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ras-mediated cell cycle arrest is altered by nuclear oncogenes to induce Schwann cell transformation

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The cellular responses to *ras* and nuclear oncogenes were investigated in purified populations of rat Schwann cells. v-Ha-*ras* and SV40 large T cooperate to transform Schwann cells, inducing growth in soft agar and allowing proliferation in the absence of added mitogens. Expression of large T alone reduces their growth factor requirements but is insufficient to induce full transformation. In contrast, expression of v-Ha-*ras* leads to proliferation arrest in Schwann cells expressing a temperature-sensitive mutant of large T at the restrictive temperature. Cells arrest in either the G_1 or G_2/M phases of the cell cycle, and can re-enter cell division at the permissive temperature even after prolonged periods at the restrictive conditions. Oncogenic *ras* proteins also inhibit DNA synthesis when microinjected into Schwann cells. Adenovirus E1a and c-*myc* oncogenes behave similarly to SV40 large T. They cooperate with Ha-*ras* oncogenes to transform Schwann cells, and prevent *ras*-induced growth arrest. Thus nuclear oncogenes fundamentally alter the response of Schwann cells to a *ras* oncogene from cell cycle arrest to transformation.

Key words: oncogene cooperation/*ras*/SV40 large T/cell cycle arrest/Schwann cells

Introduction

Tumorigenesis is generally believed to be a multistep process involving the accumulation of several lesions over a long period of time. There is now considerable evidence that activation of cellular proto-oncogenes plays an important role at some stages of tumour development. Activated oncogenes have been found in a wide variety of human cancers (reviewed in Bishop, 1987). Furthermore, transgenic mice carrying oncogenes show increased incidence of tumour formation (reviewed in Hanahan, 1986). *In vitro* single oncogenes usually induce only partial transformation of embryonic rodent cells, whereas certain pairs of oncogenes are able to cooperate to induce full transformation (Land *et al.*, 1983a; Ruley, 1983). In addition, DNA tumour viruses and some acutely transforming retroviruses contain two oncogenes, both of which are required for tumorigenesis *in vivo* (reviewed in Bishop, 1985). Many oncogenes can be assigned to one of two classes according to their ability to cooperate in transformation assays (Land *et al.*, 1983b,1984; reviewed in Weinberg, 1985). One class of oncogenes encode nuclear proteins, including c-*myc*, adenovirus E1a and SV40 large T antigen. The second class encode proteins associated with the cell membrane, including the *ras* gene family, *src* and polyoma middle T.

Insight into the possible mechanisms involved in oncogene cooperativity has come from studying the action of individual oncogenes in primary cell cultures and established cell lines. Nuclear oncogenes facilitate establishment of cell lines from a variety of primary cells in culture (Mougneau *et al.*, 1984; Ruley *et al.*, 1984; Land *et al.*, 1986), and are able to inhibit differentiation in some cell types (e.g. Falcone *et al.*, 1985; Coppola and Cole 1986). They can also increase cellular growth rate (Royer-Prokora *et al.*, 1978) and reduce growth factor requirements (Armelin *et al.*, 1984; Stern *et al.*, 1986). Some of them are implicated in the regulation of gene transcription (reviewed in Varmus, 1987). However, the mechanisms whereby nuclear oncogenes achieve changes in cell behaviour have not yet been elucidated.

Expression of activated *ras* oncogenes or overexpression of normal *ras* genes stimulates proliferation and induces transformation in a number of cell lines (reviewed in Barbacid, 1987). Since *ras* proteins bind and hydrolyse GTP (Gibbs *et al.*, 1984; Sweet *et al.*, 1984), it is thought that they may act as signal transducers at the cell membrane, in a similar manner to the G proteins which regulate adenylate cyclase (Gilman, 1984). Activation of *ras* proteins by point mutation has been hypothesized to enhance second messenger production in the absence of extracellular signals such as growth factors (Berridge and Irvine, 1984; Fleischman *et al.*, 1986; Wakelam *et al.*, 1986; Lacal *et al.*, 1987), and might in this way cause a constitutive intracellular growth stimulus. *ras* oncogenes also induce secretion of growth factors (Ozanne *et al.*, 1980; Sporn and Todaro, 1980; Stern *et al.*, 1986), which may allow the proliferation of cells by autocrine mechanisms.

The optimal growth of cells in culture normally requires more than one growth factor (Rozengurt, 1986; Goustin *et al.*, 1986), while a fundamental trait of tumour cells is a reduced dependence on exogenous growth factors. The properties of nuclear and *ras* oncogenes suggest that they could cooperate to transform cells by reducing or abrogating the requirement for different growth factors.

Initial studies on the cooperative action of oncogenes in rodent cells precluded detailed analysis of alterations in growth factor requirements, since they were performed on mixed cell populations (Land *et al.*, 1983a; Ruley, 1983). In order to investigate the molecular basis of oncogene action and cooperation in a homogenous, non-established cell population, we chose to use rat Schwann cells as a model system. Schwann cells are the glial cells of the vertebrate peripheral nervous system. During embryogenesis, they grow along peripheral nerve axons, and eventually ensheathe or myelinate them (Webster, 1975). 99.5% pure primary populations of Schwann cells can be obtained from sciatic nerves (Brockes *et al.*, 1979), and these cells respond to only

a very restricted number of mitogenic stimuli (Raff *et al.*, 1978; Ratner *et al.*, 1985). In contrast to the large number of growth factors known to stimulate fibroblast cell lines, the only defined polypeptide so far found to act as a Schwann cell mitogen is Glial Growth Factor (GGF; Lemke and Brockes, 1984). The expression of a large number of antigenic markers on Schwann cells has been studied in detail (Raff *et al.*, 1979; Brockes *et al.*, 1980; Cornbrooks *et al.*, 1983; Jenssen and Mirsky, 1984; Noble *et al.*, 1985), so the cells can be easily identified in culture, and any changes in antigen expression followed. Finally, Schwann cells passaged for several months in culture retain the growth factor responsiveness and surface antigen expression characteristic of early-passage cells, and are still able to form myelin when cocultivated with neurites (Porter *et al.*, 1986). These unique properties make it possible to compare the behaviour of normal Schwann cells with those expressing single or multiple oncogenes.

We have introduced Ha-*ras* and SV40 large T oncogenes into Schwann cells *in vitro*, and find that each oncogene induces a distinct cellular response. Expression of large T reduces the growth factor requirements of Schwann cells, whereas oncogenic *ras* proteins induce a proliferation arrest. Co-expression of both oncogenes leads to cellular transformation. Thus a nuclear oncogene alters the phenotype induced by *ras* oncogenes from growth inhibition to proliferation and transformation.

Results

SV40 large T and v-Ha-ras cooperate to transform Schwann cells

Rat Schwann cells were purified from cultures of neonatal sciatic nerves as described (Brockes *et al.*, 1979). They expressed antigenic markers characteristic for Schwann cells, including N-CAM (Noble *et al.*, 1985), laminin (Cornbrooks *et al.*, 1983) and collagen IV (Carey *et al.*, 1983).

Schwann cells divided very slowly, if at all, in the presence of fetal bovine serum (FBS) alone (Figure 1a). Their growth rate in FBS-DMEM was stimulated by addition of conditioned medium from the glioma cell line IN/259 (259 CM, Figure 1a), which appears to secrete GGF (M. Noble, B.Watkins and J.Brockes, unpublished observations—see Materials and methods). Schwann cells have previously been shown to be stimulated synergistically by a combination of GGF and 2 μM forskolin (Porter *et al.*, 1986).

In order to investigate the effects of oncogenes on the growth properties and growth factor requirements of Schwann cells, recombinant retroviruses were used to introduce the SV40 large T gene and v-Ha-*ras* into cells. Initially, cells were infected with ZipSV40 6 (Jat *et al.*, 1986), a recombinant retrovirus derived from ZipNeoSV(X) (Cepko *et al.*, 1984). ZipSV40 6 carries the SV40 large T gene and the neomycin acetyl transferase gene (*neo*^{r}) conferring resistance to the aminoglycoside G418 (Southern and Berg, 1982). Clones of cells (LT cells) were selected in G418 and shown to express the large T antigen by immunofluorescence (not shown). LT cells were superinfected with Zipras 6 (Dotto *et al.*, 1985), which was also derived from ZipNeoSV(X) and carries the v-Ha-*ras* gene. Foci of morphologically altered, rapidly growing cells (LT-ras cells) appeared in the cultures within 10 days, and were either ring-cloned or populations were pooled. The growth

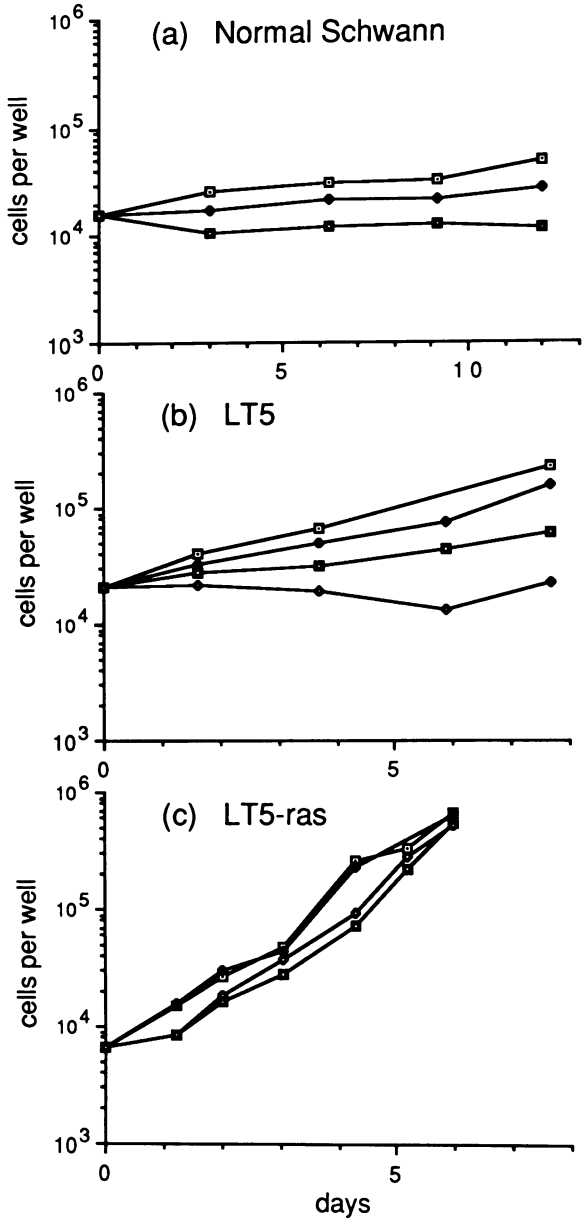

Fig. 1. Comparison of the proliferation rates of normal Schwann cells, LT5 and LT5-ras cells in different media. Cells were grown in B-S (●), B-S supplemented with 10% 259 CM (-■-), 3% FBS-DMEM (-●-), or 3% FBS-DMEM supplemented with 10% 259 CM (-□-). Cell numbers were determined at the times indicated.

characteristics of LT cells and LT-ras cells were then compared to those of normal Schwann cells under identical conditions. Results are shown for a typical large T clone LT5, and for LT5-ras, a pooled population of Zipras 6-infected cells derived from the clone LT5. Four other analogous pairs of LT and LT-ras populations gave similar results.

Expression of large T increased the growth rate of Schwann cells in both FBS-DMEM alone or FBS-DMEM with 259 CM. In addition, LT cells were able to grow in chemically-defined medium (B-S: see Materials and methods) supplemented with 259 CM in the absence of FBS, but were unable to grow in the defined medium alone. Thus large T reduced the growth requirements of Schwann cells (Figure 1b).

Expression of v-Ha-*ras* in addition to large T eliminated requirements for exogenous growth factors, allowing growth of Schwann cells in defined medium without FBS or 259 CM (Figure 1c). LT-ras cells were also able to grow in DMEM supplemented only with transferrin (100 μg/ml) (A.Ridley, unpublished observations). In addition, the v-Ha-*ras* oncogene increased the growth rate of LT cells several-fold. For example, the doubling time for LT5 cells in FBS-DMEM was ~72 h, whereas under the same conditions the LT5-ras population doubled every 12 h. Furthermore, LT-ras cells were able to grow in soft agar, whereas neither normal or LT cells exhibited this property (not shown).

Expression of the antigens N-CAM, laminin and collagen IV was retained on both LT and LT-ras cells, suggesting that changes in the growth characteristics of Schwann cells were not accompanied by a substantial alteration in their specific antigenic phenotype. In addition, LT and LT-ras cells showed no gross karyotypic changes. Schwann cells therefore provide a model system in which oncogene cooperation occurs, and where oncogenes cause defined changes in cellular growth properties.

Ha-ras oncogenes inhibit outgrowth of normal Schwann cells

The previous results suggested that Schwann cells expressing a *ras* oncogene alone would also exhibit reduced growth requirements. However, attempts to express Ha-*ras* oncogenes in Schwann cells were unsuccessful. Infection of primary cells with Zipras 6 followed by G418 selection consistently did not generate any G418-resistant colonies (Table I). This was not due to poor cloning efficiency of the cells, since infection of cells from the same population with ZipNeoSV(X) led to colony outgrowth under G418 selection. Since the virus titres of both ZipNeoSV(X) and Zipras 6 were similar when assayed on NIH-3T3 cells, these observations suggested that expression of v-Ha-*ras* was inhibiting outgrowth of Schwann cells in selective medium. Table I shows that transfection assays yielded similar results. No G418-resistant colonies were obtained after co-transfection of early-passage Schwann cells with pEJ6.6, a plasmid carrying an activated human Ha-*ras* oncogene (Shih and Weinberg, 1982), together with pSV2-neo, a plasmid carrying the *neo*^{r} gene (Southern and Berg, 1982). However, transfection of pPVU-O, a plasmid containing the SV40 large T gene (Kalderon *et al.*, 1982), either with pSV2-neo alone or in combination with pEJ6.6 led to colony formation. Thus a Ha-*ras* oncogene in the absence of large T appeared to inhibit the clonal outgrowth of Schwann cells, whereas in the presence of large T it contributed to transformation.

Expression of a temperature-sensitive large T antigen together with v-Ha-ras

In order to analyse the effect of v-Ha-*ras* expression in the absence of a cooperating oncogene, a temperature-sensitive large T gene (tsA58, Tegtmeyer *et al.*, 1975) was introduced into Schwann cells together with v-Ha-*ras*. Primary cells were infected with the recombinant retrovirus LJ-tsSVLT (Figure 2), and clones of cells (LTts cells) carrying the provirus were isolated under G418 selection. Four distinct LTts cell clones were super-infected with Zipras 6 at the permissive temperature of 33°C, and foci of transformed cells (LTts-ras cells) were cloned or populations were pooled.

Table I. Inhibition of Schwann cell outgrowth by Ha-*ras* oncogenes

	G418-resistant colonies/10^6 cells	
	NIH-3T3	Schwann
Infection		
Zipras 6	3.8×10^5	0
Zip SV40 6	2.0×10^5	1.8×10^3
ZipNeoSV(X)	5.0×10^5	1.1×10^3
	LT5	Schwann
Transfection		
pPVU-0/pSV2-neo	N/D	3.2×10^3
pEJ6.6/pSV2-neo	1.1×10^3	0
pEJ6.6/pPVU-0/ pSV2-neo	N/D	3.7×10^3

Infection: Primary Schwann cells were infected by cocultivation with Ψ-2 cells producing the recombinant retroviruses shown. The titres of virus stocks from the same Ψ-2 cells, as determined by ability to confer G418 resistance in NIH-3T3 cells, is also shown.
Transfection: Normal Schwann cells or the SV40 large T-expressing Schwann cell clone LT5 were transfected with the plasmids shown. Cultures were selected in medium containing 400 μg/ml G418, and colonies were counted after 2–3 weeks.

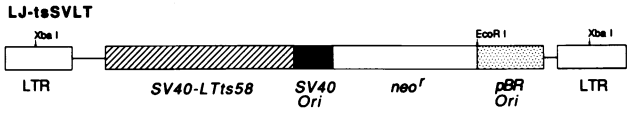

Fig. 2. Schematical representation of the recombinant retrovirus LJ-tsSVLT. LTR, long terminal repeat; SV40-LTts58, temperature sensitive SV40 large T gene derived from mutant tsA58; SV40 ori, origin of SV40; *neo*^{r}, neomycin acetyl transferase gene derived from transposon 5; pBR ori, origin of pBR322. The map is not to scale.

The behaviour of several LTts-ras clones and polyclonal populations derived from each LTts clone were found to be similar. Four LTts-ras clones, one derived from each of the four LTts clones, have been analysed in detail. Results for two of these clones, 33-10ras3 and 33-11ras2, and the LTts clones from which they were derived, 33-10 and 33-11, are shown.

Analysis of the retroviral integration patterns of LJ-tsSVLT proviruses by Southern blotting showed that LTts and LTts-ras cells were indeed clonally related. 33-10 and 33-10ras3 carried one integrated copy of LJ-tsSVLT (Figure 3a, lanes 5 and 3, respectively); while 33-11 and 33-11ras2 contained two proviruses (lanes 2 and 1, respectively). Both LTts-ras clones carried only one copy of Zipras 6 (Figure 3b, lanes 1 and 2), and all virus integrants were of the expected size (not shown).

Expression of SV40 large T antigen and p21$^{v\text{-Ha-}ras}$ in these cell clones was determined by immunoprecipitations. A large T-specific polyclonal antiserum (Simanis and Lane, 1985) detected SV40 large T and various breakdown products in 33-10ras3 and 33-11ras2 cells grown at the permissive temperature of 33°C, but not at the restrictive temperature of 39.5°C (Figure 4a). p21$^{v\text{-Ha-}ras}$ expression in 33-10ras3 (Figure 4b, lane 4) and 33-11ras2 (lane 5) was similar to the level of p21$^{c\text{-Ha-}ras}$ in normal or LTts Schwann cells (lanes 1 and 2, respectively), both at 33°C (data not shown) and at 39.5°C (Figure 4b). This also indicated that SV40 large T did not alter v-Ha-*ras* expression. p21$^{v\text{-Ha-}ras}$ migrated with a slightly slower mobility (see Furth *et al.*,

1982), as clearly seen for the LTts-ras clone 33-4 ras2 (Figure 4b, lane 3), which expressed high levels of $p21^{v\text{-}Ha\text{-}ras}$.

LTts and LTts-ras cells retained antigenic expression of N-CAM, laminin and collagen IV at 33°C and 39.5°C.

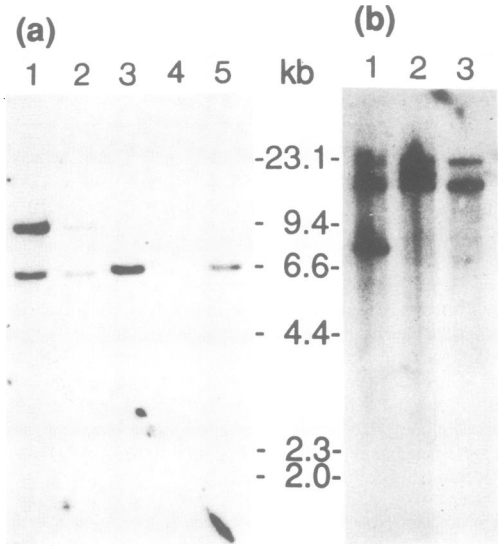

Fig. 3. Proviral integration analysis of LJ-tsSVLT and Zipras 6 in clones of Schwann cells. Southern blots show (**a**) two copies of LJ-tsSVLT in 33-11 (lane 2) and in 33-11ras2 (lane 1) and a single proviral copy in 33-10 (lane 5) and 33-10ras3 (lane 3). Normal Schwann cells are represented in lane 4. (**b**) Single Zipras 6 proviruses were detected in 33-11ras2 (lane 1), 33-10ras3 (lane 2). The two bands detected in normal Schwann cell DNA (lane 3) represent c-Ha-*ras*-specific cross-hybridization of the probe. Hybridizations were carried out with ^{32}P-labelled probes specific to the large T gene of SV40tsA58 (in a) and the v-Ha-*ras* gene (in b).

v-Ha-ras induces reversible growth arrest

The growth of pairs of LTts and LTts-ras clones was analysed at the permissive (33°C) and restrictive (39.5°C) temperatures for the temperature-sensitive large T mutant. Preliminary characterization of LTts cells showed that their behaviour was comparable to LT cells at 33°C, in that they were able to grow in FBS-DMEM without 259 CM. At 39.5°C they exhibited the growth properties of normal Schwann cell populations. They required FBS-DMEM supplemented with 259 CM for growth, proliferated in 2FF medium, but did not grow in FBS-DMEM alone (data not shown). This reversion of growth behaviour at the restrictive temperature was observed both at early generation numbers and after 30–40 generations at the permissive temperature. Expression of large T therefore induced reversible changes in the growth requirements of Schwann cells, but was not essential for the continuous growth of LTts cells.

The proliferation rates of the LTts clones 33-10 and 33-11 and of their derivative LTts-ras clones 33-10ras3 and 33-11ras2 were measured in 2FF medium at 33°C and 39.5°C. Cell populations for all clones were tested at the same time and at an equivalent number of cell doublings (30–40 generations after the initial infection with LJ-tsSVLT), in order to control for any possible changes of cellular growth properties during clonal expansion. 33-10 and 33-11 cells proliferated at both temperatures (Figure 5a and c). In contrast, the proliferation rate of 33-10ras2 and 33-11ras3 cells fell dramatically after 2 days at 39.5°C, and the cell number remained approximately constant (33-10ras3) or declined slightly (33-11ras2) over the following 8 days (Figure 5b and d). While normal Schwann cells reached saturation density at $\sim 5 \times 10^4$cells/cm^2, growth arrest of LTts-ras cells could occur at densities of 2.5×10^3 cells/cm^2. Hence it is unlikely that the arrest was due to contact inhibition.

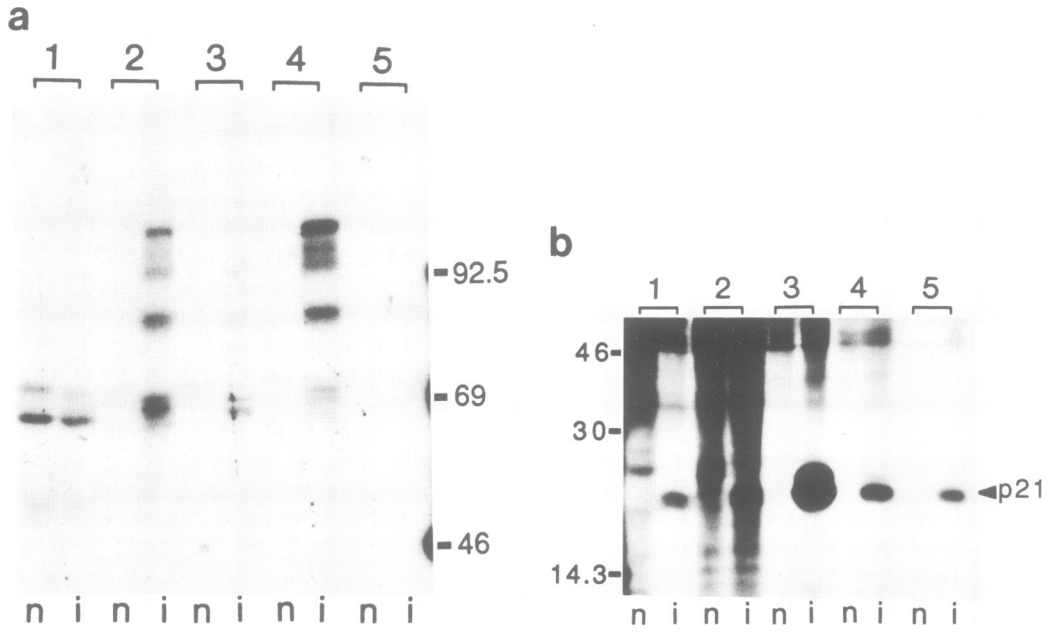

Fig. 4. Co-expression of SV40-ts58 large T antigen and $p21^{v\text{-}Ha\text{-}ras}$ in clones of Schwann cells. (**a**) Immunoprecipitations with an SV40 large T-specific antiserum were carried out on lysates of normal Schwann cells at 33°C (lane 1), 33-10ras3 cells at 33°C (lane 2) or 39.5°C (lane 3), and 33-11ras2 cells at 33°C (lane 4) or 39.5°C (lane 5). (**b**) Immunoprecipitations with the $p21^{ras}$ specific monoclonal rat antibody Y13-259 were carried out on lysates of cells grown at 39.5°C: normal Schwann cells (lane 1), tsLT clone 33-4 (lane 2), clone 33-4ras2 (lane 3), clone 33-10ras3 (lane 4) and clone 33-11ras2 (lane 5). Tracks representing cell lysates incubated with specific antibodies are labelled (i), non-immune sera controls are labelled (n). Mol. wt standard sizes are given in kd.

Further evidence for a growth arrest of LTts-ras cells at the restrictive temperature was obtained from time-lapse cinemicroscopy of the clones 33-10ras3 and 33-11ras2. Cells were filmed while maintained at 33°C for 1 day, then for 9 days at 39.5°C, and finally shifted back to 33°C. Figure 6 shows that no cells undergoing mitosis were detected in cultures of 33-10ras3 cells after 3 days at the restrictive temperature. Most cells remained viable at 39.5°C for 9 days, and 50–80% of cells underwent mitosis within 4 days of shifting the cultures back to 33°C. As observed for other cell types expressing SV40-tsA58 large T (Brugge and Butel, 1975; Brockman, 1978), changes in growth behaviour on altering the temperature occurred slowly, over a period of days. Similar results were obtained with 33-11ras2 cells (not shown). Thus v-Ha-*ras* expression in the absence of functional large T led to a complete block in cell division. The majority of cells were not irreversibly arrested, and re-expression of functional large T could stimulate the division of cells which had been in growth arrest for 6 days or more.

v-Ha-*ras*-induced growth arrest could be prevented in Schwann cells by introduction of wild-type SV40 large T antigen. LTts-ras cells were infected with Zip SV40 or ZipNeoSV(X) at 33°C, then maintained at 39.5°C. Foci of rapidly growing cells appeared within 7 days in cultures infected with Zip SV40, but not those infected with ZipNeoSV(X) (Table II). These foci were morphologically indistinguishable from LT-ras cells.

v-Ha-ras arrests Schwann cells in G_1 and G_2/M phases of the cell cycle

In order to determine at what point in the cell cycle the LTts-ras cells arrested when cultivated at 39.5°C, LTts and LTts-ras cells were analysed for DNA content using a FACS flow cytometer, at the same time as their growth rates were measured. On shifting from 33°C to 39.5°C, the LTts clones 33-10 and 33-11 showed a slight increase in the percentage of cells in G_1, and a decrease in G_2/M (Figure 7a and c). A completely different pattern of behaviour was observed for the LTts-ras clones 33-10ras3 and 33-11ras2. Clone 33-10ras3 showed a decrease in the number of cells in S phase to <3% after 2 days at 39.5°C, but the size of the G_2/M peak remained approximately constant during the 6-day experimental period. This suggested that, at the restrictive temperature, cells in G_1 were not able to enter S, and cells in G_2/M were unable to progress through mitosis to G_1. The percentage of 33-11ras2 cells in S phase also decreased at 39.5°C, while the proportion of cells in G_2/M increased to ~46% after 6 days. The DNA content of four other LTts-ras clones was analysed under similar conditions, and they also showed decreases in the number

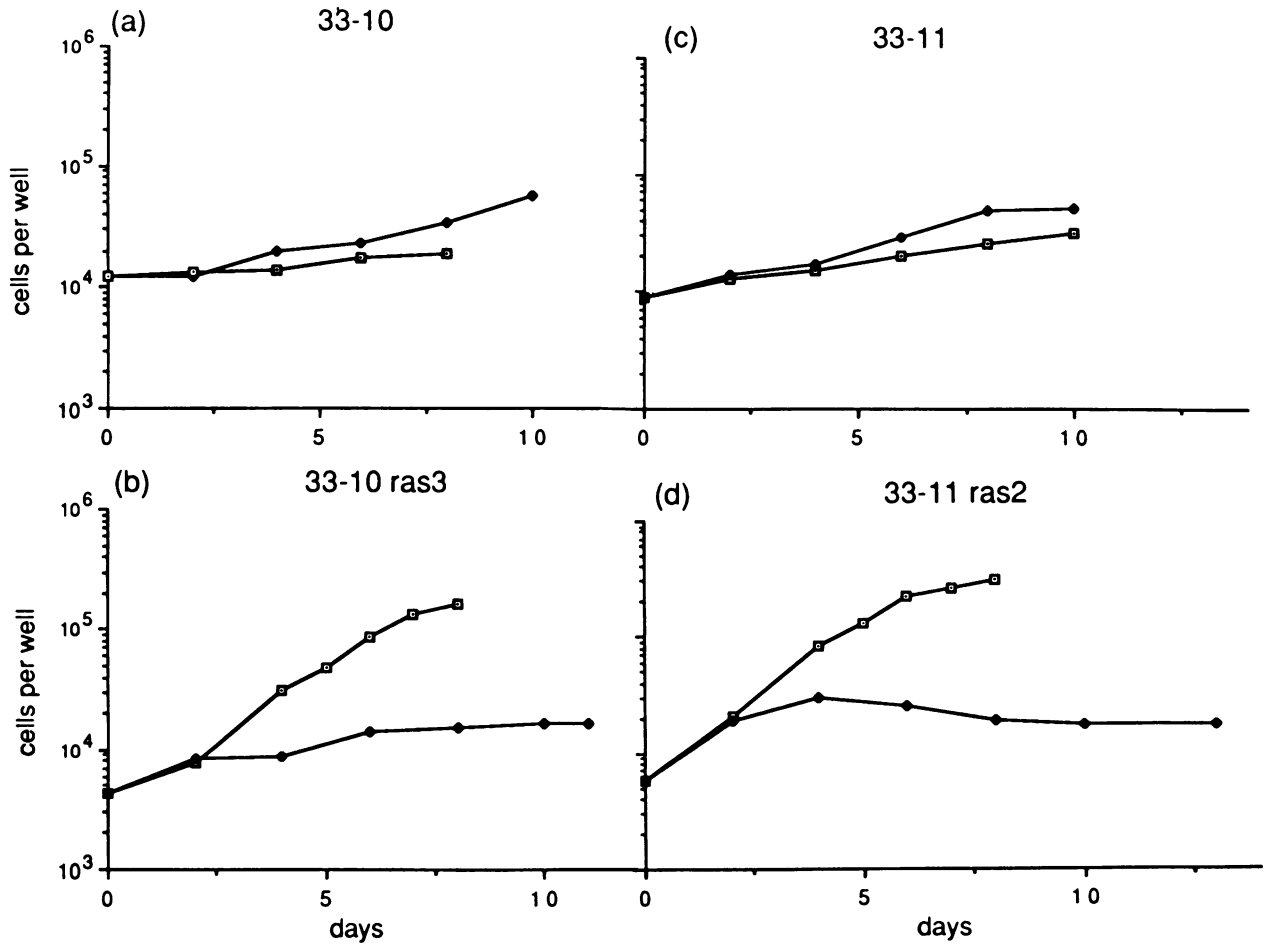

Fig. 5. Growth curves of Schwann cell clones expressing SV40-ts58 large T (33-10 and 33-11), and of clones co-expressing SV40-ts58 large T and $p21^{v\text{-Ha-}ras}$ (33-10ras3 and 33-11 ras2) were recorded at 33°C (-⊡-) and 39.5°C (-◆-). Cells were grown in 2FF medium. Cell numbers were determined at the times indicated.

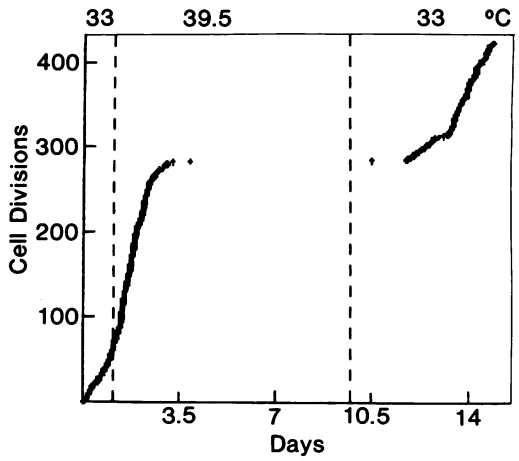

Fig. 6. Proliferation of Schwann cell clone 33-10ras3 as analysed by time-lapse cinemicroscopy. Cell divisions of an initial population of 72 cells were followed for a 15-day period. After 1 day at 33°C, the temperature was shifted to 39.5°C and kept for 9 days at this level. During the remaining 5 days the cells were observed at 33°C again. Cell divisions were plotted against time. Each division is represented by a cross.

Table II. Prevention of *ras*-induced growth arrest by wild-type SV40 large T or Adenovirus E1a

	Foci/10^6 cells: 33-10ras3	$G418^r$ col/10^6 cells: NIH-3T3
Zip SV40	8.96×10^3	2.0×10^5
ZipE1a 12S	2.40×10^3	2.4×10^5
ZipNeoSV (X)	0.05×10^3	5.0×10^5

33-10ras3 cells were infected at 33°C with the recombinant retroviruses shown, and subsequently maintained at 39.5°C. Foci were counted 14 days after infection. The titres of virus stocks, as determined by ability to confer G418 resistance in NIH-3T3 cells, is shown for comparison.

of cells in S phase at 39.5°C, with unchanged or accumulating numbers in G_2/M (data not shown). From these results we conclude that *ras* oncogene expression in the absence of functional large T caused two blocks in progression through the cell cycle, one in G_1 inhibiting entry into S and one in G_2/M inhibiting cell division. In contrast, early-passage Schwann cells accumulated in G1 both at high density and under growth factor starvation (A.Ridley, unpublished observations).

Microinjection of ras p21 proteins inhibits DNA synthesis in normal Schwann cells

The results so far show that oncogenic *ras* proteins are able to inhibit the proliferation of Schwann cells carrying the temperature-sensitive large T gene at the restrictive temperature. To investigate whether oncogenic *ras* proteins could also inhibit the proliferation of normal Schwann cells at physiological temperatures, subconfluent Schwann cells were microinjected with purified *ras* proteins. Cells were subsequently assayed for DNA synthesis 48 h after microinjection. Cultures were incubated with the thymidine analogue bromodeoxyuridine (BUdR) for 12 h, to determine the proportion of cells synthesizing DNA. Table III and Figure 8 show that DNA synthesis was 3- to 4-fold less in cells injected with either Val^{12}Ha-*ras* p21 or Lys^{61}N-*ras* p21, when compared to uninjected cells or those injected with a control mutant protein, $Val^{12}Ser^{186}$Ha-*ras* p21. This *ras* protein carries both an activating mutation at position 12, and a mutation at position 186 which prevents palmitoylation. The protein is not localized to the cell membrane, and is therefore not transforming (Willumsen *et al.*, 1984). The amount of injected Val^{12}Ha-*ras* p21 or Lys^{61}N-*ras* p21 which inhibited DNA synthesis in Schwann cells was of the same order as was required to stimulate DNA synthesis and morphologically transform NIH-3T3 cells (Trahey *et al.*, 1987).

As well as inhibiting DNA synthesis, microinjection of Val^{12}Ha-*ras* p21 or Lys^{61}N-*ras* p21 induced a dramatic change in morphology within 15–20 h of microinjection, as shown by time-lapse video microscopy. Very flat cells with an extensive cytoplasmic area became hyper-refractile with little visible cytoplasm (Figure 8a), similar in appearance to LTts-ras clones expressing high levels of v-Ha-*ras* p21 at 39.5°C (not shown). This morphology change was reversible, and cells began to return to their original appearance ~3 days after microinjection. This is in accordance with the time-dependent effect of *ras* p21 proteins in NIH-3T3 cells (Trahey *et al.*, 1987), and indicates that the inhibition of DNA synthesis observed was not due to cell death.

myc and E1a cooperate with v-Ha-ras in Schwann cells

Our results show that the nuclear oncogene SV40 large T cooperates with *ras* oncogenes to transform Schwann cells, and prevents *ras*-induced growth arrest. To determine whether other nuclear oncogenes had similar properties, plasmids carrying a *myc* oncogene (pSVc-myc 1, Land *et al.*, 1983a) or the adenovirus E1a 12S cDNA (pJF12, Schneider *et al.*, 1987) were cotransfected into Schwann cells together with pEJ6.6. Colonies with morphology similar to LT-ras cells were obtained, and representative clones were analysed for expression of *myc* (Figure 9a) or E1A mRNA (not shown). Expression of *myc*-specific sequences was at least 20-fold higher in cells transfected with pSVc-myc 1 compared to untransfected Schwann cells. This was principally due to expression of pSVc-myc 1. c-*myc* exon 1-specific sequences unique to the endogenous c-*myc* gene were not over-expressed, as shown by RNase protection experiments (L.Penn and A.Ridley, unpublished observations).

In addition, LTts-ras cells were infected with a recombinant retrovirus containing the E1a 12S cDNA (ZipE1a-12S, Roberts *et al.*, 1985). Expression of E1a enabled the outgrowth of transformed colonies at 39.5°C (Table II, Figure 9b). The Northern blots shown in Figure 9 were rehybridized with a γ-actin-specific probe to confirm that approximately equal amounts of RNA were present in each sample (not shown). These results show that c-*myc* and E1a oncogenes, as well as SV40 large T, are able to prevent *ras*-induced growth arrest in Schwann cells.

Discussion

We have used Schwann cells as a model system to investigate the mechanisms whereby oncogenes cooperate to transform cells. Coexpression of the SV40 large T and v-Ha-*ras* oncogenes transforms Schwann cells, concomitantly

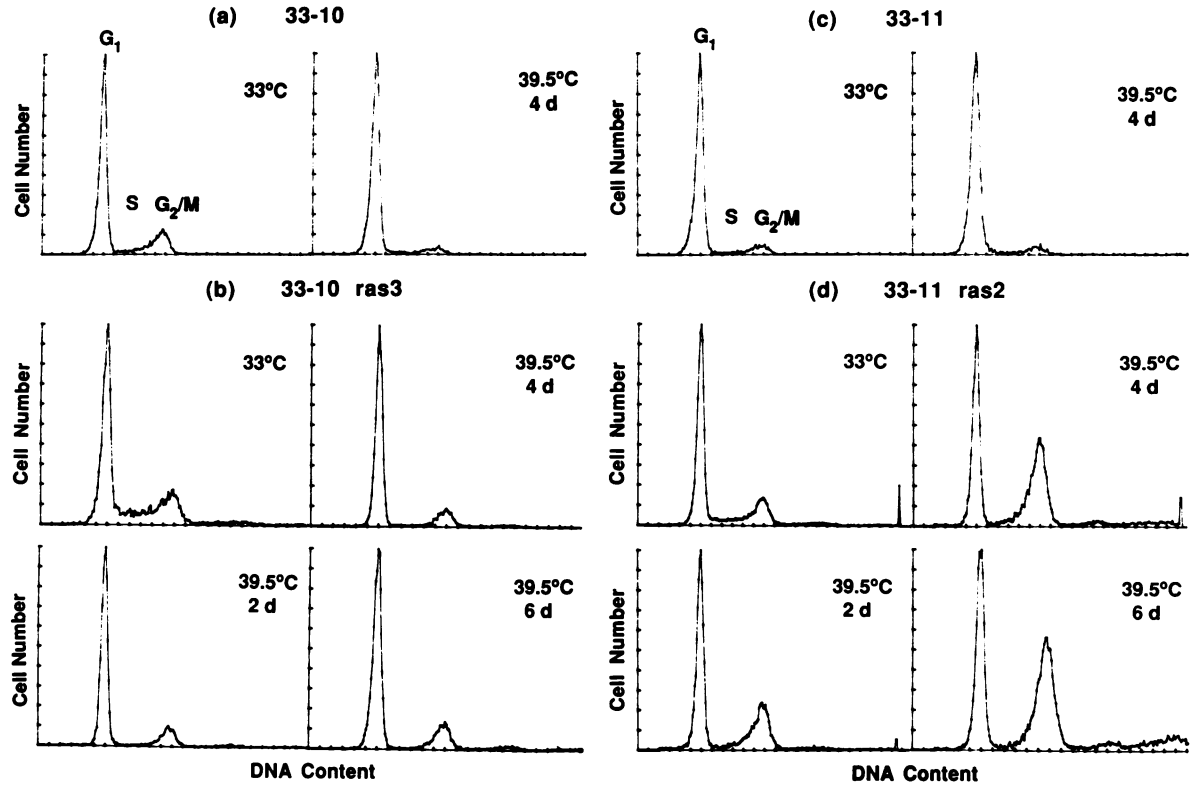

Fig. 7. Flow cytometric analysis of DNA content in Schwann cell clones 33-10, 33-10ras3, 33-11 and 33-11ras grown at 33°C or 39.5°C for 2, 4 or 6 days.

eliminating their requirement for exogenous growth factors, increasing their growth rate several-fold, inducing growth in soft agar and leading to focus formation. Expression of SV40 large T alone does not fully transform the cells, but reduces their stringent growth factor requirements. In contrast, expression of oncogenic *ras* proteins in the absence of functional large T inhibits cell division. Thus the response of Schwann cells to *ras* oncogenes is changed by a nuclear oncogene from cell cycle arrest to proliferation and transformation.

In general, cells from primary cultures have a limited lifespan *in vitro*. However, introduction of a nuclear oncogene into primary cell populations can often promote the establishment of cell lines with prolonged or indefinite replicative potential. Rat Schwann cells are unusual in that the conditions which have been optimized for their growth *in vitro* allow proliferation for several months without crisis (Porter *et al.*, 1986). During this time, no significant changes occur in their growth factor responsiveness, antigen expression or their ability to myelinate nerve axons. Therefore in these cells a nucelar oncogene would not be expected to play a role in prolonging their lifespan in culture. We have confirmed this using a temperature-sensitive mutant of large T. Schwann cells expressing SV40-tsA58 large T continue to proliferate at both the permissive and restrictive temperatures, even after 40 population doublings. However, SV40 large T does have a clear effect on Schwann cells in reducing their growth factor requirements, and in altering their response to *ras* oncogenes.

The proliferation arrest induced by activated *ras* proteins both in LTts-ras cells at the restrictive temperature and in normal Schwann cells upon microinjection is in marked contrast to the induction of proliferation observed in several cell lines (Feramisco *et al.*, 1984; Trahey *et al.*, 1987). Microinjection of activated *ras* proteins has also been reported to stimulate DNA synthesis in early-passage rodent embryo fibroblasts (Sullivan *et al.*, 1986). However, in cells from the pheochromocytoma line PC12, microinjection of oncogenic *ras* proteins induced differentiation, and concomitantly inhibited cell division (Bar-Sagi and Fermamisco, 1985). We have no evidence that the *ras*-induced proliferation arrest of Schwann cells is accompanied

Table III. BUdR uptake and incorporation in Schwann cells following microinjection with *ras* p21

	No. exp.	No. cells injected	No. BUdR-labelled nuclei	% BUdR-labelled nuclei
a) Val^{12} Ha-*ras* p21				
Val^{12} Ha	I	589	86	13.2
	II	596	70	
$Val^{12}ser^{186}$ Ha	I	664	302	46.7
	II	447	217	
Uninjected	I	642	314	46.3
	II	593	258	
b) Lys^{61}N-*ras* p21				
Lys^{61} N	I	310	42	10.1
	II	514	41	
$Val^{12}ser^{186}$Ha	I	427	177	34.1
	II	662	194	
Uninjected	I	416	168	32.1
	II	619	164	

Normal Schwann cells were microinjected with purified *ras* proteins in two separate experiments. 48 h after microinjection, BUdR was added to the cultures for a 12 h period. The number of BUdR-labelled nuclei was determined by immunofluorescence using a BUdR-specific antibody.

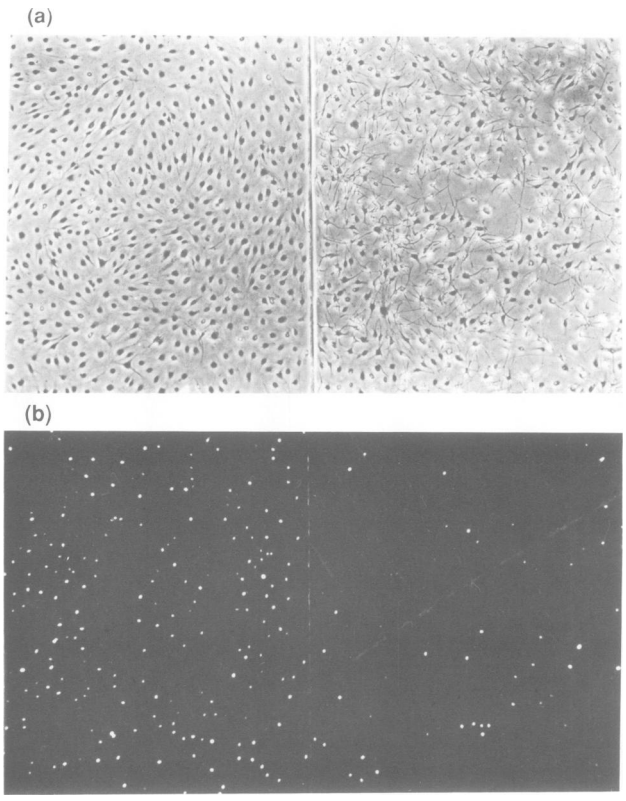

Fig. 8. Inhibition of DNA synthesis by Val^{12}Ha-*ras* p21. Normal Schwann cells were microinjected with Val^{12}Ha-*ras* p21 (right of the central line) or $Val^{12}Ser^{186}$Ha-*ras* p21 (left of the central line). **(a)** Phase-contrast micrograph of cells 48 h after microinjection. **(b)** Fluorescence micrograph of the same field of cells stained for BUdR incorporation with an anti-BUdR antibody.

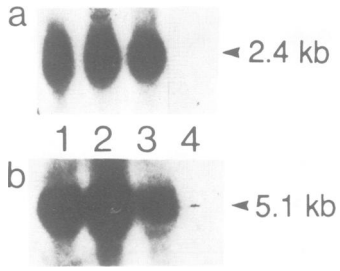

Fig. 9. Cooperation of *myc* and adenovirus E1A oncogenes with *ras* oncogenes in Schwann cells. Northern analysis of RNA derived from transformed cell clones obtained **(a)** after co-transfection of Schwann cells with pEJ6.6 and pSV c-myc 1, and **(b)** after infection of LTts-ras cells with the retrovirus ZipE1A-12S and subsequent cultivation at 39.5°C. Lane 4 represents RNA from normal Schwann cell populations in a and b. The samples were hybridized with ^{32}P-labelled probes specific for murine c-*myc*, exons 2 and 3 (a) and for adenovirus E1A (b).

by expression of differentiation-specific antigens, but we cannot exclude the possibility that the cells may acquire a more differentiated phenotype.

At the restrictive temperature, LTts-ras cells arrest in either the G_0/G_1 or G_2/M phases of the cell cycle, whereas cultured cells normally arrest in G_0/G_1 when they stop dividing at high cell density or through starvation (Pardee *et al.* 1978; Zetterberg and Larsson, 1985). However, there is some evidence that a G_2 control point may exist in the cell cycle of higher eukaryotic cells, as has been well characterized in fission yeast (Nurse, 1985). For example, lymphocytes have been shown to arrest in G_1 or G_2 depending on the growth factors in the medium (Melchers and Lernhardt, 1985). In addition, while NGF inhibits the division of PC12 cells, it causes an increase in the number of cells in G_2/M from 15% to 45%, along with the appearance of octaploid cells (Ignatius *et al.*, 1985). This observation may reflect a physiological phenomenon, since some neuronal cells are tetraploid *in vivo* (Lapham, 1968; Bregnard *et al.*, 1975; Bohm *et al.*, 1981). Finally, when cells of the chronic myeloid leukaemia line K-562 are treated with phorbol esters, division is inhibited due to cell cycle blocks in G_1 and G_2 (Colamonici *et al.*, 1985). However, while microinjection of antibodies to *ras* proteins inhibits transition from G_1 to S (Mulcahy *et al.*, 1985), indicating that *ras* proteins are required during G_1, little is known about their activity during G_2 or M. Our results indicate that oncogenic *ras* proteins may also act at this later stage in the cell cycle, but whether normal *ras* proteins play a role in regulating transition through G_2 or M remains to be established.

ras-induced growth arrest is prevented by other nuclear oncogenes apart from SV40 large T. c-*myc* and adenovirus E1a oncogenes also cooperate with Ha-*ras* oncogenes to transform Schwann cells, indicating that they are able to provide a rescuing function similar to large T. Hence the interaction between *ras* and SV40 large T oncogenes may reflect a general mechanism for oncogene cooperation in transformation.

Expression of *ras* oncogenes alone clearly alters the growth factor responsiveness of Schwann cells, apparently desensitizing them to their normal mitogenic stimuli. In contrast, expression of nuclear oncogenes allows *ras* oncogenes to stimulate proliferation. There are several models which could explain these observations. In one model, oncogenic *ras* proteins would inhibit the cellular responsiveness to one or more growth factors, whereas nuclear oncogenes would abrogate the requirement for these same growth factors. Thus the growth-inhibitory effect of *ras* expression would no longer be of consequence. In an alternative model, expression of *ras* oncogenes would either stimulate secretion of growth-inhibitory factors, or sensitize Schwann cells to such factors. Nuclear oncogenes would desensitize cells to these factors, so that Schwann cell proliferation would no longer be inhibited. In both of these scenarios, it would be necessary for oncogenic *ras* proteins to have a second, independent action stimulating uncontrolled growth, in order to achieve full transformation in the presence of a nuclear oncogene.

Establishment of cell lines involves as yet uncharacterized changes in cell properties, and these may include altering the cellular response to *ras* oncogenes. It would therefore be of interest to investigate the action of *ras* proteins in other normal cell populations, and to determine how general *ras*-induced effects on growth might be. Proliferating haematopoietic cell populations have been isolated from both spleen and bone marrow cultures infected with retroviruses carrying the v-Ha-*ras* oncogene. (Pierce and Aaronson, 1985; Rein *et al.*, 1985; Kahn *et al.*, 1986). However, there is some evidence that high levels of *ras* oncogene expression may inhibit the proliferation of baby rat kidney cells (Kelekar and Cole, 1987) and REF52 cells (Franza *et al.*,

1986). We have observed that proliferation arrest of Schwann cells *in vitro* occurs at levels of $p21^{v\text{-}Ha\text{-}ras}$ expression equivalent to normal levels of $p21^{c\text{-}Ha\text{-}ras}$. Therefore activation of one Ha-*ras* allele *in vivo* might be sufficient to cause a similar effect.

Our results suggest that certain cell types could harbour activated *ras* genes *in vivo* for long periods of time without any signs of abnormal growth. This idea is supported by several observations. For example, if small numbers of cells expressing a *ras* oncogene are introduced into a reconstituted organ, they participate normally in the development of both epithelial and mesenchymal tissues, without signs of aberrant proliferation (T.C.Thompson and H.Land, in preparation). In addition, infection of mouse skin cells with Harvey sarcoma virus does not lead to neoplastic growth until tumour promoters are applied (Brown *et al.*, 1986). Cells carrying a mutated *ras* gene *in vivo* could be stimulated to divide transiently by specific mitogens or hormones. Subsequent activation of a second, cooperating oncogene could lead to rapid tumour progession if, as in Schwann cells, coexpression of *ras* and nuclear oncogenes caused autonomous growth and increased proliferation rate.

Materials and methods

Preparation of primary Schwann cells

Sciatic nerves from 2–3 day-old Wistar rats were prepared and Schwann cells purified as described by Brockes *et al.* (1979). Briefly, sciatic nerves were excised, treated with collagenase and trypsin and plated on 60 mm dishes (Corning) coated with poly-L-lysine (Sigma). Cells for continuous culture were initially maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS (Gibco) for 48–72 h, and subsequently treated with the antimitotic drug cytosine arabinoside for 48 h to eliminate dividing fibroblasts. Most remaining fibroblasts were removed using complement-mediated lysis with monoclonal anti-Thy-1 antibody (Lake *et al.*, 1979). For infection, cells were plated together with retrovirus-producing Ψ-2 cells (see below), 2 days prior to treatment with anti-Thy-1 and complement.

Cell culture

IN/259 cells originate from a human glioma cell line, and appear to secrete GGF, since conditioned medium from IN/259 cells stimulates Schwann cell growth through a molecule with the physicochemical properties of GGF (M.Noble, B.Watkins and J.Brockes, unpublished observations). IN/259 cells were grown to confluency in 10% FBS-DMEM, incubated in DMEM for 48 h, then conditioned DMEM (259 CM) was harvested from them every 48 h. Schwann cells were grown on tissue culture dishes coated with poly-L-lysine, in DMEM with 1.5 mg/ml glucose. Normal, LT and tsLT cells were maintained in 2FF: DMEM with 3% FBS supplemented with 10% 259 CM and 2 μM forskolin (Calbiochem). LT-ras and LTts-ras cells were maintained in 3% FBS-DMEM, except where indicated differently. The serum-free medium B-S has been described previously (Raff *et al.*, 1985). It consisted of DMEM with bovine insulin (0.5 mg/ml), human transferrin (100 μg/ml), bovine serum albumin (100 μg/ml), Progesterone (0.06 ng/ml), putrescine (16 μg/ml), selenium (0.04 ng/ml), thyroxine (0.4 ng/ml) and triiodothyronine (0.3 ng/ml).

For soft agar assays, 5×10^4 cells were seeded in DMEM containing 0.3% low-melting agarose (Seaplaque) and 3% FBS. Colony growth was assessed 14 days after seeding.

Construction of the retrovirus LJ-tsSVLT

The *Avr*II (5234)–*Hpa*I (2666) fragment carrying the large T gene derived from SV40-tsA58 DNA (Tegtmeyer *et al.*, 1975) (provided by Dr Feunteun) was inserted into the retroviral vector pLJR using *Bam*H1-linkers to form LJ-tsSVLT. pLJR was derived from pLJ (Korman *et al.*, 1987) by replacing the Polyoma virus-specific backbone with a portion of pBR322 containing the amp^r gene and the replication origin (J.Morgenstern, unpublished data). LJ-tsSVLT was transfected into the packaging cell line Ψam-22 (Cone and Mulligan 1984) and transiently produced amphotrophic virus was harvested 20 h later. This virus stock was used to infect Ψ-2 cells (Mann *et al.*, 1983), a packaging cell line giving rise to ecotrophic virus particles. G418-resistant colonies of infected Ψ-2 cells were subsequently isolated. The clone Ψ2-SVts58III/6 produced titres of $\sim 5 \times 10^5$ c.f.u./ml when tested for the ability to confer G418 resistance in NIH-3T3 cells (see below). The virus stock was shown to be free of helper virus contamination. Southern blot analysis demonstrated that Ψ2-SVts58III/6 contained a single copy of a provirus carrying the cDNA encoding large T ts58. The small T-specific intron had been lost, probably due to a splicing event in the viral RNA prior to stable infection of the Ψ-2 cells. These experiments have been carried out in accordance with the British ACGM/HSE guidelines, note 5.

Infection of Schwann cells

Primary Schwann cells were infected by cocultivation with Ψ-2 cells producing the recombinant retroviruses Zip SV40 and LJ-tsSVLT. Ψ-2 cells were pretreated with mitomycin C (20 μg/ml, Sigma) for 2 h, trypsinized and seeded with Schwann cells at a ratio of 1:2. 48–72 h after seeding, cells were transferred to selective medium containing 400 μg/ml G418 (Gibco). LT, LTts and LTts-ras cells were infected by incubating subconfluent cultures with supernatant from Ψ-2 producer cells for 2 h in the presence of polybrene (8 μg/ml). Titres of virus stocks were determined by infecting NIH-3T3 cells with limiting dilutions of supernatant from each Ψ-2 cell line, followed by G418 selection.

Transfection of Schwann cells

2×10^5 Schwann cells were transfected with 10 μg of each oncogene-bearing plasmid, 1 μg pSV2-neo and 30–40 μg rat embryo fibroblast carrier DNA using the calcium phosphate precipitation method of Graham and Van der Eb (1973). 48 h after transfection, cells were trypsinized and split 1:4, and 24 h later selective medium containing 400 μg/ml G418 was added.

Immunofluorescence

Laminin antiserum was obtained from Gibco, collagen IV antiserum was a gift from R.Mirsky, and SV40 large T-specific antiserum was a gift from D.Lane. Cells were grown on 12 well multitest slides (Flow laboratories) coated with poly-L-lysine. Cells to be stained for cell surface antigens N-CAM, laminin and Collagen IV were incubated for 30 min at 20°C with the appropriate antiserum diluted in HHM (Hepes-buffered Hank's medium containing 5% calf serum and 0.02% sodium azide). They were then washed 5 times with HHM, and incubated with fluorescein-coupled goat anti-rabbit immunoglobulin (Southern Biotechnology) at a dilution of 1:100 in HHM for 30 min. Cells were fixed for 20 min in methanol at −20°C. Cells to be stained for large T were prefixed in methanol than stained as above.

Growth curves

Normal, LT and LTts Schwann cells were seeded at 10^4 cells/well, LT-ras and LTts-ras cells at 2×10^3 cells/well on 24-well dishes (Falcon). 2 days after seeding, cells were fed with the appropriate medium and shifted to 39.5°C where required. Subsequently cells were fed every 48 h. To count cells, duplicate wells were trypsinized and counted with a Coulter counter.

Nucleic acid analysis

For analysis of proviral integrations, 10 μg aliquots of cellular DNA were digested with *Eco*RI and electrophoresed in 1% agarose gels. *Eco*RI cleaves at a unique site in the proviral DNA of both LJ-tsSVLT and Zipras 6. For Northern blotting, total cellular RNA was extracted by the guanadinium isothiocyanate method (Maniatis *et al.*, 1982) and electrophoresed in 1% agarose/formaldehyde gels. Nucleic acids were transferred to GeneScreen *Plus*™ (NEN), and hybridized with ^{32}P-labelled probes. Filters were autoradiographed using Kodak XAR-5 film.

Immunoprecipitation

Semi-confluent cells were labelled with [^{35}S]methionine (200 μCi/60 mm dish; Amersham) for 14 h ($p21^{Ha\text{-}ras}$) or 3 h (SV40 large T) in DMEM lacking methionine and containing 1% FBS. Cells were washed in phosphate-buffered saline and lysed in phospholysis buffer [10 mM Na_2HPO_4 (pH 7.5), 0.1 M NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS]. A normalized amount of tricholoroacetic acid-precipitable radioactivity (5×10^6 c.p.m.) was used for each immunoprecipitation. Cell extracts were precleared by incubation with normal rabbit serum and then with *Staphylococcus aureus* Cowan 1. To detect SV40 large T, extracts were incubated with 2 μl of polyclonal rabbit anti-SV40 large T antigen antiserum (a gift from D.Lane) or with 2 μl of rabbit serum overnight at 4°C. For detection of $p21^{Ha\text{-}ras}$, each extract was incubated with 10 μl of the rat anti-*ras* p21 monoclonal antibody Y13-259 (Oncogene Science) or with 2 μl of rat serum overnight at 4°C. Five microlitres of goat anti-rat IgG (Cappel) was added and extracts incubated for 20 min. Extracts were finally incubated for 20 min with 40 μl of *S.aureus*, and cell pellets were washed sequentially with the following buffers: (i) 0.1 M NaCl, 1mM EDTA, 0.1 M Tris–Cl (pH 8.0), 1% NP40, 0.3% SDS (ii) 1 M NaCl,

0.1% Tris−Cl (pH 8.0), 0.1% NP40 (iii) 10 mM Tris−Cl (pH 8.0), 0.1% NP40. Pellets were resuspended in 20 μl SDS−PAGE sample buffer, boiled for 5 min and centrifuged. Supernatants were run on 8% (large T) or 13% ($p21^{ras}$) SDS−polyacrylamide gels as described by Laemmli (1970).

Gels were fixed in 10% acetic acid, 50% methanol, incubated for 30 min in Amplify™ (Amersham), dried and exposed to Kodak XAR-5 film.

Time-lapse cinemicroscopy

The procedures used have been described in detail elsewhere (Riddle, 1979; Brooks *et al.*, 1983). Briefly, 5×10^4 cells were plated on poly-L-lysine-coated 60 mm Petri dishes (Corning), in 2FF medium. Cultures were filmed on Olympus IMT inverted microscopes using Bolex cameras with Olympus controls and Kodak Infocapture AHU 16-mm film. A frame interval of 4 min was used. Cultures were maintained in 10% humidified CO_2 in air at 33°C or 39.5°C, and refed with 2FF every 3 days. Films were analysed using an L.W. Analytical Projector.

DNA flow cytometry

Cells for flow cytometric analysis were fixed in 80% ethanol for 30 min at 4°C, and stained in 50 μg/ml mithromycin (Pfizer), 25% ethanol, 20 mM $MgCl_2$ for 30 min. The relative fluorescence of $\sim 2 \times 10^4$ cells was measured in a flow cytometer (FACS-1). The percentage of cells within the cell cycle phases G_0/G_1, S and G_2+M was calculated from the DNA histogram.

Microinjection

Subconfluent Schwann cells cultured on poly-L-lysine-coated 60 mm Petri dishes in 2FF were microinjected intracytoplasmically according to the method of Graessmann and Graessmann (1983). Approximately 2×10^{-11} ml of *ras* p21 protein solution was injected into every cell within marked areas of the culture dish. *Escherichia coli*-produced, purified *ras* p21 proteins (a gift from A.Hall) were diluted to a concentration of 1.5 mg/ml with phosphate-buffered saline. Injected cultures were subsequently monitored using a Panasonic NV8051 time-lapse video recorder connected to a Nikon Diaphot microscope with incubator jacket.

DNA synthesis assay

10 μM 5-bromodeoxyuridine (BUdR) was added to the culture medium of microinjected cells at 48 h after injections for 15−16 h. Cells were fixed *in situ* in 70% ethanol for 30 min, and allowed to air-dry. Dishes were immersed in 0.07 N NaOH for 2 min and neutralized with 0.1 M $Na_2B_4O_7$ at pH 8.5. Cells were then labelled with rat anti-BUdR antibody (a gift from M.Ormerod) in 0.5% Tween20 for 30 min at 37°C. After washing in phosphate-buffered saline, cells were further incubated with rhodamine-conjugated rabbit anti-rat IgG (Sigma) diluted 1:100 in 0.5% Tween20. DNA synthesis was determined from the number of BUdR-positive fluorescent nuclei scored using a Zeiss fluorescent microscope.

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References

Armelin,A., Armelin,M.C.S., Kelly,K., Stewart,T., Leder,P., Cochran, B.H. and Stiles,C.D. (1984) *Nature*, **310**, 655−660.
Barbacid,M. (1987) *Annu. Rev. Biochem.*, **56**, 779−827.
Bar-Sagi,D. and Feramisco,J.R. (1985) *Cell*, **42**, 841−848.
Berridge,M.J. and Irvine,R.F. (1984) *Nature*, **312**, 315−321.
Bishop,J.M. (1985) *Cell*, **42**, 23−38.
Bishop,J.M. (1987) *Science*, **235**, 305−310.
Bohm,N., Kroner,B. and Kaiser,E. (1981) *Cell tissue Kinet.*, **14**, 433−444.
Bregnard,A., Knusel,A. and Kuenzle,C.C. (1975) *Histochemistry*, **43**, 59−61.
Brockes,J.P., Fields,K.L. and Raff,M.C. (1979) *Brain Res.*, **165**, 105−118.
Brockes,J.P., Raff,M.C., Nishiguchi,D.J. and Winter,J. (1980) *J. Neurocytol.*, **9**, 67−77.
Brockman,W. (1978) *J. Virol.*, **25**, 860−870.
Brooks,R.F., Riddle,P.N., Richmond,F.N. and Marsden,J. (1983) *Exp. Cell Res.*, **148**, 127−142.
Brown,K., Quintanilla,M., Ramsden,M., Kerr,I.B., Young,S. and Balmain,A. (1986) *Cell*, **46**, 447−456.
Brugge,J.S. and Butel,J.S. (1975) *J. Virol.*, **15**, 619−635.
Carey,D.J., Eldridge,C.F., Cornbrooks,C.J., Timpl,R. and Bunge,R.P. (1983) *J. Cell Biol.*, **97**, 473−479.
Cepko,C.L. Roberts,B.E. and Mulligan,R.C. (1984) *Cell*, **37**, 1053−1062.
Colamonici,O.R., Trepel,J.B. and Neckers,L.M. (1985) *Cytometry*, **6**, 591−596.
Cone,R.D. and Mulligan,R.C. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 6349−6353.
Coppola,J.A. and Cole,M.D. (1986) *Nature*, **320**, 760−763.
Cornbrooks,C.J., Carey,D.J., McDonald,J.A., Timpl,R. and Bunge,R.P. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 3850−3854.
Dotto,G.P., Parada,I.F. and Weinberg,R.A. (1985) *Nature*, **318**, 472−475.
Falcone,G., Tato,F. and Alema,S. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 426−430.
Feramisco,J.R., Gross,M., Kamata,T., Rosenberg,M. and Sweet,R.W. (1984) *Cell*, **38**, 109−117.
Fleischman,L.F., Chahwala,S.B. and Cantley,L. (1986) *Science*, **231**, 407−410.
Franza,B.R.,Jr, Maruyama,K., Garrels,J.I. and Ruley,H.E. (1986) *Cell*, **44**, 409−418.
Furth,M.E., Davies,L.J., Fleurdelys,B. and Scolnick,E.M. (1982) *J. Virol.*, **43**, 294−304.
Gibbs,J.B., Sigal,I.S., Poe,M. and Scolnick,E.M. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 5704−5708.
Gilman,A.G. (1984) *Cell*, **36**, 577−579.
Goustin,A.S., Leof,E.B., Shipley,G.D. and Moses,H.L. (1986) *Cancer Res.*, **46**, 1015−1029.
Graessmann,M. and Graessmann,A. (1983) *Methods Enzymol.*, **101**,. 482−492.
Graham,F.L. and Van der Eb,A.J. (1973) *Virology*, **52**, 456−467.
Hanahan,D. (1986) In Kahn,P. and Graf,T. (eds), *Oncogenes and Growth Control*. Springer-Verlag, Berlin, pp. 349−363.
Ignatius,M.J., Chandler,C.R. and Shooter,E.M. (1985) *J. Neurosci.*, **5**, 343−351.
Jat,P.S., Cepko,C.L., Mulligan,R.C. and Sharp,P.A. (1986) *Mol. Cell Biol.* **6**, 1204−1217.
Jenssen,K.R. and Mirsky,R. (1984) *J. Neurocytol.*, **12**, 923−934.
Kahn,P., Frykberg,L., Brady,C., Stanely,I., Beug,H., Vennstrom,B. and Graf,T. (1986) *Cell*, **45**, 349−356.
Kalderon,D., Oostra,B.A., Ely,B.K. and Smith,A.E. (1982) *Nucleic Acids Res.*, **10**, 5161−5171.
Kelekar,A. and Cole,M.D. (1987) *Mol. Cell. Biol.*, **7**, 3899−3907.
Korman,A.J., Frantz,J.D., Strominger,J.L. and Mulligan,R.C. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 2150−2154.
Lacal,J.C., Fleming,T.P., Warren,B.S., Blumberg,P.M. and Aaronson,S.A. (1987) *Mol. Cell. Biol.*, **7**, 4146−4149.
Laemmli,U.K. (1970) *Nature*, **227**, 680−682.
Lake,P., Clark,E.A., Khorshidi,M. and Sunshine,G.H. (1979) *Eur. J. Immunol.*, **9**, 875−886.
Land,H., Parada,L. and Weinberg,R. (1983a) *Nature*, **304**, 596−602.
Land,H., Parada,L.F. and Weinberg,R.A. (1983b) *Science*, **222**, 771−778.
Land,H., Parada,L.F. and Weinberg,R.A. (1984) *Cancer Cells*, **2**, 473−479.
Land,H., Chen,A.C., Morgenstern,J.P., Parada,L.F. and Weinberg,R.A. (1986) *Mol. Cell. Biol.*, **6**, 1917−1925.
Lapham,L.W. (1968) *Science*, **159**, 310−312.
Lemke,G.E. and Brockes,J.P. (1984) *J. Neurosci.*, **4**, 75−83.
Maniatis,T., Fritsch,P. and Sambrook,J. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Mann,R., Mulligan,R.C. and Baltimore,D. (1983) *Cell*, **33**, 153−159.
Melchers,F. and Lernhardt,W. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 7681−7685.
Mougneau,E., Lemieux,L., Rassoulzadegan,M. and Cuzin,F. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 5758−5762.
Mulcahy,L.S., Smith,M.R. and Stacey,D.W. (1985) *Nature*, **313**, 241−243.
Noble,M., Albrechsten,M., Meller,C., Lyles,J., Bock,E., Goridis,C., Watanabe, M. and Rutishauser,U. (1985) *Nature*, **316**, 725−728.
Nurse,P. (1985) *Trends Genet.*, **1**, 51−55.
Ozanne,B.R., Fulton,R.J. and Kaplan,P.L. (1980) *J. Cell Phys.*, **105**, 163−180.
Pardee,A.B., Dubrow,R., Hamlin,J.L. and Kletzien,R.F. (1978) *Annu. Rev. Biochem.*, **47**, 715−750.

Pierce,J.H. and Aaronson,S.A. (1985) *Mol. Cell. Biol.*, **5**, 667–674.
Porter,S., Clark,M.B., Glaser,L. and Bunge,R.P. (1986) *J. Neurosci.*, **6**, 3070–3078.
Raff,M.C., Abney,E., Brockes,J.P. and Hornby-Smith,A. (1978) *Cell*, **15**, 813–822.
Raff,M.C., Fields,K.L., Hakamori,S.I., Mirsky,R., Pruss,R. and Winter,J. (1979) *Brain Res.*, **174**, 283–308.
Raff,M.C., Miller,R.H. and Noble,M. (1985) *Nature*, **303**, 390–396.
Ratner,N., Bunge,R.P. and Glaser,L. (1985) *J. Cell Biol.*, **101**, 744–754.
Rein,A., Keller,J., Schultz,A.M., Holmes,K., Medicus,R. and Ihle,J.N. (1985) *Mol. Cell. Biol.*, **5**, 2257–2264.
Riddle,P.D. (1979) *Time-lapse Cinemicroscopy*. Academic Press, London.
Roberts,B.E., Miller,J.S., Kimelman,D., Cepko,C.L., Lemischka,I.R. and Mulligan,R.C. (1985) *J. Virol.*, **56**, 404–413.
Royer-Prokora,B., Beng,H., Claviez,M., Winkhardt,H.-J., Friis,R.R. and Graf,T. (1978) *Cell*, **13**, 751–760.
Rozengurt,E. (1986) *Science*, **234**, 161–166.
Ruley,H.E. (1983) *Nature*, **304**, 602–606.
Ruley,H.E., Moomaw,J.F. and Maruyama,K. (1984) *Cancer Cells*, **2**, 481–486.
Schneider,J.F., Fisher,F., Goding,C.R. and Jones,N.C. (1987) *EMBO J.*, **6**, 2053–2060.
Shih,C. and Weinberg,R.A. (1982) *Cell*, **29**, 161–169.
Simanis,V. and Lane,D. (1985) *Virology*, **144**, 88–100.
Southern,P.J. and Berg,P. (1982) *J. Mol. Appl. Genet.*, **1**, 327–341.
Sporn,M.B. and Todaro,G.J. (1980) *N. Engl. J. Med.*, **303**, 878–880.
Stern,D.F., Roberts,A.B., Roche,N.S., Sporn,M.B. and Weinberg,R.A. (1986) *Mol. Cell. Biol.*, **6**, 870–877.
Sullivan,N.F., Sweet,R.W., Rosenberg,A. and Feramisco,J.R. (1986) *Cancer Res.*, **46**, 6427–6432.
Sweet,R.W., Yokoyama,S., Kamata,T., Feramisco,J.R., Rosenberg,M. and Gross,M. (1984) *Nature*, **311**, 273–275.
Tegtmeyer,P., Schwartz,M., Collins,J.K. and Rundell,K. (1975) *J. Virol.*, **16**, 168–178.
Trahey,M., Milley,R.J., Cole,G.E., Innis,M., Paterson,H., Marshall,C.J., Hall,A. and McCormick,F. (1987) *Mol. Cell Biol.*, **7**, 541–544.
Varmus,H.E. (1987) *Science*, **238**, 1337–1339.
Wakelam,M.J.O., Davies,S.A., Houslay,M.D., McKay,I., Marshall,C.J. and Hall,A. (1986) *Nature*, **323**, 173–176.
Webster,H. de F. (1975) In Dyck,P.J., Thomas,P.K. and Lambert,E.H. (eds) *Peripheral Neuropathology*. W.B.Saunders Co., Philadelphia, pp. 37–61.
Weinberg,R.A. (1985) *Science*, **230**, 770–776.
Willumsen,B.M., Norris,K., Papageorge,A.G., Hubert,N.L. and Lowy, D.R. (1984) *EMBO J.*, **3**, 2581–2585.
Zetterberg,A. and Larsson,O. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 5365–5369.

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