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Transformation and stimulation of DNA synthesis in NIH-3T3 cells are a titratable function of normal p21N-*ras* expression

Ian A.McKay, Christopher J.Marshall, Carmela Calés and Alan Hall

Chester Beatty Laboratories, Institute of Cancer Research, Fulham Road, London SW3 6JB, UK

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A plasmid has been constructed which contains the normal human N-*ras* proto-oncogene under the transcriptional control of the steroid-sensitive promoter of the mouse mammary tumor virus long terminal repeat. This plasmid has been introduced into NIH-3T3 cells producing a clone of cells, T15, which is phenotypically normal in the absence of the transcription inducer, dexamethasone, and transformed when treated with high levels of the inducer. At lower levels of dexamethasone, both morphological transformation and stimulation of DNA synthesis are titratable functions of p21N-*ras* levels. T15 cells have been used to demonstrate that: (i) a 20- to 50-fold over-expression of normal p21*ras* is required for complete cellular transformation, (ii) p21N-*ras* expression induces DNA synthesis and the effect can be amplified by epidermal growth factor, (iii) moderate increases in normal p21*ras* expression can influence cell behaviour.

Key words: N-*ras*/inducible proto-oncogene/transformation/DNA synthesis

Introduction

The human *ras* gene family has three active members, N-*ras*, c-Ha-*ras*-1 and c-Ki-*ras*-2 (Chang *et al.*, 1982a; Hall *et al.*, 1983; Taparowsky *et al.*, 1983). Each gene encodes a 21 kd protein product, p21*ras*, which has been localized to the inner surface of the plasma membrane (Willingham *et al.*, 1983). Point mutations in each of these genes give rise to altered protein products which are capable of transforming NIH-3T3 cells to a fully malignant phenotype (Santos *et al.*, 1982; Tabin *et al.*, 1982; Capon *et al.*, 1983; Brown *et al.*, 1984). Such point mutations have been detected in *ras* genes in ~20% of human tumour DNAs covering a wide variety of tissue types (Der *et al.*, 1982; Pulciani *et al.*, 1982). Around 1% of human tumours have been found to contain an amplified *ras* gene (Fasano *et al.*, 1984; Pulciani *et al.*, 1985; Bos *et al.*, 1986; Miyaki *et al.*, 1985; Yokota *et al.*, 1986) and it appears from this and other work that high levels of expression of normal p21 products can also lead to transformation (Chang *et al.*, 1982b; Pulciani *et al.*, 1985). More moderate increases in p21*ras* levels have been reported in various tumour types (Gallick *et al.*, 1985; Viola *et al.*, 1986); Thor *et al.*, 1984; DeBortoli *et al.*, 1985), although it is not clear if these apparent changes contribute to the tumour phenotype.

Very little is known about how amino acid substitutions in p21*ras* cause cells to be transformed. The known biochemical properties of p21*ras*, namely GTP/GDP binding (Finkel *et al.*, 1984) and GTPase activities (McGrath *et al.*, 1984), and some sequence homology to G proteins (Hurley *et al.*, 1984) have led to speculation that the p21*ras* proteins may be regulatory proteins associated with growth factor receptors. The identity of these putative receptors, however, is completely unknown. One known biological effect of mutant p21*ras* expression in rodent fibroblasts is the production of a transforming growth factor TGFα (DeLarco *et al.*, 1978; Marshall *et al.*, 1985). This mitogen is known to act through the EGF receptor and it has been suggested that this receptor may interact, directly or indirectly, with the p21*ras* (Todaro *et al.*, 1976; Kamata and Feramisco, 1984).

We describe here the isolation of NIH-3T3 cells containing a normal N-*ras* proto-oncogene attached to a steroid-inducible transcription unit. In one cell line we have derived, T15, the levels of normal p21N-*ras* can be titrated. We find that induction of the protein leads to an incremental stimulation of DNA synthesis and that this effect can be augmented in the presence of EGF. Eventually at high levels of expression the cells become fully transformed.

Results

Transfection of NIH-3T3 cells with a steroid-inducible N-ras proto-oncogene

A recombinant plasmid (pLFe) was constructed which contained a normal N-*ras* gene, placed downstream of the steroid-inducible MMTV-LTR promoter (see Materials and methods). The position of the N-*ras* promoter is known and is separated from the coding exons by a *Bgl*II site (Hall and Brown, 1985). pLFe was constructed in such a way that the N-*ras* promoter was replaced by that in the MMTV-LTR. Published results using other genes linked to the MMTV-LTR (Huang *et al.*, 1981) led us to expect that in the presence of a steroid inducer, such as dexamethasone, transcription would initiate from the glucocorticoid-sensitive promoter. Transcripts which initiate from this start and terminate with exon VIa of the N-*ras* gene should be ~2.2 kb in length.

pLFe was co-transfected onto NIH-3T3 cells with pSV2neo using a calcium phosphate co-precipitation technique and morphologically transformed G418-resistant colonies were picked 2 weeks later. pHT, a plasmid containing a full length activated N-*ras* gene under its own promoter was used as a positive control (Brown *et al.*, 1984). The percentage of neo resistant colonies that were morphologically transformed was 100-fold less with pLFe in the presence of 2 μM dexamethasone than with pHT. Although other workers have reported transformation of NIH-3T3 cells with the c-Ha-*ras*-1 proto-oncogene attached to a strong promoter (Chang *et al.*, 1982b), this is, to our knowledge, the first time that transformation of cells has been obtained with a normal *ras* gene attached to the MMTV-LTR. A representative clone of NIH-3T3 cells transformed with pLFe was picked and subcloned as described in Materials and methods. This clone, T15, was further characterized.

Characterization of inducible clone

We have tested the sensitivity of NIH-3T3 cells to concentrations of dexamethasone up to 2×10^{-6} M and found no cytotoxicity over 2 weeks. Therefore, in the experiments reported here, we used 2 μM inducer except where indicated. T15 cells maintained

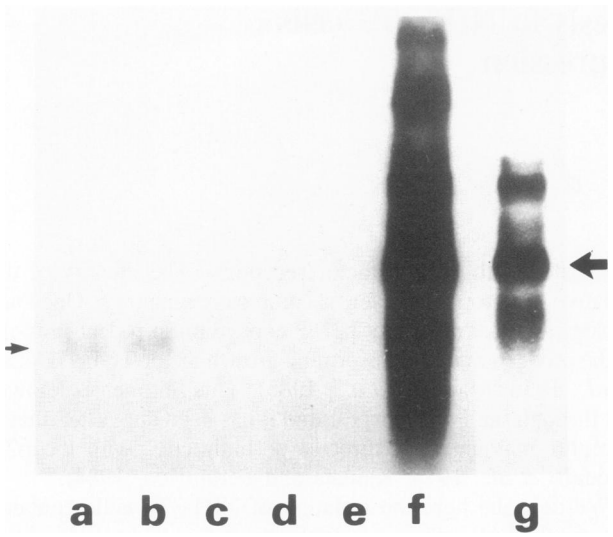

Fig. 1. Northern blot analysis of total RNA (50 μg) isolated from NIH-3T3 **(c,d)**, T15 **(e,f)** and 149/169 **(a,b)** cells grown in the presence **(b,d,f)** or absence **(a,c,e)** of 2 μM dexamethasone. Blot probed with nick-translated N-*ras* cDNA from pCDNI (Hall and Brown, 1985). **Track g** is a reduced exposure of **track f**.

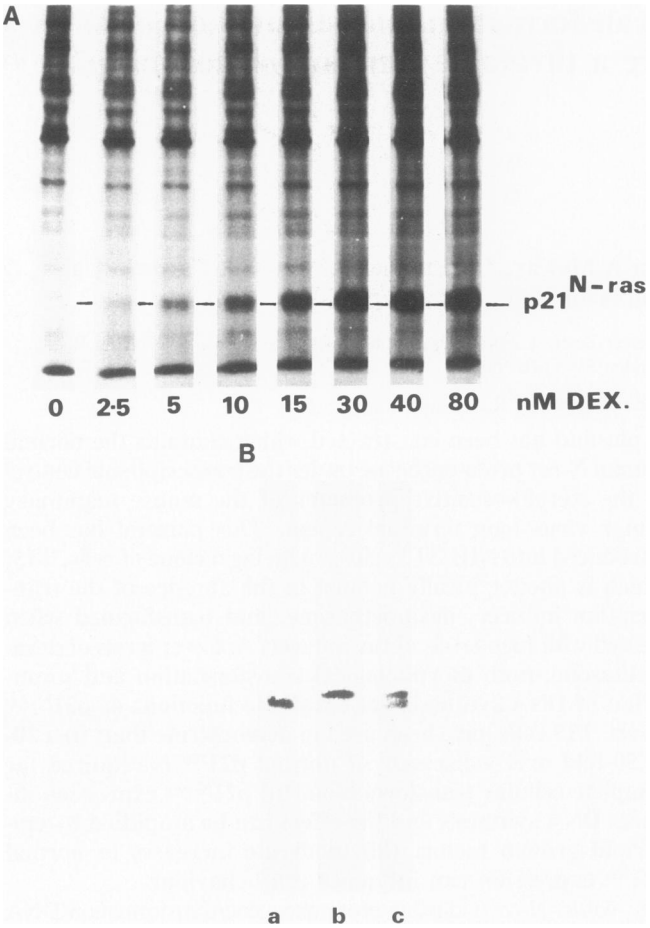

Fig. 2. (A) p21$^{N\text{-}ras}$ levels in T15 cells incubated with increasing concentrations of dexamethasone. **(B)** Western blot on membrane proteins isolated from T15 in the presence of 2 μM dexamethasone. **Lane a**, 20 μg of membrane protein from T15 grown in 2 μM dexamethasone; **lane b**, 0.2 μg of purified p21$^{N\text{-}ras}$ from an *E. coli* expression system; **lane c**, a mixture of **a** and **b**.

in the absence of inducer are indistinguishable from NIH-3T3 cells. In 2 μM dexamethasone, however, they are no longer contact inhibited for growth, and they achieve a higher saturation density than NIH-3T3 cells. They evince long processes which overlap other cells and, after prolonged incubation in the inducer, tend to form clumps. T15 cells do not grow in soft agar in the absence of inducer but in 2 μM dexamethasone they produce colonies of growing cells after 4 weeks and with comparable efficiency (~2%) to NIH-3T3 cells transformed by a constitutively expressed activated N-*ras* gene. None of these changes is observed when dexamethasone is added to NIH-3T3. After removal of dexamethasone the transformed T15 cells revert to a flat morphology similar to NIH-3T3, they are contact inhibited for growth and do not grow in soft agar. The transformed state is therefore completely reversible and dependent on N-*ras* expression. We conclude that T15 cells are fully transformed in the presence of 2 μM dexamethasone.

A Northern blot analysis of RNA extracted from T15 cells in the presence and absence of inducer is shown in Figure 1. Note that the predominant transcript is the expected 2.2 kb in length (heavy arrow). Under the stringent washing conditions used, endogenous mouse N-*ras* transcripts are not detected. As a control for non-specific effects of dexamethasone on N-*ras* transcription, the cell line 149/169 was used. Treatment of this cell line, which contains a constitutively expressed activated human N-*ras* gene, has no effect on N-*ras* expression (Figure 1, lanes a and b) or on morphology. Note that the level of the N-*ras* transcript in T15 is ~50- to 100-fold higher than in 149/169. This very high level of expression is probably due to maximal induction from the many integrated copies of pLFe identified on Southern blots. Oligonucleotide probe analysis shows in fact the presence of 4–5 complete copies of the N-*ras* gene (data not shown).

Induction of p21$^{N\text{-}ras}$ in response to increasing concentrations of dexamethasone

The results of the analysis shown in Figure 1 would lead us to expect a large induction of p21$^{N\text{-}ras}$ in T15 when compared with NIH-3T3 cells. Immunoprecipitations of p21 with monoclonal antibody 259 reveal that this is indeed the case (Figure 2a). To investigate whether the levels of p21$^{N\text{-}ras}$ in T15 cells could be varied by altering the amount of inducer, immunoprecipitations were carried out but on cells labelled in the presence of different concentrations of dexamethasone. Maximal induction of p21$^{N\text{-}ras}$ was seen at ~60 nM dexamethasone and half-maximal induction at 15 nM (Figure 2a).

In order to quantitate accurately the amount of p21 in the cells, we used a Western blotting technique both on plasma membranes (Figure 2b) and on total cell lysates isolated from NIH-3T3 and non-induced and fully induced T15 cells. As a standard, p21$^{N\text{-}ras}$ purified from an *Escherichia coli* expression system was included (M.Trahey, R.J.Milley, G.E.Cole, M.Innis, H.Paterson, C.J.Marshall, A.Hall and F.McCormick, unpublished results). It can be seen from Figure 2b that 20 μg of membrane proteins from induced T15 cells (lane a) contain 200 ng of p21$^{N\text{-}ras}$, i.e. 1%. The mobility of this species is slightly faster than the *E. coli* product (lane b) because of a palmitic acid attached to the carboxy terminus of the mammalian product (Weeks *et al.*, 1985). Since the antibody used to detect p21 in this system also recognizes mouse p21^{ras} products, we can also determine the normal level of endogenous p21^{ras} in NIH-3T3 using the *E. coli* product as standard. In this case ~0.02% of the membrane protein is p21^{ras} (data not shown). Using Western analysis we have shown that p21$^{N\text{-}ras}$ appears in the membrane ~20 h after addition of dexamethasone.

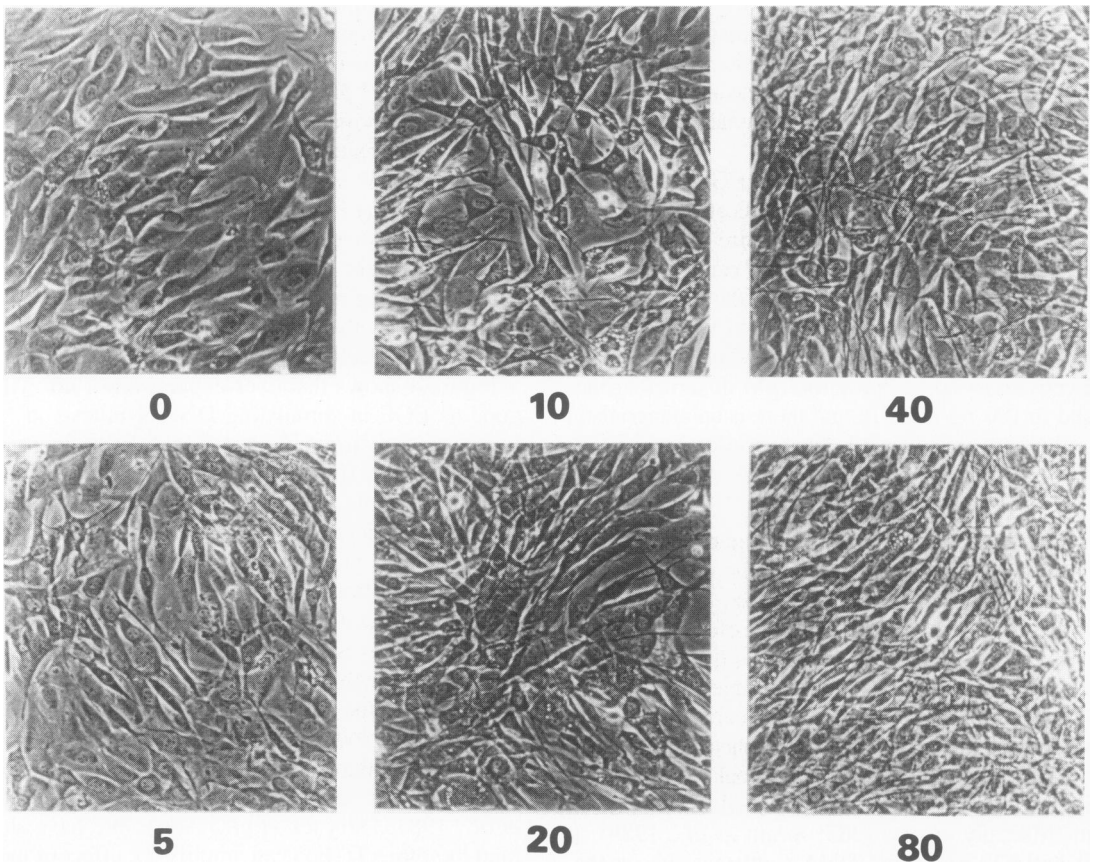

Fig. 3. Titration of morphology of T15 cells against indicated concentrations (nM) of dexamethasone.

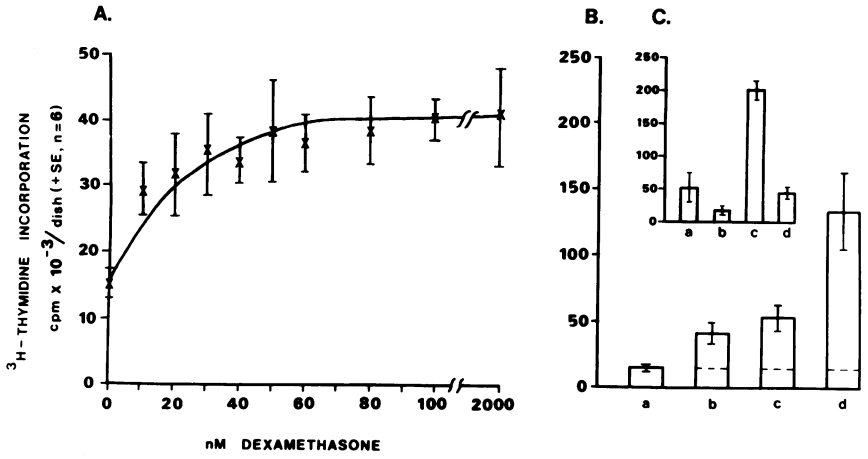

Fig. 4. (A) Incorporation of [^{3}H]thymidine into DNA of quiescent T15 cells after addition of increasing concentrations of dexamethasone. **(B)** Incorporation of [^{3}H]thymidine into T15 cells. **a**, background incorporation; **b**, 2 μM dexamethasone; **c**, 100 ng/ml EGF; **d**, both. **(C)** Incorporation of [^{3}H]thymidine into NIH-3T3 cells as in **(B)**.

Incremental transformation of T15 cells

T15 cells exhibit a striking change in morphology upon addition of dexamethasone as described earlier. In order to examine the effects of different levels of normal p21$^{N\text{-}ras}$ on cell morphology, flat, untransformed T15 cells were treated for 4 days with different concentrations of dexamethasone (Figure 3). At concentrations of inducer <5 nM, very little effect is observed. However, increasing the levels to >10 nM has a dramatic effect on cell morphology and density. We estimate that $\sim 40-80$ nM dexamethasone is sufficient for complete morphological transformation, which also corresponds to the maximum level of p21^{ras} produced in these cells (see Figure 2a). A similar effect is observed when the ability of T15 to grow in soft agar is measured with different amounts of dexamethasone. Again, half-maximum growth is observed at ~ 15 nM (data not shown).

Effect of p21$^{N\text{-}ras}$ on DNA synthesis

The effect of N-*ras* induction on DNA synthesis in quiescent T15 cells has been investigated by measuring the incorporation of [^{3}H]thymidine in response to increasing doses of dexamethasone.

The results of these experiments are shown in Figure 4A. It can be seen that in the presence of insulin and transferrin, normal $p21^{N\text{-}ras}$ is capable of stimulating DNA synthesis. Half-maximal incorporation occurs at ~15 nM dexamethasone and is maximal at 50 nM. 2 μM dexamethasone also gives maximal incorporation (not shown).

In the presence of insulin and transferrin, EGF will induce DNA synthesis in NIH-3T3 cells. Titrations indicate that 100 ng/ml EGF induces maximal DNA synthesis. Figure 4B shows that either 2 μM dexamethasone or 100 ng/ml EGF can induce DNA synthesis in T15 cells and to approximately the same extent. However, 2 μM dexamethasone plus 100 ng/ml EGF produces an additive effect on incorporation of [^{3}H]thymidine. In comparison, NIH-3T3 cells show no response to 2 μM dexamethasone. They do respond to 100 ng/ml EGF and there is an antagonistic response when they are treated with dexamethasone and EGF.

Discussion

Several groups have used microinjection techniques to introduce purified $p21^{ras}$ into cells and then examined the effects on cell behaviour (Stacey and Kung, 1984; Feramisco *et al.*, 1984). Microinjection of mutant *ras* proteins has a profound effect on the morphology of rodent fibroblast cell lines and on DNA synthesis and mitosis. Normal $p21^{ras}$, when microinjected at similar concentrations, has little or no observable effect and at higher concentrations becomes cytotoxic. This technique has not, therefore, been useful for examining the role of normal $p21^{ras}$. Others have microinjected into quiescent NIH-3T3 cells antibodies to the *ras* protein (Mulcahy *et al.*, 1985; Smith *et al.*, 1986). It was found that anti-p21 prevented DNA synthesis after stimulation with exogenous growth factors, the conclusion being that $p21^{ras}$ is essential for entry into S phase. This is consistent with the proposed model for $p21^{ras}$ action which is that it acts as a regulatory protein associated with one or possibly a spectrum of growth factor receptors. It is assumed that after stimulation of a receptor, $p21^{ras}$ would become activated and in turn stimulate other membrane proteins leading to the generation of an intracellular signal. As yet there is no evidence for what the receptors might be though there is some evidence that phosphonositol lipid turnover may be the intracellular signal (Fleischman *et al.*, 1986).

In this paper we describe the isolation of a cell line, T15, which exhibits steroid-sensitive expression of normal $p21^{N\text{-}ras}$. A high level of induction of the normal protein, ~50-fold compared with endogenous NIH-3T3 levels, is observed after addition of 2 μM dexamethasone. This cell line can be used, therefore, for titrating the biological and biochemical effects of normal $p21^{N\text{-}ras}$. We find that both morphological transformation and the ability to grow in agar alter in an incremental fashion with increasing synthesis of $p21^{N\text{-}ras}$, and a fully transformed phenotype is observed at ~60 nM dexamethasone. Interestingly, this also corresponds to maximum p21 synthesis obtainable in this cell line.

Using Western blot analysis on membranes isolated from NIH-3T3 cells and from fully induced T15 cells, we estimate that 1% of the membrane proteins in T15 are $p21^{N\text{-}ras}$. Total $p21^{ras}$ in NIH-3T3 is ~0.02%. This reflects an increase in $p21^{ras}$ molecules from $\sim 2 \times 10^5$ molecules/cell to 10^7 molecules/cell. We conclude from these results that a 20- to 50-fold increase in the expression of normal $p21^{ras}$ is required to elicit a fully transformed phenotype. The levels of *ras* gene amplification reported so far in some human tumour cell lines, i.e. ~50-fold (Fasano *et al.*, 1984; Pulciani *et al.*, 1985; Bos *et al.*, 1986; Yokota *et al.*, 1986), would, therefore, be sufficient to account for transformation, assuming that the level of transcription increases accordingly. It is interesting to note that apparently no increase in expression is required for transformation by mutant $p21^{ras}$.

In addition, using T15 cells, we have been able to titrate the effect of $p21^{ras}$ on DNA synthesis. We find that increasing the levels of the normal protein stimulates DNA synthesis in an incremental fashion and parallels the changes seen in morphology and agar growth. Western blot analysis of T15 shows that $p21^{N\text{-}ras}$ begins to appear in the membrane around 20 h after the addition of dexamethasone. Time-lapse video recordings of contact-inhibited quiescent T15 cells maintained in 10% serum show that cell division begins ~36 h after addition of dexamethasone. This is consistent with $p21^{N\text{-}ras}$ inducing quiescent cells to leave G0 and enter the cell cycle.

Figure 4 shows that over-expression of normal $p21^{N\text{-}ras}$ is as good as EGF in stimulating DNA synthesis in NIH-3T3 cells. It can be seen from Figures 3 and 4 that increasing $p21^{ras}$ by a factor of only five to ten (~5–10 nM dexamethasone) results in an observable effect on morphology and DNA synthesis. It is possible, therefore, that the moderate increases in normal $p21^{ras}$ levels observed in a variety of tumour tissues (Thor *et al.*, 1984; DeBortoli *et al.*, 1985; Gallick *et al.*, 1985; Viola *et al.*, 1986) could contribute to their transformed phenotype. This would be especially true if the cells were also producing their own growth factors. TGFα, for example, which binds and competes for the EGF receptor (Todaro *et al.*, 1976) has been found both in rodent fibroblasts transformed by *ras* genes (DeLarco and Todaro, 1978; Marshall *et al.*, 1985) as well as in human tumour cell lines (Sporn and Roberts, 1985; Marquardt *et al.*, 1983; Derynck *et al.*, 1984). We have shown that EGF (and therefore TGFα) can amplify the effect of increased normal $p21^{N\text{-}ras}$ expression on DNA synthesis. We are currently looking at the levels of *ras* RNA and protein in tumour cells to examine more closely whether moderate changes in $p21^{ras}$ may be related in any way to the proliferation of tumour cells. It may be that the *ras* proteins play a more important role in tumorigenesis than even the frequency of mutational activations would suggest.

In summary, T15 cells have been used to examine the effects of increasing levels of normal $p21^{N\text{-}ras}$ on cell behaviour. We have shown that increasing the levels of normal $p21^{N\text{-}ras}$ is mitogenic for cells and at least one growth factor, namely EGF, can complement the *ras* protein in stimulating DNA synthesis. We are currently extending this analysis to other growth factors as a first step in delineating the possible interaction of $p21^{N\text{-}ras}$ with growth factor receptors.

Materials and methods

Cell culture

NIH-3T3 cells clone D4 were isolated from stocks kindly supplied by Dr G.Cooper and maintained in DMEM with 10% newborn calf serum (NCS).

Plasmids

pLFe contains the normal N-*ras* gene under the control of the MMTV-LTR and was constructed as follows. The MMTV-LTR sequence (GR strain), contained within a 1.4 kb *Pst*I fragment in plasmid pVC27 (generously donated by Dr G. Peters) was subcloned into pUC9 to generate pUC-LTR(+). The N-*ras* gene was introduced into this plasmid in two parts. First, a 4 kb *Bgl*II–*Eco*RI fragment derived from pAT 7.8 Fe (Hall *et al.*, 1983) and containing exons I and II of the normal N-*ras* gene was subcloned downstream of the MMTV-LTR into the *Bam*HI–*Eco*RI sites of pUC-LTR(+). The 7.0 kb *Eco*RI insert of pAT 7.0 (Brown *et al.*, 1984) containing the remaining two coding exons and termination sequences of N-*ras* was then subcloned into the regenerated *Eco*RI site. pSV2neo confers on cells resistance to the antibiotic G418.

Chemicals

Dexamethasone (Sigma) was dissolved in absolute ethanol, stored at +4°C and diluted to 2 μM, except where otherwise stated, in culture media. EGF (Sigma) was diluted in sterile deionized water and stored at −20°C. Antibiotic G418

(Gibco) was dissolved in sterile deionized water to a concentration of 100 mg/ml and subsequently diluted to 1 mg/ml in culture media.

Transfections

NIH-3T3 cells were prepared for transfection as previously described (Marshall *et al.*, 1982). 100 ng of plasmid pLFe and 10 ng of pSV2neo were co-transfected onto each 60 mm dish. After selection of G418, colonies were picked into duplicate wells in a 24-well plate (Costar): one well with 2 μM dexamethasone and one well without dexamethasone. One clone, which was morphologically untransformed without inducer and transformed with dexamethasone, was picked and the cells seeded at low density on 100 mm dishes. Individual colonies were ring cloned and tested for their response to dexamethasone. One subclone, T15, gave 100% transformed colonies with inducer (2 μM) and 0% without.

Growth of cells in soft agar

10^5 cells were seeded into 0.2% agar and tested for their ability to form colonies with or without the concentrations of dexamethasone indicated in the text. After 4 weeks, colonies were stained with 1% MTT (Sigma) in phosphate-buffered saline (PBS) and counted.

Extraction of cellular RNA and Northern blotting

Cellular RNA was extracted from cells treated with or without 2 μM dexamethasone as described (Hall *et al.*, 1983) and electrophoresed through 1% agarose/formaldehyde gels (Thomas, 1980) before transfer to nylon membranes (Biodyne) for probing.

Immunoprecipitations

Monoclonal antibody 259 which is known to precipitate all human *ras* proteins (Furth *et al.*, 1982) was used to precipitate p21 from lysates of cells labelled with [^{35}S]methionine as described (Furth *et al.*, 1982). The precipitates were separated on a discontinuous Laemmli gel.

Immunoblotting

Plasma-enriched membranes were isolated using hypo-osmotic borate-EDTA (Thom *et al.*, 1977). Membrane proteins were run on a discontinuous Laemmli gel and transferred to nitrocellulose by electroblotting. P21 was visualized by treatment of the nitrocellulose with anti-p21 antibody 259 for 16 h at 4°C, followed by sheep anti-rat immunoglobulin conjugated to horseradish peroxidase (Amersham) for 1 h at room temperature. Protein was visualized using tetramethylbenzidene.

DNA synthesis assay

2×10^5 cells were seeded onto 30 mm dishes and left for 4 days when the cells were washed twice with PBS before the addition of 2 ml of serum-free medium (DMEM/Ham's F12, 50/50 with 10 μg/ml transferrin and 1 μg/ml insulin). Dexamethasone (2 μM) and EGF (100 ng/ml) were added as indicated. [^{3}H]Thymidine (Amersham) was diluted in cold 100 μM thymidine to 100 μCi/ml and included in the incubation medium at 1 μCi/ml. After 40 h cells were washed twice in PBS, and then extracted three times with 5% trichloroacetic acid (TCA). The TCA-insoluble material was dissolved in 1 ml 2% Na_2CO_3/0.1 N NaOH solution and 0.4 ml of this solution counted in acidified scintillant.

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