tumours. In one case the inoculated culture came from REF cells exposed to DNA of the EJ *ras* and *Ecogpt* clones and selected with mycophenolic acid: 80 resulting colonies, which contained in aggregate 5×10^4 transformed cells, were mixed with 5×10^6 untransfected 3° REFs before inoculation. This inoculum yielded only small, subcutaneous, cartilaginous nodules (average diameter 3 mm) 3 weeks after injection. In contrast, when 5×10^4 Rat-1 cells carrying the EJ *ras* oncogene were inoculated together with 2×10^6 untransfected Rat-1 cells,

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the mixture induced rapidly growing fibrosarcomas which were easily observable after 1 week, and reached a size of 4 cm after 3 weeks.

It was clear that the EJ *ras* oncogene had only circumscribed powers. It was unable to impel transfected REFs into focal expansion in dense monolayer culture; the transfected cells had only limited proliferative ability; and they were not tumorigenic when seeded into host animals. However, activities that the oncogene lacked could be supplied by cellular functions existing in the Rat-1 cells prior to transfection. We concluded that the process of *in vitro* establishment provided the Rat-1 cells with functions that collaborate with the EJ *ras* oncogene to create a competent tumour cell.

Cooperative effects of *myc* and *ras*

We next wished to determine whether specific cellular functions, and by implication cellular genes, could work together with EJ *ras* to create a tumour cell. The cellular *myc* gene represented a candidate for such a cellular gene. As mentioned above, altered versions of this gene coexist with an active EJ *ras* oncogene in at least two different human tumour cell lines[12].

We tested the *myc* gene in the form of the oncogene carried by the avian MC29 virus. A molecular clone of its provirus, termed pv-*myc* (Fig. 2), was provided by J. M. Bishop[30]. The high degree of evolutionary conservation of *myc*[31-33] suggested that the MC29 oncogene, while originating from the avian genome, might nevertheless be able to function in rat cells. A complete provirus clone was generated by joining a circularly permuted provirus clone[29] to an additional proviral segment at its right (3′-proviral) end. This created tandemly duplicated long terminal repeat (LTR) segments at the left (5′-proximal) and at the right (3′-proximal) ends of the provirus (termed psVv-*myc*; see Fig. 2). Initial experiments were designed to test the biological properties of these v-*myc* clones. Each DNA was applied to NIH 3T3 cells, to Rat-1 cells, or to 2° REFs, either alone or together with DNA of the Ecogpt clone. We observed no obvious effect on these transfected cultures.

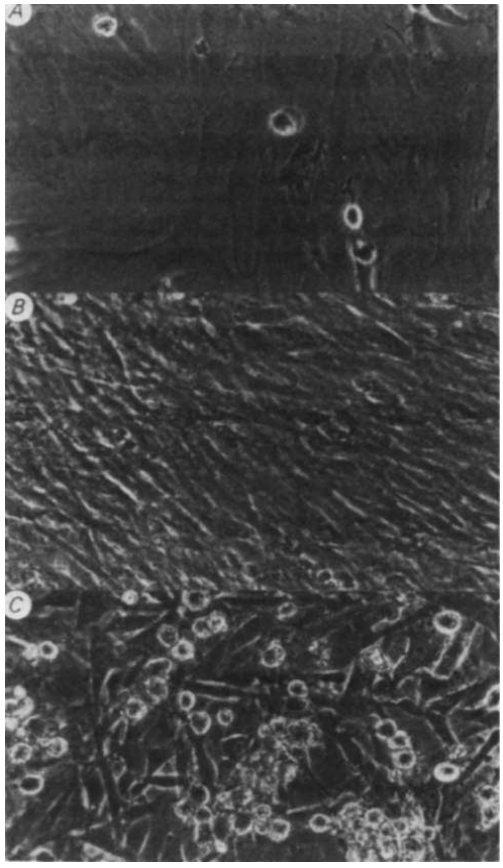

Fig. 1 Phase-contrast photomicrographs of REFs. (×70). *A*, Untransfected non-dividing 3° REFs forming a sparse monolayer in *Ecogpt* selection medium. *B*, Mycophenolic acid-resistant clone of REFs after transfection of pSV2gpt. *C*, Mycophenolic acid-resistant clone of REFs after cotransfection of pEJ6.6 and pSV2gpt, showing morphologically transformed cells. The DNA transfection was performed as described in Table 1 legend. Photographs were taken 14 days after transfection.

Table 2 Focus-forming ability of transfected Rat-1 cells or REFs after seeding into large excess of untransfected 3° REFs

Origin of cell clones seeded into REF monolayer			No. of foci per 100 seeded cells	
Original recipient cell	Introduced oncogene	Clone designation	Normal medium	*Ecogpt* selection medium
Rat-1	*ras*	1	20	9
Rat-1	*ras*	3	28	40
REF	*ras*	1 S	0	0
REF	*ras*	4 S	0	0
REF	*ras*	6 S	0	0
REF	*ras*	7 S	0	0
REF	*ras*	9 S	0	0
Rat-1	*ras–myc*	1	19	50
Rat-1	*ras-myc*	2	19	41
Rat-1	*ras-myc*	3	26	6
REF	*ras-myc*	1 N	38	38
REF	*ras–myc*	7 N	44	0
REF	*ras–myc*	3 S	55	68
REF	*ras-myc*	8 S	30	42

500 cells of a clone of Rat-1 cells or REFs which had been isolated after cotransfection of pSV2gpt and the human EJ c-Ha-*ras*1 clone, pEJ6.6 (designated *ras* clones), or after cotransfection of pSV2gpt, pEJ6.6 and pSVv-*myc* (designated *ras–myc*) (see also Tables 1 and 3) were mixed with 7.5×10^5 untransfected 3° REFs and seeded onto 10-cm dishes in either normal medium or *Ecogpt* selection medium. Focus formation was observed 6 days after plating. Foci were counted 12 days after seeding. N, Cell clone derived from a focus of transformed cells that had originally been isolated from a dish containing normal medium. S, Cell clone derived from a focus of transformed cells that had originally been isolated from a dish containing *Ecogpt* selection medium. Cells from all these clones were carried briefly in normal medium before being used in this assay (see also Table 1).

Subsequent experiments tested the effects of the *myc* and EJ *ras* oncogenes introduced together into 2° REFs. In conditions in which either EJ *ras* or *myc* alone had no obvious effect on the monolayer cultures, the two genes together achieved a dramatic alteration of phenotype. Rapidly growing foci of morphologically altered cells (Fig. 3) became apparent within 8 days after transfection, whether or not the Ecogpt-cotransfection/mycophenolic acid selection protocol was followed. Such foci were able to expand into the surrounding monolayer, in all culture conditions.

When foci carrying the EJ *ras* and *myc* genes were picked, they yielded rapidly growing cultures (doubling time <24 h) of morphologically transformed cells. This contrasted with the poor growth of cells carrying only the EJ *ras* gene (see above). These cotransfected cells were tumorigenic when introduced into nude mice or 12-day-old Fisher rats, yielding tumours that grew to a diameter of 1 cm 2 weeks after inoculation. Southern blot analysis of the DNAs from five of these lines confirmed the presence of multiple copies of both the transfected EJ *ras* and v-*myc* segments in these DNAs (data not shown).

These results led us to the tentative conclusion that the effects of *in vitro* establishment could be mimicked, at least in part, by introduction of the active *myc* oncogene. Both establishment and *myc* acquisition made the REFs highly reactive to the transforming effects of the EJ *ras* gene. However, a subtle and potentially significant difference emerged. The EJ *ras–myc* cotransfectants formed a tumour that reached a static size of 2 cm after 3 weeks of rapid expansion in the nude mouse hosts. In contrast, the established Rat-1 cells carrying the EJ *ras* gene induced tumours that expanded until they killed the host. We

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interpret this as follows: the *myc* gene greatly enhances the phenotype created by the EJ *ras* oncogene, but is nevertheless unable to fully mimic the cellular traits conferred by establishment and immortalization *in vitro*.

Further characterization of *myc* gene

The above results caused us to examine in more detail those properties of the *myc* clone that allowed it to cooperate with the EJ *ras* oncogene. We showed that the LTRs, each one of which carried promoter and enhancer sequences[34], were not alone responsible for the observed activity of the clone, by deleting a 1.6-kilobase (kb) *Sac*I fragment that lies in the protein-coding sequences of the *gag–myc* viral oncogene within pSV-v-*myc* (Fig. 2). When cotransfected with the EJ *ras* oncogene DNA, this modified provirus induced no detectable phenotype. Thus, activity of the *myc* clone depended on synthesis of part or all of the *gag–myc* protein.

Because the v-*myc* gene originated from the chicken genome, we thought that its activities might reflect the idiosyncracies of an avian gene acting in a foreign cellular environment. Therefore, we developed an active clone of a rodent *myc* gene. A mouse *myc* clone, isolated by Shen-Ong and colleagues[35], originated from DNA of the mouse plasmacytoma MOPC 315, in which a chromosomal translocation caused a *myc*–immunoglobulin gene juxtaposition. This immunoglobulin–*myc* hybrid clone was modified by removing the immunoglobulin gene and placing the remaining *myc* segment under control of an early simian virus 40 (SV40) transcriptional promoter present in the SV2 *gpt* vector[27]. This construction (termed pSVc-*myc*-1; Fig. 2) placed the transcriptional promoter and translation initiation codon 40 nucleotides apart.

This SV40–*myc* chimaera was indistinguishable from the avian MC29 provirus in its ability to cooperate with a cotransfected EJ *ras* oncogene (Table 3). Optimal expression of this activity seemed to require that the transcription of the *myc* gene be driven directly by the SV40 promoter. When this promoter was introduced downstream of the gene, and in the opposite transcriptional polarity (pSVc-*myc*-2; Fig. 2), the number of colonies induced on cotransfection with a *ras* clone decreased by a factor of 10. A c-*myc* clone (termed pc-*myc*; Fig. 2) lacking any added promoter/enhancer showed no biological activity at all in the cotransfection assay (Table 3). Because of the functional equivalence of the avian and murine *myc* clones, we used the avian MC29-derived construct in further studies (see below).

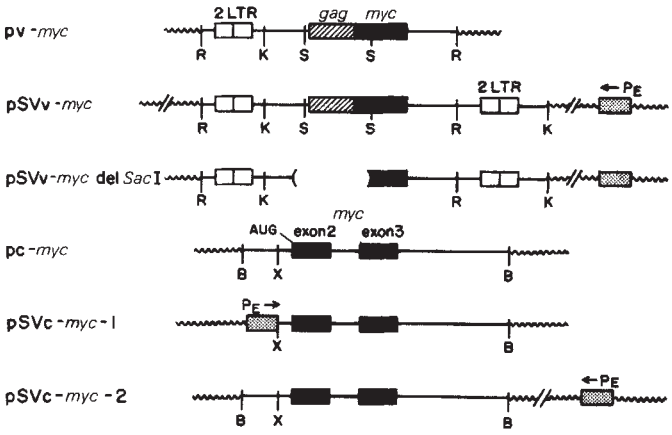

Fig. 2 Schematic diagram of plasmids containing various *myc* genes used in the cotransfection of 2° REFs: pv-*myc* contains a permuted copy of the avian MC29 provirus DNA (5.5 kb)[30] (given by J. M. Bishop). To provide a polyadenylation signal, the circularly permuted provirus clone was completed by duplication of the 5′-proximal 1.1-kb *Eco*RI–*Kpn*I restriction fragment at the 3′-proximal end of the provirus. To achieve this, the *Eco*RI–*Kpn*I fragment, which contains the two LTRs, was subcloned into the *Eco*RI and *Kpn*I sites of pSV2gpt[27]. In a second step, the *Eco*RI fragment containing the entire permuted proviral DNA was ligated into the modified vector. In the resulting plasmid, termed pSVv-*myc*, the SV40 early promoter (P_E) has opposite polarity to the direction of transcription of the proviral DNA. The pSVv-*myc* del plasmid, a further modification of the pSVv-*myc* described above, had a 1.6-kb *Sac*I fragment deleted by cleavage with this enzyme and recircularization by ligation. This deletion removes a large portion of the sequences which encode the *gag–myc* fusion protein. pc-*myc* (given by M. Cole) contains the second and third exons of the cellular mouse *myc* gene within a 5.6-kb *Bam*HI fragment. It originated from DNA of the mouse plasmacytoma MOPC 315, in which this *myc* gene has been translocated into the immunoglobulin C_α locus[35]. These immunoglobulin sequences were removed during construction of pc-*myc*. To express the mouse cellular *myc* gene of the pc-*myc* plasmid, we created a plasmid, termed pSVc-*myc*-1, in which transcription of the *myc* gene is driven by the SV40 early promoter (P_E). By linker ligation, an *Xba*I site was added to the *Hin*dIII site of pSV2gpt[27], then the 4.8-kb *Xba*I–*Bam*HI fragment of the pc-*myc* insert was cloned into this vector, separating the transcriptional start and AUG codon by 40 nucleotides. pSVc-*myc*-2 contains the *Bam*HI insert of pc-*myc* in pSV_2gpt in opposite transcriptional orientation to the SV40 early promoter/enhancer sequences.

Complementation groups

The ability of the EJ *ras* and *myc* genes to cooperate with one another led us to test whether other genes could be placed in complementation groups based on their activities in cooperating with either EJ *ras* or *myc* in the REF focus assay. An initial test measured the ability of the N-*ras* oncogene to behave like the Ha-*ras* oncogene (EJ *ras*) in its ability to cooperate with *myc*. A biologically active clone of this gene was isolated recently in this laboratory from HL 60 cells (J. M. Cunningham, in preparation). The two genes encode similar proteins. As expected, the N-*ras* gene behaved identically to the Ha-*ras* gene in its ability to cooperate with *myc* (Table 3). The two *ras* genes were therefore placed in the same complementation group.

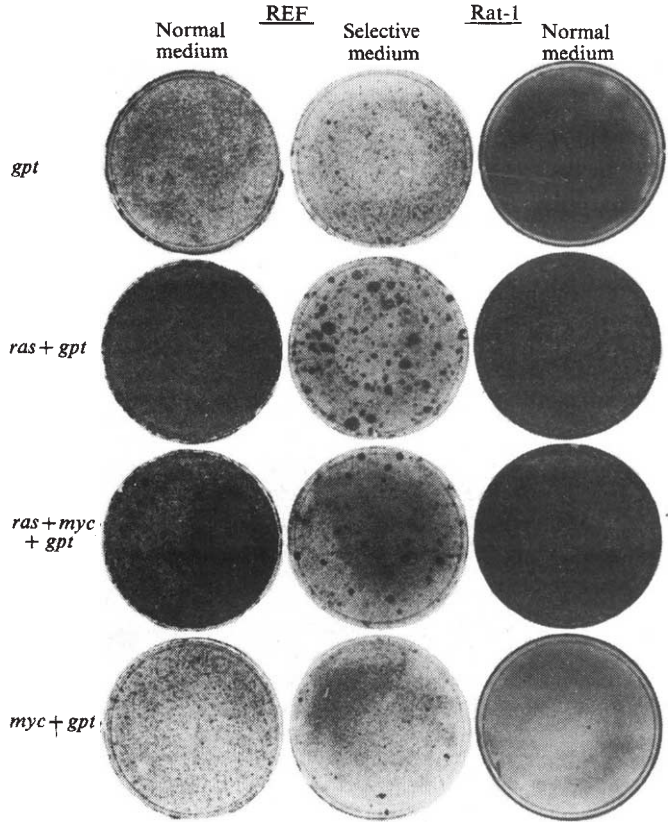

Fig. 3 Crystal violet-stained 3° REF and Rat-1 cell cultures photographed 14 days after transfection. Examples of the focus assays described in Tables 1 and 3 are shown. *gpt* Represents transfection of pSV2gpt DNA; *ras*, transfection of pEJ6.6 DNA; *myc*, transfection of pSVv-*myc* DNA.

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Table 3 Complementation of transformation by cotransfection of different oncogenes into REFs

Transfected oncogene clones	Normal medium	*Ecogpt* Selection medium		Tumorigenicity in nude mice (no. of tumours/ no. of injections)
	Foci per 10^6 cells	No. of colonies per 10^6 cells	% Of colonies with morphologically transformed cells	
pSVv-*myc*	0	100	0	0/7
pSVc-*myc*-1	0	120	0	0/6
pEJ6.6+pSVv-*myc*	200	200	80	10/10
pEJ6.6+pSVv-*myc* del *Sac*I	0	200	80	0/9
pEJ6.6+pv-*myc*	80	200	80	7/7
pEJ6.6+pc-*myc*	0	200	80	0/11
pEJ6.6+pSVc-*myc*1	220	220	80	9/9
pEJ6.6+pSVc-*myc*2	25	200	80	6/6
Φ N-*ras**	0	140	15	0/5
Φ N-*ras**+pSVv-*myc*	25	140	20	5/5
pPyMT1	0–10	75	15	0/6
pLT214	0	150	0	0/5
pPyMT1+pSVv-*myc*	60	100	15	2/5
pLT214+pSVv-*myc*	0	100	0	0/3
pEJ6.6+pPyMT1	0–10	70	10	0/5
pEJ6.6+pLT214	200	200	80	6/6

Cultures of 2° REFs were transfected by combinations of different cloned oncogenes as indicated. In each transfection, 1 μg of pSV2gpt per 2×10^6 cells was transfected together with 10 μg of each oncogene-carrying plasmid. Focus, colony, and tumorigenicity assays are described in detail in Table 1 legend. The data represent a mean of several experiments for each test.

* 2 μg of phage DNA were used.

We then tested the two viral genes encoding the middle- and large-T antigens of polyoma virus, isolated as separate molecular clones by Kamen and colleagues[36,37]. As reported by Rassoulzadegan *et al.*[6], these genes confer distinct and separable phenotypes on rat cells. The middle-T antigen induces morphological alteration and anchorage independence, while the large-T antigen affects serum dependency and cell immortalization.

Transfection of the middle T clone pPyMT1 either alone or together with the EJ *ras* DNA induced no obvious foci in our culture conditions. In contrast, cotransfection of middle -T and *myc* allowed outgrowth of dense foci. When these cultures were tested for tumorigenicity, they were found to induce tumours in only two of five animals. The remainder had only small nodules at the site of inoculation. Thus, middle-T behaved, in this experiment, similarly to but not identically with EJ *ras*, and can tentatively be assigned to the same complementation group as the *ras* oncogenes.

Manipulation of the large-T oncogene clone was more difficult. Initial experiments showed that it strongly inhibited establishment of cotransfected genes (data not shown), which may have been due to the presence of the polyoma replication origin together with the gene for the entire large-T antigen. This might allow the large-T antigen to trigger repeated rounds of viral DNA replication and in turn create a cytopathic effect. To avoid this problem, we chose a clone, termed pLT214, that carries a truncated version of the large-T antigen[6]. This clone, a gift from R. Kamen and colleagues, allowed synthesis of the N-proximal half of the large-T antigen implicated in alteration of serum dependency and immortalization[6]. The encoded large-T antigen lacked the C-proximal half, which is required for mediating viral DNA replication.

Transfection of the truncated large-T-antigen clone alone had no obvious effect on morphology of the 3° REF monolayer. The same result was obtained on cotransfection of this clone with the *myc* clone, pSVv-*myc*. However, when this altered large-T clone was cotransfected with EJ *ras*, dense foci appeared after 10 days. When cotransfected cultures were inoculated into six nude mice, rapidly growing tumours were found in all of them. These tumours reached a diameter of 4 cm after 3 weeks. Unlike the *myc*–EJ *ras*-induced tumours which stopped growing after reaching a diameter of 2 cm, these tumours continued to grow until they killed the host animals.

These data indicate that the truncated large-T gene behaves like *myc* in its ability to cooperate with a *ras* gene. However, the two genes do not function identically: *myc* allows growth of a large tumour of limited size, while the truncated large-T allows unlimited tumour growth.

Generality of experimental model

The present results raise several questions. Do these results apply only to fibroblast transformation induced by an arbitrarily chosen set of oncogenes? Or are the present results representative of processes that lead to many kinds of tumours?

One suggestion of possible generality comes from examination of the cellular oncogenes used here. *ras* and *myc* genes have been implicated as determinants in a wide variety of tumours. Thus active *ras* oncogenes have been found in bladder, colon, lung, pancreatic and skin carcinomas, neuroblastomas, sarcomas, and at least three types of haematopoietic malignancies[17–19,23–25,38]. The cellular Ki-*ras* and Ha-*ras* genes encode almost identical gene products[39,40]. Preliminary sequence analysis of a third member of this gene family, termed N-*ras*[12,24,25], indicates encoded amino acid sequences very similar to those of the other two members of the gene family (J. M. Cunningham, in preparation). We consider it likely that all members of this gene family act in a similar, if not identical fashion, and that the EJ c-Ha-*ras*1 allele used in most of the present experiments represents a good model of all members of this gene family.

The *myc* gene is similarly involved in a range of tumours. It is associated with haematopoietic diseases such as myelocytomatosis[41], myeloid leukaemia[42–44], bursal lymphomas[9,10], Burkitt's lymphomas[42,45,46] and plasmacytomas[35,42,46–48]. Its involvement in non-haematopoietic disease is also well documented. Viruses that transduce the v-*myc* oncogene are known to induce kidney, pancreatic and liver carcinomas, and mesotheliomas[41], and recent work shows the gene to be present in amplified copy number in a neuroendocrine tumour of the colon[49].

These diverse data suggest that neither type of gene is a 'tissue-specific' oncogene having a narrow range of tissue tropism. Instead, each type of gene appears to be competent in a variety of cellular environments. Moreover, the creation of disparate types of tumours seems to depend on common

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molecular mechanisms frequently involving *ras* and/or *myc* oncogene activation. Because of the parallel behaviours of many tissues to these oncogenes, we believe that the fibroblasts used in this study represent credible models for a variety of target cells present in many tissues throughout the body.

Validity of the focus assay

The particular REF monolayer culture conditions used here do not allow focus induction by a *ras* oncogene (EJ *ras* or HL 60 N-*ras*) acting alone. However, by varying conditions, such as the frequency of passage and seeding density, we have been able to reveal focus-inducing ability by *ras* or polyoma middle-T oncogenes acting alone (unpublished results). Therefore, the conditions used in the present experiments have been chosen to demonstrate the limited powers of the singly acting oncogenes.

This choice of focus-assay conditions is vindicated by the use of other, independent measurements of oncogene activity. These other measurements—ability to grow in long-term culture and tumorigenicity—yield similar conclusions to those obtained from use of the focus assay. In all cases, the oncogenes of the *ras* group emerge as genes having potent but circumscribed abilities.

ras and *myc* act differently

The present results show that a *ras* oncogene is able to alter the morphology of REFs in monolayers in which the growth of surrounding cells has been suppressed, and can also induce colony growth in soft agar. However, the *ras* oncogene is unable to induce obvious foci in the midst of densely growing normal cells, and the REFs transformed by *ras* oncogene exhibit very limited proliferative and tumorigenic abilities. This would seem to contrast with the tumorigenic abilities of Ha- and Ki-murine sarcoma viruses, that transduce very closely related *ras* genes[50] and appear to be able to induce tumours with single-hit kinetics. We suggest that the *in vivo* steps in viral tumorigenesis are poorly understood, and may depend on additional cellular alterations beyond the acquisition of an exogenous oncogene.

The *myc* oncogene, when acting alone, has no effects on REFs that we can discern. Thus we cannot confirm other reports of rodent fibroblast transformation by the avian *myc* oncogene[51,52]. We suggest that the reported transformations may stem from rare events that depend on secondary, cooperating cellular alterations.

One point that emerges from the present work is that *ras* and *myc* oncogenes act differently, because together they are able to achieve phenotypes that neither is able to achieve alone. It would seem that their modes of action differ qualitatively, and that each impinges on a distinct cellular target. The study of the *myc* oncogene has been hampered until now by the absence of any induced phenotype in mammalian cells. The cotransfection test described here may provide a useful technique for assaying the *myc* oncogene and related genetic segments.

Effects of *in vitro* establishment

It would appear that the processes of establishment and immortalization lead to development of cellular functions that are able to cooperate with the *ras* oncogene. We do not know how many separate cellular functions are required for development of the fully tumorigenic phenotype. However, it appears that the established cells carry all necessary functions except those provided by the *ras* oncogene, whose introduction produces full transformation with single hit kinetics.

Previously we had chosen to work with the established NIH 3T3 cells because of their efficient ability to take up and fix murine leukaemia virus DNAs[14]. Now it has become apparent that these cells also possess additional traits that make them unusually reactive to introduced oncogenes. Thus these cells are useful as indicators of the presence of certain transforming oncogenes; they cannot be considered good models of normal target cells in the animal.

In experiments not described here we have introduced the (EJ) c-Ha-*ras*1 oncogene into Sprague–Dawley 2° REFs and only subsequently undertaken establishment of the transformed cells *in vitro*. These cells have acquired the tumorigenic phenotype. We conclude that the temporal order of the two events—*ras* oncogene acquisition and establishment—is not important for the final result.

Designation of separate functional classes

The *myc* oncogene introduction yields a phenotype which mimics in part the effects of *in vitro* establishment; in either case the addition of a *ras* oncogene creates the traits of focus formation and tumorigenesis. This does not mean that the introduction of *myc* and establishment create identical changes in cellular behaviour. Thus, we have no evidence that *myc* is able to immortalize cells, nor do we know whether the process of establishment/immortalization depends on activation of *myc*-like cellular genes. Nonetheless, the two factors are operationally very similar. This allows us to construct a complementation group in which we place *myc* and those functions subsumed under the term 'establishment'. The further inclusion of the large-T in this class might have been anticipated, even before the present results, since this gene had been implicated previously in establishment/immortalization functions[6].

Recently, we have been informed of experiments indicating cooperation between a *ras* oncogene and the *E1a* oncogene of adenovirus 5 (ref. 62). Our own, preliminary experiments confirm that. Such a result is consistent with an earlier report showing that *E1a* acts, like polyoma large-T, to induce immortalization[7]. We assign *E1a* to the functional class that includes *myc*, large-T antigen, and establishment.

The other class of transforming oncogenes presently includes Ha-*ras*, N-*ras* and the polyoma middle-T. We note that this class, implicated in morphological alteration and anchorage independence, encodes gene products that are localized to the plasma membrane[53–55]. In contrast, the identified genes of the other class (*myc*, large-T and *E1a*) specify proteins that bind to nuclear structures[56–59]. This suggests that these functional groupings may reflect underlying common molecular mechanisms, including interaction with common cellular targets.

Finally, how does the activation of multiple oncogenes relate to the observed multi-step process of carcinogenesis? The activation of each oncogene appears to derive from a genetic alteration of low probability. Two of the steps normally required for tumour formation may represent the activation of a *ras*-like and a *myc*-like gene. It is not yet obvious whether these two steps will suffice to convert a normal cell into a fully competent tumour cell. Other steps may be required also. Thus, the cells carrying *myc* and *ras* oncogenes seed tumours that usually reach a large but static size. This contrasts with the behaviour of cells that have acquired a *ras* oncogene and have undergone establishment. Such cells seed tumours of apparently unlimited growth capacity. Perhaps the process of establishment/immortalization yields cellular functions beyond those achieved by *myc* alone. In that case, a third distinct gene may collaborate with *ras* and *myc* in creating the total tumorigenic phenotype. It would seem that the number of distinct steps in tumorigenesis is limited, and that each of these may soon be described at the molecular level.

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Adenovirus early region 1A enables viral and cellular transforming genes to transform primary cells in culture

H. Earl Ruley

Cold Spring Harbor Laboratory, Cold Spring Harbor, PO Box 100, New York 11724, USA

The polyoma virus middle-T and the T24 Harvey ras*1 genes are individually unable to transform primary baby rat kidney cells. Adenovirus early region 1A provides functions required by these genes to transform primary cells following DNA-mediated gene transfer. These results suggest that separate establishment and transforming functions are required for oncogenic transformation of primary cells in culture.*

THE oncogenic DNA viruses encode proteins that are able to transform cells grown in tissue culture[1]. Virus-transformed cells, which can be isolated by their ability to form dense foci on monolayers of untransformed cells, typically retain and express all or some of the viral genes that are expressed early during lytic infection. Both primary and established cells can be transformed by polyoma virus (Py) and human adenovirus (Ad). However, the viral functions required to transform these cell types are different, at least in some cases.

Transformation of primary cells requires at least two separate functions. The first, an establishment function, is concerned with immortalization of cells, while the second, the transformation function, is required for full expression of an oncogenic phenotype[2–5]. Thus, establishment functions expressed by adenovirus early region 1A (E1A) or portions of the polyoma large-T antigen lead to the ability of primary cells to grow indefinitely in culture[2,3]. Additional functions expressed by adenovirus early region 1B (E1B) or the polyoma virus middle-T antigen result in phenotypic changes characteristic of oncogenic transformation[2–5] such as anchorage-independent cell growth and the ability to form tumours when cells are transplanted into syngeneic animals.

By contrast, transformation of established cell lines can require fewer viral functions. Thus, expression of the polyoma middle-T antigen alone is sufficient to transform a variety of cell lines[5]. Apparently, such cell lines constitutively express establishment functions that can substitute for those of the virus. The interaction between establishment and transforming functions is poorly understood, as is the mechanism by which they combine to elicit the transformed phenotype.

Recently, several genes have been isolated from cell lines established from human tumours, that have the ability to cause morphological transformation of the mouse NIH 3T3 cell line[6–10]. However, the ability of cellular oncogenes to transform cultured primary cells has not been critically addressed. Given the requirement for at least two viral functions for transformation of primary cells by polyoma and adenoviruses, it seemed quite likely that cellular oncogenes might also require additional functions. A failure to transform primary cells could be explained if oncogenes that score in the NIH 3T3 assay carry transforming functions but lack establishment functions.

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