Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 1519–1523, March 1988
Cell Biology

Rescue of cells from *ras* oncogene-induced growth arrest by a second, complementing, oncogene

(simian virus 40 large tumor antigen/chemotherapy)

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Communicated by Philip A. Sharp, October 2, 1987

ABSTRACT **Established REF52 cells (rat embryo fibroblasts) completely resist stable transformation by *ras* oncogenes, and simian virus 40 large tumor (T) antigen collaborates with *ras* to convert REF52 cells to tumorigenic state. A temperature-sensitive simian virus 40 large T antigen (encoded by *tsA58*) allowed the T24 Ha-*ras* oncogene to transform REF52 cells in a temperature-dependent manner. Two thirds of the clones transformed with *tsA58* and *ras* became arrested in G_2 or late S phase when shifted to a nonpermissive temperature for T antigen stability. Thus, *ras* induced growth arrest rather than stable transformation in the absence of a functional collaborating oncogene. These results indicate that collaborating oncogenes can regulate cellular responses to *ras* and have implications regarding therapeutic strategies to control tumor cells expressing activated *ras* oncogenes.**

Oncogenes are genes implicated in carcinogenesis by virtue of associations with oncogenic viruses, tumor-specific chromosome abnormalities, and DNA sequences that transform cultured cells to a tumorigenic state (1). Mutations and virus associations enable oncogenes to alter cell phenotypes by circumventing regulatory mechanisms that control protooncogene function and expression in normal cells. As carcinogenesis is generally a multistep process, malignant phenotypes may frequently require multiple alterations affecting several levels of growth control (2). This notion is supported by gene transfer experiments demonstrating that two or more oncogenes, acting in concert, are often required to convert normal cells to a tumorigenic state (3–5).

Collaborations between oncogenes indicate that regulatory mechanisms which preclude transformation by one oncogene can be circumvented by a second oncogene. This implies that certain oncogenes can actively regulate cellular responses to other oncogenes. To examine this possibility, we have investigated the mechanism whereby a collaborating oncogene enables activated *ras* oncogenes to transform REF52 cells (rat embryo fibroblasts). Previous studies have shown that neither *ras* nor adenovirus early region 1A (*E1A*) transforms REF52 cells, whereas *E1A* allowed *ras* to convert REF52 cells to a tumorigenic state (6). While REF52 cells transfected with the T24 Ha-*ras* oncogene were morphologically normal, the cells expressed only low levels of the oncogene-encoded p21 protein (i.e., 10–30% of the endogenous c-Ha-*ras* p21). By contrast, cells transformed by T24 Ha-*ras* and *E1A* expressed 10- to 100-fold higher levels of T24 Ha-*ras* p21 (6).

Higher *ras* expression could result either if *E1A* enhanced *ras* expression or if *E1A* enabled cells to tolerate higher p21 levels. To distinguish between these possibilities, a means to regulate transformation by *ras* was developed. The method exploited the ability, reported here, of simian virus 40 (SV40) large tumor (T) antigen to enable *ras* to transform REF52 cells. By using an allele encoding a temperature-sensitive large T protein, transformation by *ras* was made to rely on a collaborating oncogene, *tsA58* (7), whose activities could be regulated simply by shifting cells from one temperature to another. If the collaborating oncogene activates *ras* expression, then p21 levels could decline in *tsA/ras* transformants transferred from a permissive to a nonpermissive temperature. Alternatively, an intolerance to *ras* could be revealed by phenotypic changes that accompany loss of collaborating activities.

MATERIALS AND METHODS

Plasmids and DNA Transfections. Plasmids were as follows. pKOneo contains a gene for bacterial aminoglycoside phosphotransferase (*aph*) expressed from the SV40 early region promoter (8); pT24 contains the Harvey *ras* oncogene (T24 Ha-*ras*) from T24 bladder carcinoma cells (9); pT24neo contains T24 Ha-*ras* inserted into pKOneo; pZipSVLT (10) and pZiptsA58 (P. Jat and P. A. Sharp, personal communication) contain SV40 early regions from wild-type and *tsA58* viruses (7), respectively, inserted into pZipneoSV(X)1 (11). pZipneoSV(X)1 expresses inserted genes and *aph* from the Moloney murine leukemia virus long terminal repeat. Plasmid DNAs were used at 10 μg per dish to transfect subconfluent cultures of REF52 cells by the calcium phosphate coprecipitation method (6). Transfected cultures were passaged to six 10-cm dishes in medium containing G418 (GIBCO; 50% active) at 400 μg/ml, fixed, and stained with Giemsa stain after 2 or 6 weeks. To derive lines from G418-resistant colonies, cells were trypsinized within cloning cylinders, expanded to mass culture, and subcloned by limiting dilution. Lines were analyzed 30–35 population doublings after transfection.

Immunoprecipitation. Extracts were prepared from 10^6 cells, labeled for 24 hr with 100 μCi (1 Ci = 37 GBq) of [^{35}S]methionine in Dulbecco's modified Eagle's medium without unlabeled methionine and containing 10% fetal bovine serum. Immunoprecipitations employed monoclonal antibodies (12, 13) against SV40 T antigen (421) or *ras* p21 (Y13-259) and control sera; previously described methods (14) were used.

Flow Cytometry. Cells were fixed in ethanol, treated with ribonuclease A, and stained with propidium iodide as described (15) and analyzed with an Ortho Diagnostics Cytofluorograf.

RESULTS

Plasmids expressing the T24 Ha-*ras* oncogene and SV40 early regions were transfected into REF52 cells individually

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Abbreviations: SV40, simian virus 40; T antigen, tumor antigen.

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and in combinations as shown in Table 1. Morphologically transformed cells were identified after selection for a gene (*aph*) that conferred resistance to the antibiotic G418 (Fig. 1). As reported previously (6), the T24 Ha-*ras* oncogene rarely transformed REF52 cells, and these rare transformants were unstable and failed to grow beyond 23 population doublings. By contrast, wild-type (pZipSVLT) and temperature-sensitive (pZiptsA58) SV40 large T antigen genes enabled T24 Ha-*ras* to transform; approximately 10% of the colonies displayed morphologies characteristic of *ras*-transformed cells (Fig. 1*C* and 1*E*). As SV40 early region plasmids induced morphological changes (Fig. 1*A*), transformation by *ras* and T antigen together was scored conservatively. Only those clones transfected with T antigen and *ras* expressing clearly distinguishable phenotypes were counted.

*tsA*58 enabled T24 Ha-*ras* to transform REF52 cells in a temperature-dependent manner. Thus, the percent of *ras*-transformed colonies was nearly 10-fold greater at a temperature permissive for T antigen stability (33°C) than at a nonpermissive temperature (39°C). Moreover, infrequent transformants obtained at 39°C were unstable and typically lost *ras*-induced morphologies when subcultured at 39°C (data not shown). Therefore, collaboration between T24 Ha-*ras* and SV40 early region plasmids required a functional large T antigen.

T antigen and *ras* levels were measured in transfected cells by immunoprecipitation using monoclonal antibodies (12, 13). An analysis of this type is shown in Fig. 2. Oncogene p21 was expressed at about 2–5 times the levels of the endogenous c-Ha-*ras* p21 in transformed cells cotransfected with T24 Ha-*ras* and either wild-type large T antigen gene or *tsA*58. *ras* oncogene expression was not appreciably altered

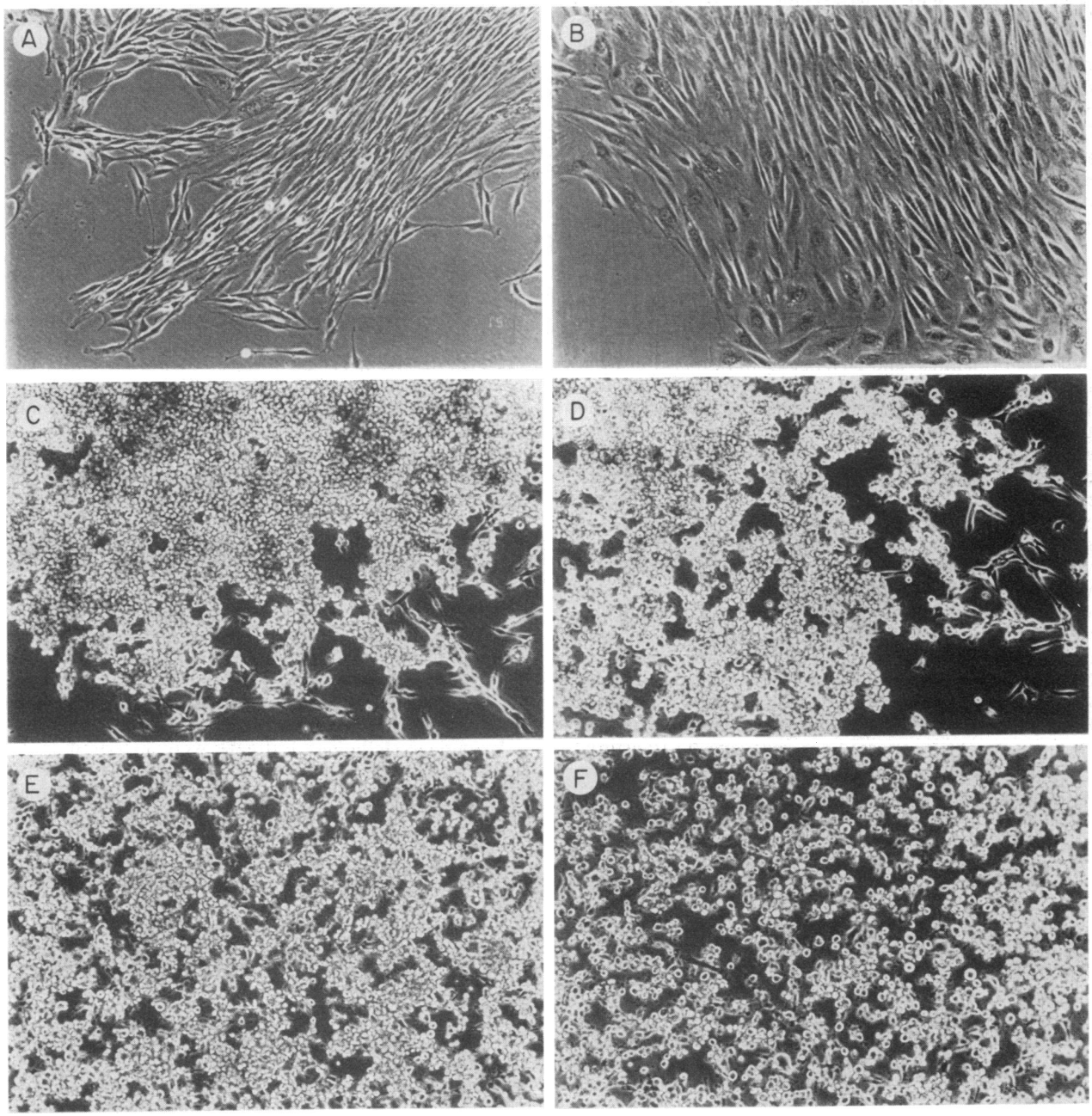

FIG. 1. Photomicrographs of transfected REF52 clones. (×28.) Cells from clones induced by the following plasmids are shown. (*A* and *B*) pZiptsA58, clone TS-4; (*C* and *D*) pZipSVLT + pT24, clone WSR-1; (*E* and *F*) pZiptsA58 + pT24, clone TSR-5. Cell lines, maintained at 33°C for 30–35 population doublings, were passaged and cultured for 4 days at 33°C (*A*, *C*, and *E*) or 39°C (*B*, *D*, and *F*) prior to photomicroscopy.

Table 1. SV40 early region plasmids enable the T24 Ha-*ras* oncogene to transform REF52 cells

Plasmids	Temp., °C	G418-resistant colonies	% transformed
pKOneo (10 μg)	37	1400	0
pT24neo (10 μg)	37	1600	0.06
pZipSVLT (10 μg)	37	850	0
pZiptsA58 (10 μg)	33	720	0
pZiptsA58 (10 μg)	39	950	0
pZipSVLT (1 μg) + pT24 (9 μg)	37	600	10
pZiptsA58 (1 μg) + pT24 (9 μg)	33	850	9
pZiptsA58 (1 μg) + pT24 (9 μg)	39	900	1

Plasmid DNAs (total of 10 μg per dish, as indicated) were transfected onto each of four 10-cm dishes of REF52 cells. Transfected cultures were passaged to six 10-cm dishes in medium containing G418 (GIBCO; 50% active) at 400 μg/ml, maintained at the indicated temperature, and fixed and stained after 2 (37°C and 39°C) or 6 (33°C) weeks. The number of G418-resistant colonies and the percent of colonies that were morphologically transformed (excluding T antigen-induced morphologies, see Fig. 1) are indicated.

by shifting the temperature from 33°C to 39°C. By contrast, T antigen levels decreased dramatically in *tsA*58/T24 Ha-*ras* cotransformants within 2 days after shifting to the nonpermissive temperature. This result is consistent with the previous demonstration that *tsA*58 is temperature sensitive for T antigen stability (16). T antigen levels did not decline in cells expressing the wild-type early region.

Approximately two-thirds (6 of 10) of the clones induced by pZiptsA58 alone reverted to a normal morphology (Fig. 1*B*) when shifted to the nonpermissive temperature. Transformants induced by *ras* and either wild-type T antigen gene or *tsA*58 at 33°C did not revert at 39°C (Fig. 1 *D* and *F*), although several T24 Ha-*ras*/*tsA*58 cotransformants became slightly flatter.

While Ha-*ras*/*tsA*58 cotransformants did not revert in terms of cell morphology, three of nine clones tested (TSR-1, TSR-3, and TSR-5) stopped growing within 48 hr after transfer to the nonpermissive temperature (Fig. 3*A* and data not shown). An additional three clones stopped growing within 5 days after transfer to 39°C, and the remainder were not temperature sensitive for cell growth (data not shown).

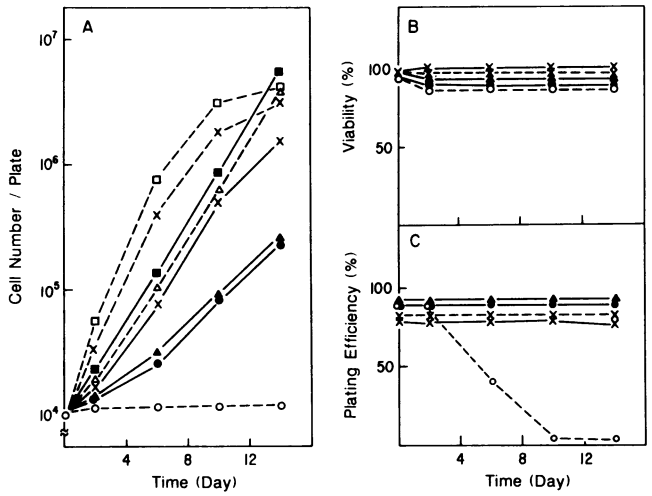

FIG. 3. Temperature-dependent growth and survival of transfected REF52 cells. (*A*) Growth potential of transfected clones. Subconfluent cultures maintained at 33°C were trypsinized and 10^4 cells were inoculated into 20 10-cm dishes. Every other day, cells from replica cultures maintained at either 33°C (—) or 39°C (---) were trypsinized and counted. Growth of REF52 cells (×) and derivative lines expressing *tsA*58 alone (clone TS-1; □, ■), *tsA*58 and T24 Ha-*ras* (clone TSR-5; ○, ●); and wild-type large T antigen gene and T24 Ha-*ras* (clone WSR-1; △, ▲) are plotted. (*B*) Viability of transfected clones. Cells harvested for counting in *A* were stained with 0.02% trypan blue. Cell viability, expressed as the percent of dye-excluding cells, is plotted for each clone as in *A*. (*C*) Irreversible loss of growth potential of TSR-5 cells maintained at 39°C. Two hundred cells, harvested for counting in *A*, were seeded into 60-mm dishes and cultured at 33°C. After 7 days, colonies were fixed, stained with crystal violet, and counted. Plating efficiencies, expressed as the percent of cells forming colonies at 33°C, are plotted for each clone as in *A*.

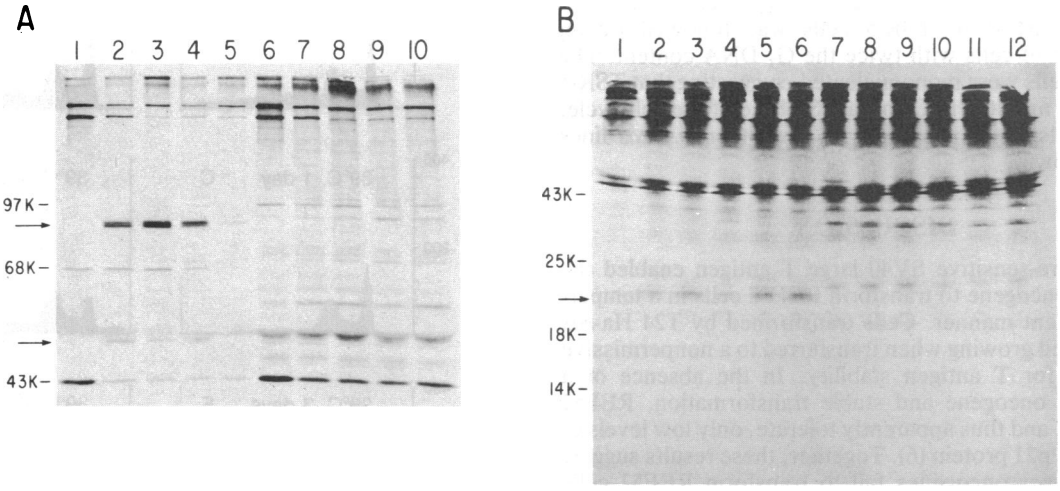

FIG. 2. SV40 large T antigen and *ras* p21 protein expression in REF52 clones. (*A*) Temperature-dependent expression of large T antigen was monitored by immunoprecipitation with an anti-T monoclonal antibody (lanes 1–5) or normal rat serum (lanes 6–10). Precipitates were analyzed after electrophoresis in 10% polyacrylamide gels and fluorography. REF52 (lanes 1 and 6); clone WSR-1, transformed with wild-type large T antigen gene and T24 Ha-*ras* (lanes 2, 3, 7, and 8); and clone TSR-5, transformed with *tsA*58 and T24 Ha-*ras* (lanes 4, 5, 9, and 10), are shown. Cells were cultured for 2 days at either 33°C (lanes 2, 4, 7, and 9) or 39°C (lanes 3, 5, 8, and 10) prior to analysis. The mobilities of T antigen and associated cellular p53 proteins (upper and lower arrows, respectively) and protein size standards (K = kDa) are indicated. (*B*) *ras* p21 proteins in REF52 (lanes 1, 4, 7, and 10), WSR-1 (lanes 2, 5, 8, and 11), and TSR-5 (lanes 3, 6, 9, and 12) cells were analyzed by immunoprecipitation using a rat monoclonal antibody to *ras* p21 (lanes 1–6) or control serum (lanes 7–12). Cells were cultured for 2 days at either 33°C (lanes 1–3 and 7–9) or 39°C (lanes 4–6 and 10–12) prior to analysis.

The growth kinetics of representative clones are shown in Fig. 3. The TSR-5 clone transformed by T24 Ha-*ras* and *tsA*58 grew continuously at 33°C but rapidly ceased growing when shifted to 39°C (Fig. 3*A*). By contrast, wild-type/T24 Ha-*ras* cotransformants grew faster at the higher temperature. Likewise, REF52 cells and REF52 cells expressing *tsA*58 also grew faster at 39°C, although *tsA*58 transfectants that morphologically reverted grew to lower saturation densities. In short, growth arrest was seen only when cells transformed with *tsA*58 and *ras* were shifted to the nonpermissive temperature. Thus, growth arrest of *tsA*/T24 Ha-*ras* transformants did not result from the sudden temperature shift, toxic breakdown products of T antigen, or a simple addiction to T antigen. These results indicate that *ras* oncogenes can induce growth arrest when complementing T antigen is removed.

While it is not clear why some *tsA*/*ras*-transformed clones were not temperature sensitive for cell growth, similar clonal variation has been observed in other studies using *tsA* alleles (17). On average, T antigen levels declined to a greater extent and to a lower level in susceptible clones than in nonresponding clones. However, a decline in T antigen levels, while perhaps necessary, was not always sufficient for temperature sensitivity (data not shown). T24 Ha-*ras* p21 levels were similar in all clones tested and therefore did not correlate with the temperature-sensitive phenotype (data not shown). Failure to express temperature-sensitive phenotypes could result from secondary changes in transfected cells, rendering certain clones independent of T antigen. Altered T antigen genes generated during transfection might also enable *ras* to transform while losing sequences necessary to express temperature-sensitive phenotypes.

The ability of cells maintained at 39°C to form colonies at 33°C (Fig. 3*C*) was measured to assess whether *ras*-induced growth arrest was reversible. Growth arrest was reversible for 2 days, but recovery declined thereafter, and arrest was largely irreversible after 10 days. Plating efficiencies declined only among T24 Ha-*ras*/*tsA*58 cotransformants maintained at 39°C; however, arrested cells remained metabolically viable for at least 2 weeks, as judged by trypan blue dye exclusion (Fig. 3*B*). Thus, growth arrest did not result simply from cell death.

Cell cycle analysis by flow cytometry (Fig. 4) indicates that growth arrest of TSR-5 cells was followed by an accumulation of cells with twice the G_1 DNA content. The majority of cells were mononucleated, suggesting that TSR-5 cells become arrested in G_2 or late S phase of the cell cycle. Similar results were obtained with two other *tsA*/*ras* lines (data not shown).

DISCUSSION

A temperature-sensitive SV40 large T antigen enabled the T24 Ha-*ras* oncogene to transform REF52 cells in a temperature-dependent manner. Cells transformed by T24 Ha-*ras* and *tsA* ceased growing when transferred to a nonpermissive temperature for T antigen stability. In the absence of a collaborating oncogene and stable transformation, REF52 cells express, and thus apparently tolerate, only low levels of the oncogene p21 protein (6). Together, these results suggest (*i*) activated *ras* oncogenes fail to transform REF52 cells because the oncogene inhibits cell proliferation when expressed above a low threshold level, and (*ii*) SV40 large T antigen and adenovirus *E1A* products regulate transformation by protecting from *ras*-induced growth arrest.

Like E1A, SV40 T antigen enables REF52 cells to tolerate higher oncogene p21 protein levels but does not appear to enhance *ras* expression directly. Thus, p21 levels did not decline in *tsA*/*ras* transformants shifted to 39°C. However, *ras* may be repressed in nontransformed cells, since stable lines transfected with T24 Ha-*ras* invariably express only low levels of the oncogene p21. Moreover, *aph* plasmids that express *ras* do not induce appreciably fewer G418-resistant colonies, as compared with plasmids that lack *ras* (see Table 1 and ref. 1). Therefore, most cells appear to have accommodated transfected *ras* oncogenes by restricting *ras* expression and not by the selective loss of clones expressing higher p21 levels.

Mechanisms limiting *ras* expression are not known. The Ha-*ras* promoter is not simply defective in REF52 cells since rare clones, abortively transformed by *ras*, express extremely high levels of the oncogene p21 protein (6). The Ha-*ras* promoter is also not required for repression, since several other promoters and enhancers affect neither p21 levels nor the ability of *ras* to transform (ref. 6; M. Worrell, A. Kuo, and H.E.R., unpublished results). The lack of *ras* expression may be related to the repression of coselected genes reported in other systems (18).

By analogy with GTP-regulated signal transducers, *ras* p21 may regulate intracellular levels of second-messenger signals (19). In theory, SV40 large T antigen and E1A product could influence *ras* function or enable REF52 cells to respond to *ras*. However, the inhibition of cell growth by *ras* indicates a cellular response to functionally active p21 protein. Instead, large T antigen and E1A product appear to complement *ras*-induced growth defects that preclude transformation. While underlying biochemical mechanisms are unknown, we suspect that *ras* represses activities necessary for growth, and the viral early region proteins either reactivate these activities or provide biochemical substitutes. Gene transfer experiments may identify cellular targets repressed, either directly or indirectly, by *ras*. Such targets could be encoded by cellular genes, presumably activated to escape repression, that enable *ras* to transform REF52 cells.

Microinjected p21 induces density-arrested REF52 cells to progress through the cell cycle and engage in DNA synthesis (20). While initial responses to microinjected p21 are similar in REF52, NIH 3T3, and NRK cells (21, 22), steady-state

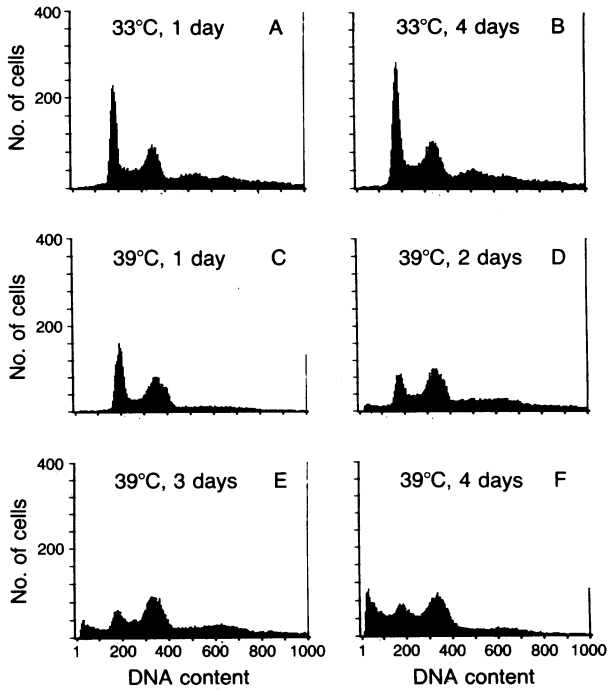

FIG. 4. Cell cycle analysis of *ras*-induced growth arrest. TSR-5 cells were maintained at 33°C for 1 (*A*) and 4 (*B*) days or at 39°C for 1 (*C*), 2 (*D*), 3 (*E*), and 4 (*F*) days prior to analysis. DNA content is in terms of relative fluorescence.

responses clearly differ, as *ras* transforms NIH 3T3 and NRK but not REF52 cells. This suggests that growth arrest stems from a secondary response to *ras*, involving, for example, a homeostatic mechanism to down-regulate signal transduction pathways in which *ras* p21 is thought to participate (19).

The regulation of cellular responses to *ras* does not appear to be an idiosyncrasy of REF52 cells. Thus, two other lines, resembling REF52 in their responses to transfected T24 Ha-*ras* and *tsA* genes, have recently been characterized in our laboratory (A. Lamond and H.E.R., unpublished results). Furthermore, several lines of evidence suggest that REF52 cells maintain a level of growth control normally lost during the isolation of established rodent cell lines. First, SV40 T antigen and E1A product also enable *ras* to transform cultured primary cells (22, 23) and facilitate the growth of primary cells into established lines (17, 24). Second, *tsA*/*ras*-transformed REF52 cells resemble primary rat embryo fibroblast lines conditionally established by *tsA*58 in that both cell types accumulate in G_2 or late S phase when shifted from 33°C to 39°C (P. Jat and P. A. Sharp, personal communication). Finally, *ras* oncogenes fail to transform secondary rat kidney cells, in part, because the cells tolerate only low levels of *ras* expression (25).

In summary, *ras* oncogenes can promote either oncogenic transformation or growth arrest, depending on the physiological state of the cell. This study demonstrates that one oncogene can regulate cellular responses to a second oncogene by complementing oncogene-induced growth defects. While "toxicity" has been reported to interfere with transformation by *src* and *abl* (26, 27), it remains to be determined if these inhibitory effects can be mended by other oncogenes. It will also be interesting to assess if oncogenes activated later in tumor progression enable cells to tolerate higher levels of oncogenes activated earlier (and vice versa). For example, while increased *ras* expression may accompany tumor progression (28, 29), selection need not have favored cells expressing higher *ras* levels until after other cellular functions were altered.

Finally, the problem of specificity is central to any therapeutic strategy to treat malignant disease. Thus, the sensitivity of tumor cells to treatment must exceed that of normal cells. Unfortunately, inhibitors of oncogene products are likely to affect similar, although unactivated, activities expressed in normal cells. However, the present study demonstrates that the consequences of suppressing one oncogene can depend on whether other oncogenes have been activated in the same cell. This was observed when cells expressing *tsA* alone, unlike *tsA*/*ras* transformants, did not arrest their growth when transferred to 39°C. Theoretical strategies to control tumor cells expressing *ras* oncogenes should target cellular analogues of *tsA* (assuming these exist) and not *ras*, since normal cells lacking activated *ras* genes could be more resistant to such therapies.

We thank Parmjit Jat, Michael Wigler, and Douglas Hanahan for providing plasmid DNAs, Ed Harlow and Mark Furth for providing monoclonal antibodies, and Eileen Feldman and Karen Traite for help preparing this manuscript. This work was supported by grants from the National Cancer Institute (CA40602 and CA42063 to H.E.R. and Cancer Center Core Grant 14051 to P. A. Sharp). H.E.R. is a Rita Allen Foundation Scholar.

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