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Participation of p53 cellular tumour antigen in transformation of normal embryonic cells

Daniel Eliyahu, Avraham Raz*, Peter Gruss†, David Givol & Moshe Oren

Departments of Chemical Immunology and *Cell Biology, The Weizmann Institute of Science, Rehovot 76100, Israel
†ZMBH, Universität Heidelberg, D-6900 Heidelberg 1, FRG

The cellular tumour antigen p53 is found at elevated levels in a wide variety of transformed cells (for reviews see refs 1, 2). Very little is yet known about the precise relationship of p53 to malignant transformation. Although the increase in p53 levels could be a secondary by-product of the transformed state, it is equally possible that p53 is actively involved in altering cellular growth properties, especially as it has been implicated in the regulation of normal cell proliferation[3-6]. We sought to test whether p53 could behave in a manner similar to known genes in a biological test system, and we demonstrate here that p53 can cooperate with the activated Ha-*ras* oncogene to transform normal embryonic cells. The resultant foci contain cells of a markedly altered morphology which produce high levels of p53. Cell lines established from such foci elicit tumours in syngeneic animals.

Recent findings have suggested certain similarities between p53 and the product of the oncogene *myc*. Both are DNA binding proteins (ref. 7 and D. Lane, personal communication) that accumulate in the nuclei of transformed cells[7,8]. Both are regulated with the cell cycle[6,9,10] and are induced at an early stage following the treatment of resting cells with mitogens[3,5,9,10]. Cycloheximide-treated cells accumulate p53[11] and show increased *myc* messenger RNA levels[9]. Detailed analysis of the amino acid sequences predicted for the two proteins shows weak similarities in both the overall molecular organization and the positioning of charged residues within distinct domains of the molecules[12,13]. Hence, if p53 can function like known oncogenes, it is likely to do so in a manner similar to *myc*.

Table 1 Transformation of rat and Chinese hamster embryo fibroblasts by various gene combinations

Transfected DNA	Foci per 10^6 cells: REF expt 1	REF expt 2	CHEF expt 3	Tumorigenicity
Carrier (BALB/c DNA)	0	0	0	
pEJ6.6	0	0	0	
pMSVp53G	0	0	ND	
pPyp53c	ND	ND	0	
pMSVp53G + pEJ6.6	13	5	ND	15/15
pPyp53c + pEJ6.6	ND	ND	20	
pLSVmyc + pEJ6.6	ND	21	ND	9/9
pLA8 + pEJ6.6	68*	ND	165*	
pMSVE + pEJ6.6	ND	0	ND	
None				0/8

Primary Fisher rat or Chinese hamster embryo fibroblasts were prepared[30] and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 4 mM L-glutamine (maintenance medium). 10^6 cells were seeded per 90-mm dish, and were transfected with the indicated DNA combination after 1 day (10 μg of each plasmid, made up to a total of 25 μg with sheared BALB/c liver DNA). pLSVmyc was constructed linking the 5.6 kb *Bam*HI fragment of the murine c-*myc* gene (see ref. 14) to the SV40 early promoter. pLA8 contains the left-end 9.1% of the adenovirus12 genome, including the EIA and part of the EIB region[16]. pMSVE is a derivative of pMSVp53G, containing only the MSV enhancer inserted in the *Bam*HI site but no p53-specific sequences (compare with Fig. 1). The cells were transfected by the calcium phosphate procedure[31] for 16 h, glycerol-shocked (10% glycerol in maintenance medium for 90 s), replenished with maintenance medium, allowed to recover for an additional day, then split and reseeded at a density of 4×10^5 (rat fibroblasts) or 2×10^5 (Chinese hamster cells) per 90-mm dish. Medium was changed every 6-7 days. Distinct foci overgrowing the monolayer were scored 16 days (expt 1), 14 days (expt 2) or 24 days (expt 3) after replating of transfected cultures. REF, rat embryo fibroblasts; CHEF, Chinese hamster embryo fibroblasts. The tumorigenicity of cell lines established from corresponding foci was determined by injecting subcutaneously 5×10^6 cells into 5-8 day-old Fisher rats whole-body irradiated with 200 rads. As a non-transfected control (bottom line) we used REF propagated in culture to obtain a sufficient number of cells. Data are derived from two different p53 + Ha-*ras* lines and one *myc* + Ha-*ras* line. ND, not determined.
* Foci possessing a distinctly different morphology from that induced by pLA8 alone.

A biological test system demonstrating the involvement of the *myc* product in malignant transformation has been established recently[14,15]; primary rat embryo fibroblasts are transformed stably by the joint action of *myc* and another oncogene such as Ha-*ras*. Similar results are obtained when another nuclear oncogene, the adenovirus-2 EIA region, is assayed by co-transfection with Ha-*ras* in an analogous system[16]. In both cases, the transformation is visualized by the appearance of dense foci capable of overgrowing the monolayer of normal cells and are dependent on the presence of both oncogenes. This system is therefore a suitable test of the oncogenic properties of p53.

Two types of recombinant DNA constructs were used as templates for efficient p53 expression in transfected cells (Fig. 1). The plasmid pMSVp53G contains the 16 kilobase (kb) *Eco*RI fragment encompassing the functional murine p53 gene[12,17] juxtaposed to the enhancer portion of the Moloney murine sarcoma virus (MoMSV) long terminal repeat. This approach utilizes the presence of a functional promoter in the 16 kb fragment (B. Bienz, unpublished results). The second construct, pPyp53c, contains a stretch of p53 cDNA linked to the polyoma virus early promoter; this cDNA contains the intact coding region for p53[17] and directs the synthesis of authentic p53 in a heterologous system[18].

Secondary Fisher rat embryo fibroblasts were co-transfected with pMSVp53G and pEJ6.6 (ref. 19), carrying an activated human c-Ha-*ras*1 gene. As a positive control, we performed a

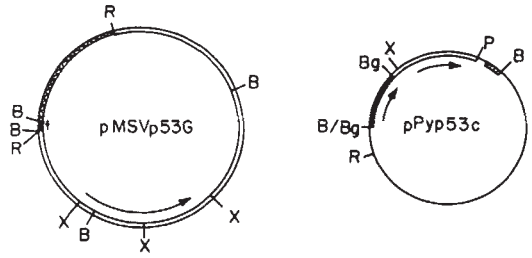

Fig. 1 p53-specific plasmids used for transfection. pMSVp53G contains the functional mouse p53 gene linked to the enhancer element of MoMSV DNA. The 16 kb *Eco*RI fragment was excised out of the recombinant phage Ch 53-7 (ref. 12) and inserted into the *Eco*RI site of plasmid pA_{10} (ref. 32). The *Hinf*I–*Xba*I fragment of the MoMSV long terminal repeat, containing the enhancer portion[33], was converted at both ends to *Bam*HI and introduced into the *Bam*HI site of the same pA_{10} plasmid. Hatched bar, pA10 DNA; full bar, MoMSV DNA; open bar, p53-specific genomic DNA. To construct pPyp53c, the *Bam*HI–*Hph*I fragment of polyoma (Py) DNA, containing the early promoter[34], was converted to *Bgl*II at both ends. This was inserted into pSVp53c17 DNA[18], cleaved previously with *Bgl*II and partially cleaved with *Bam*HI to remove the bulk of SV40 sequences. Briefly, pPyp53c consists of the following components (from 5′ to 3′) inserted within the *Bam*HI site of pBR322: first, the Py early promoter region, a segment of p53 cDNA extending from the *Ban*I site at nucleotide -67 (ref. 17) to the end of the insert of pp53-176 (nucleotide 1,188, ref. 17) and including also 25 base pairs of oligo(dG):oligo(dC); second, 125 base pairs of pBR322 DNA corresponding to the region between the *Pst*I site and the nearby *Bgl*I site[35]; finally, the *Bcl*I–*Bam*HI fragment of SV40 DNA, derived from plasmid pLSV and including the viral early polyadenylation site[36]. Thin line, pBR322 sequences; full bar, Py DNA; open bar, p53 cDNA; hatched bar, SV40 DNA, B, *Bam*HI; Bg, *Bgl*II; P, *Pst*I; R, *Eco*RI; X, *Xho*I. For clarity, only the *Pst*I site originally harbouring the cDNA insert of clone pp53-176 (ref. 17) is indicated. Arrows, normal transcriptional orientation.

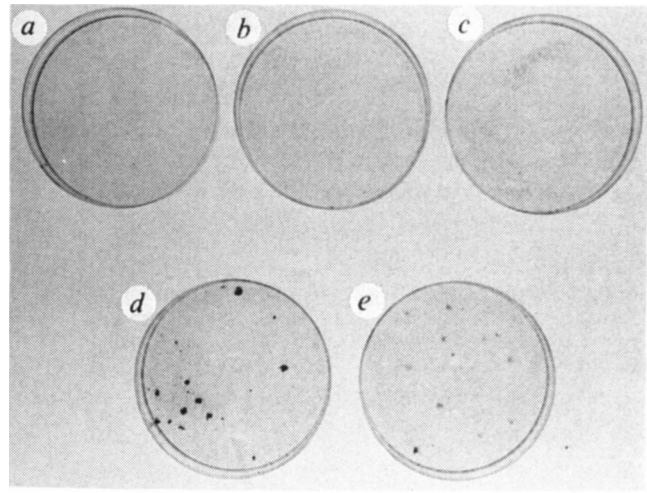

Fig. 2 Giemsa-stained rat embryo fibroblast cultures transfected with various DNA combinations. Transfection was as described in Table 1, using the following DNA: *a*, BALB/c carrier DNA alone; *b*, pEJ6.6+carrier; *c*, pMSVp53G+carrier; *d*, pLA8+pEJ6.6 carrier; *e*, pMSVp53G+pEJ6.6+carrier. Photographs were taken 16 days after replating of transfected cells.

co-transfection with pEJ6.6 and pLSVmyc. The latter plasmid contains the protein-coding part of the mouse *myc* gene, driven by the simian virus 40 (SV40) early promoter (see Table 1). In some cases, pLSVmyc was replaced by pLA8 (ref. 16), containing the left-end 9.1% of the adenovirus-2 genome.

We found that although neither p53 nor Ha-*ras* alone induced the appearance of foci, their joint action could cause this effect (Table 1). The foci generated had a distinct morphology (Fig. 2) and could usually be detected after 9–12 days. In these experiments, p53 was less efficient than *myc* and markedly less efficient than pLA8. Although pLA8 by itself is sufficient for transformation[16], the foci induced by its co-transfection with pEJ6.6 grew much more rapidly than those due to pLA8 alone and had a very different, easily recognizable morphology (data not shown).

To test that the observed co-transformation is not due to activation of unrelated genes by the enhancer in pMSVp53G[20,21], the latter plasmid was substituted by an analogue lacking p53 sequences; no foci were generated (Table 1).

The morphology of the foci cells varied with the particular gene combination, although all were clearly distinct from the surrounding non-transformed cells. Cells transformed by p53+Ha-*ras* (Fig. 3*g*) were large, refractile and more similar to the *myc*+Ha-*ras* transformants (Fig. 3*e*), whereas the foci arising in monolayers transfected with pLA8+Ha-*ras* were composed mostly of smaller cells (Fig. 3*c*). p53+Ha-*ras* foci tended to grow more slowly then *myc*+Ha-*ras* foci and often appeared to stop spreading after reaching 5–7 mm diameter, whereas *myc*+Ha-*ras* foci grew continuously until they merged and filled the dish.

Similar results were obtained using an alternative system, secondary Chinese hamster embryo fibroblasts and the cDNA construct pPyp53c (Table 1). Co-transfection with p53 and pEJ6.6 resulted in overgrowing foci of cells with transformed morphology (Fig. 3*h*,*i*). However, these experiments were hampered because the transformed cells were prone to lysis.

If p53 production is involved in the observed transformation, the protein should be made in the transformed cells. Accordingly, cells were isolated from foci, labelled with ^{35}S-methionine and assayed for p53. We used anti-p53 monoclonal antibody RA3-2C2 (refs 22, 23); this is specific for the murine form of the protein and does not cross-react with rat p53 (ref. 24), thus allowing the detection of transfection-derived murine p53 in the transformed rat cells and avoiding potential difficulties due to the presence of endogenous rat p53. Murine p53 could be immunoprecipitated easily from overgrowing foci cells (Fig. 4), sometimes in even higher concentrations than those present in Meth A mouse fibrosarcoma cells, marked overproducers of p53 (ref. 25). This suggests strongly that the actual production of the protein is involved in the transformation.

Unlike *myc*+Ha-*ras* or pLA8+Ha-*ras*, it proved difficult to establish cell lines from foci induced by p53+Ha-*ras*. Typical transformed cells exhibited a limited proliferation potential and, after repeated passaging, cultures became dominated by cells of a normal-nontransformed morphology, probably derived from the underlying monolayer. The level of murine p53 fell simultaneously to below detection after two or three passages (data not shown). These findings support the notion that p53 production, directed by the transfected DNA, is involved in the observed transformation. Almost all cells of distinct transformed morphology, when transferred into a new dish, attached to it but did not divide. On the other hand, the few normal cells transferred with the focus did start dividing once replated at a low density. Eventually, with several low-density passages, replicating normal cells became predominant. These data suggest that, unlike *myc* or EIA[26,27], p53 is not capable of efficiently immortalizing cells. Thus, the p53+Ha-*ras* transfectants, although transformed, still undergo a senescence crisis and stop multiplying. The normal underlying cells, however, are still able to resume replication once subcultured at a low density, hence they can easily overgrow the senescent transformed cells. Nevertheless, a very small proportion of transformed cells did eventually give rise to stable transformed cell lines. Thus, when foci were subcultured and cells maintained in the dish for 10–14 days without further passaging, a few overgrowing foci became evident. The cells comprising these secondary foci, while generally morphologically indistinguishable from those found in the original foci, did possess an apparently unlimited growth

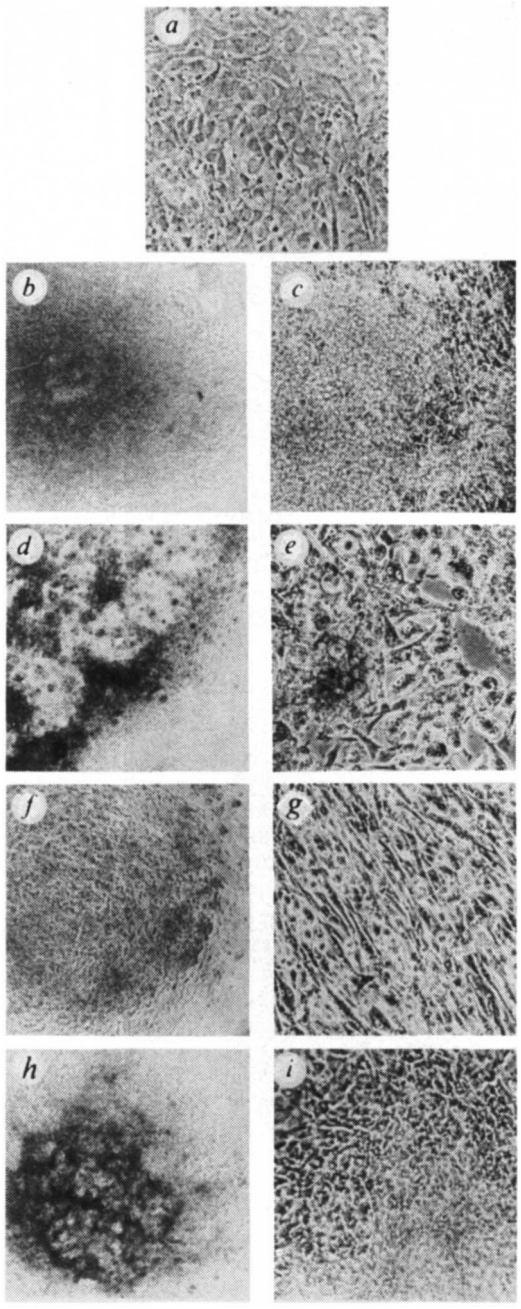

Fig. 3 Morphology of cells transformed by various plasmid combinations. Pictures were taken at two magnifications to display the typical appearance both of the foci and of individual cells within them. *a*, REF (Table 1) transfected with carrier only; *b,c*, REF transfected with pLA8+pEJ 6.6; *d,e*, REF transfected with pLSVmyc+pEJ6.6; *f,g*, REF transfected with pMSVp53G+pEJ6.6; *h,i*, CHEF transfected with pPyp53c+pEJ6.6. Normal CHEF are visible in the upper left corner of *i*. Magnification: *b*, *d*, *f*, *h*, ×40; *a*, *c*, *e*, *g*, *i*, ×100.

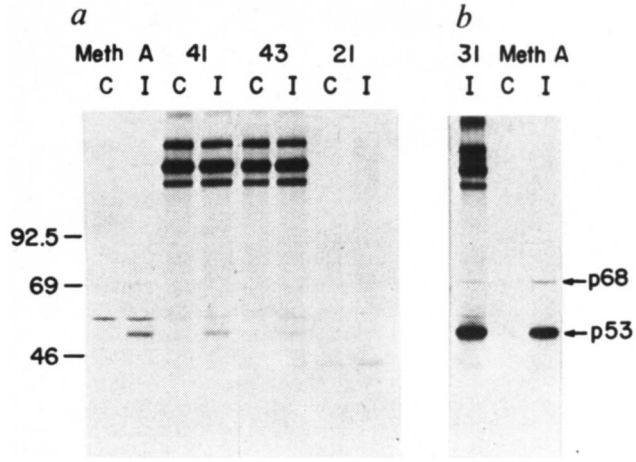

Fig. 4 Analysis of proteins produced in focus-derived rat cells. Foci arising in a monolayer of rat embryo fibroblasts following transfection with different DNA combinations were trypsinized, expanded into 35-mm Petri dish cultures and labelled with 40 μCi ^{35}S-methionine for 4 h. Extracts were prepared as described previously[37]. Equal amounts of trichloroacetic acid-insoluble radioactivity (2.8×10^6 c.p.m. per lane) were immunoprecipitated with either anti-mouse p53 monoclonal antibody RA3-2C2[22-24] or control serum, using standard procedures[38], followed by electrophoresis through a 12.5% SDS-polyacrylamide gel[38]. *a*, *b* refer to two separate experiments. *a*, Autoradiography for 2 days; *b*, for 7 days. Meth A are chemically transformed mouse fibroblasts which overproduce p53 (ref. 25); 41, 43 and 31 are derived from foci induced by pMSVp53G+pEJ6.6 while 21 is derived from a focus induced by pLA8+pEJ6.6. C, control serum, I, RA3-2C2 monoclonal autibody. Numbers on the left refer to the relative molecular mass ($\times 10^{-3}$) of co-electrophorezed markers. p68 is a polypeptide co-precipitating with mouse p53 (see ref. 18).

potential and could be propagated easily to yield homogeneous stably-transformed cell lines. When two such lines were subjected to more detailed analysis, both expressed high levels of murine p53 (data not shown). On injection into syngeneic young rats, both cell lines generated tumours at a high efficiency (Table 1) which developed significantly more slowly than those induced by pLSV*myc*+EJ6.6 transformants. In some animals, they stopped growing after reaching 6–10 mm diameter, whereas in most cases they grew continuously, reaching a very large size and eventually killing the animal. We are currently testing more lines to determine whether this is a general feature of p53+Ha-*ras* transformants.

Based on the results presented here and in ref. 28, we conclude that p53 can act as a nuclear oncogene by collaborating with Ha-*ras* in the transformation of cultured embryonic fibroblasts. Unlike *myc* or EIA, only a small proportion of the resultant transformants eventually become immortalized and give rise to stable lines, possibly through a change involving a third gene product in addition to p53 and Ha-*ras*. The initial transformed cells must be highly predisposed towards this activation event, as it occurs much more frequently than predicted for random mutations. Alternatively, the few immortalized cells may be selected for their ability to produce increased amounts of either p53 or Ha-*ras* protein. Recent data[29] suggest that massive overproduction of the Ha-*ras* p21 protein can circumvent the need for a cooperating oncogene. The rare alteration leading to immortalization may thus be quantitative rather than qualitative. Immortalization may not be a general attribute of nuclear transforming proteins. Although *myc* and EIA can efficiently immortalize cells[26,27], their ability to induce overgrowing foci in combination with Ha-*ras* may reflect an entirely different facet of their activity, the two oncogenes sharing only certain properties with p53.

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Cooperation between gene encoding p53 tumour antigen and *ras* in cellular transformation

Luis F. Parada, Hartmut Land, Robert A. Weinberg, David Wolf* & Varda Rotter*

Whitehead Institute for Biomedical Research, Center for Cancer Research and Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 20139, USA
* Department of Cell Biology, Weizmann Institute, Rehovoth 76100, Israel

The protein p53 is highly expressed in a large variety of transformed cell types originating from diverse species. These include cells transformed by Simian virus 40 (SV40), adenovirus and Abelson virus, as well as a variety of chemically transformed cells[1-6]. Substantial amounts of p53 are also present in certain non-transformed cells, for example, some embryonic tissues[7]. The protein may be localized in different cellular compartments in normal and transformed cells[8]. The strong correlation between tumorigenicity and high levels of p53 suggests an important role of p53 in tumorigenesis. We report here experiments in which we have co-transfected the murine cellular gene encoding for p53 with a *ras* gene into primary rat embryo fibroblasts. Our results indicate that the p53-encoding gene can play a causal role in the conversion of normal fibroblasts into tumorigenic cells.

A cDNA clone of the *p53* gene[9,10] was used to retrieve a homologous 16 kilobase *Eco*RI segment from a genomic library made from DNA of a murine B-cell lymphoma induced by Abelson virus[11]. This genomic clone was modified by linkage to the murine leukemia virus (MLV) promoter-enhancer long terminal repeat element. These long-terminal repeat sequences were inserted to induce a high level of expression of the cloned *p53* gene (Fig. 1).

We performed transfection experiments with clones of the modified genes (see Fig. 1) to determine whether these clones would induce foci in monolayer cultures of the Rat-1 fibroblast cell line or in cultures of secondary rat embryo fibroblasts (REFs). All transfections include the pSV2neo[33] plasmid DNA so that a portion of the cultures could be placed under G418 drug selection to control for transfection efficiencies.

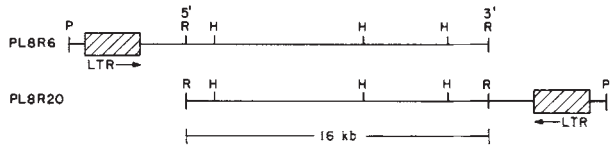

Fig. 1 Schematic diagram of *p53* encoding plasmids. (For detailed description, see ref. 15.) Briefly, the 16kb mouse DNA fragment containing all of the *p53*-encoding exons was inserted either downstream from and in the same transcriptional orientation as the MuLV long terminal repeat (PL8R6), or upstream and in the opposite transcriptional orientation (PL8R20). The vector is pBR322. R, *Eco*RI restriction sites; H, *Hin*dIII restriction sites; P, *Pst*I restriction sites.

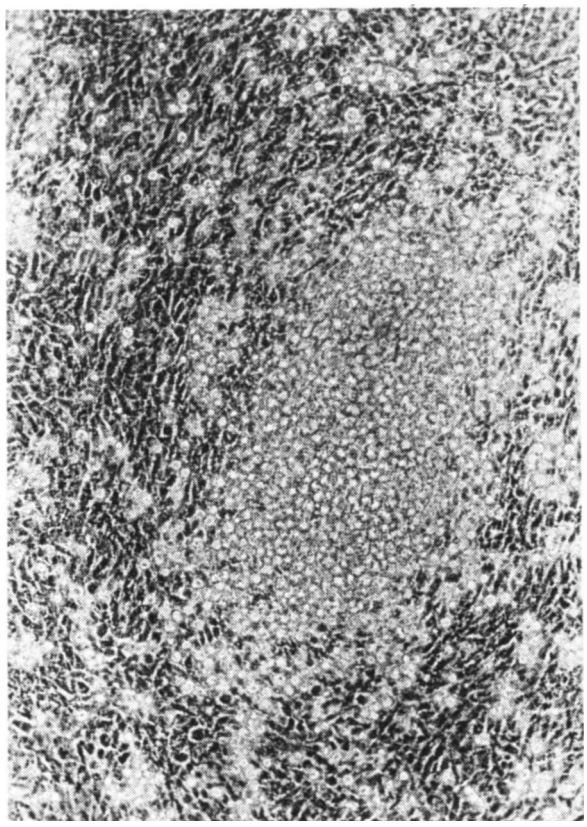

Fig. 2 Phase contrast photomicrograph of a focus arising in a dense monolayer of normal rat embryo fibroblasts after co-transfection of PL8R20 and the EJ-*ras* oncogene. The DNA transfection was performed as described in Table 1. The photograph was taken 10 days after transfection.

The various *p53* clones tested are unable to induce foci in any of the transfected Rat-1 cultures, even when such cultures are surveyed weeks after transfection (Table 1). In the same experiments, a *ras* oncogene elicits foci observable in Rat-1 cells within eight days of transfection. We conclude that the *p53* gene, unlike the *ras* oncogenes, has no apparent ability to transform readily established rodent fibroblast cells.

Earlier work had shown that a single oncogene, such as *ras* or *myc*, is unable to induce foci in REF monolayer cultures. However, when the two oncogenes are applied together, foci are induced which contain cells that are tumorigenic in young rats or nude mice[12,13]. The observed inability of *ras* or *p53* clones to yield foci in transfected REF cultures (Table 1) is consistent with these earlier results.