3 Whittenberger, B., and Glaser, L., Proc. natl Acad. Sci. *74* (1977) 2251.
4 Stallcup, K., Dawson, A., and Mescher, M., J. Cell Biol. *99* (1984) 1221.
5 Lieberman, M., Keller-McGandy, C., Woolsey, T., and Glaser, L., J. Cell Biochem. *20* (1982) 81.
6 Raben, D., Lieberman, M., and Glaser, L., J. Cell Physiol. *108* (1981) 35.
7 Peterson, S., Lerch, V., Moynihan, M., Carson, M., and Vale, R., Exp. Cell Res. *142* (1982) 447.
8 Kinders, R., Molencovic, A., Nordin, P., and Johnson, T., Biochem. J. *190* (1980) 605.
9 Wieser, R., and Brunner, G., Exp. Cell Res. *147* (1983) 23.
10 Grimstad, I., Varani, J., and McCoy, J., Exp. Cell Res. *155* (1984) 345.
11 Butters, T., Devalia, V., Alpin, J., and Hughes, R., J. Cell Sci. *44* (1980) 33.
12 Muramatsu, T., J. Biochem. *62* (1967) 487.
13 Spiro, R., Meth. Enzymol. *8* (1966) 3.
14 Muramatsu, T., J. biol. Chem. *246* (1971) 5535.
15 Enzymes and Related Biochemicals. Worthington, Freehold, NJ 1982.
16 Hunter, T., and Greenwood, F., Nature *194* (1962) 495.

0014-4754/89/040370-03$1.50 + 0.20/0

Detection of the *H-RAS* oncogene in human thyroid anaplastic carcinomas

B. M. Stringer, J. M. Rowson, M. H. Parkar, J. M. Seid, P. R. Hearn, D. Wynford-Thomas[c], S. Ingemansson[a], N. Woodhouse[b] and M. H. Goyns*

Department of Biological and Medical Research, [a] Department of Surgery, [b] Department of Medicine, King Faisal Specialist Hospital and Research Centre, P.O. Box 3354, Riyadh 11211 (Saudi Arabia), and [c] Unit of Cancer Biology, Department of Pathology University Hospital of Wales, Heath, Cardiff CF4 4XN (England)
Received 12 December 1988; accepted 19 January 1989

Summary. We have transfected high-molecular-weight DNA from human thyroid carcinomas into murine 3T3 cells. As a result we identified several foci of morphologically distinct transformed cells in each of the tumour DNA transfected cultures. After a total of three rounds of transfection, the transformed cells were shown to form tumours in nude mice. Southern blot analysis of DNA prepared from third-round transfectants demonstrated the presence of human Alu repetitive sequences and, after hybridization with probes for known oncogenes, indicated the presence of the human *H-RAS* oncogene in 3T3 cells transfected with three out of four anaplastic carcinoma DNA samples. It appears therefore that activation of *RAS* genes may be an important event in the development of the anaplastic thyroid tumours.
Key words. Anaplastic carcinoma; thyroid; *H-RAS* oncogene; transfection.

Thyroid cancer is the fourth most common form of neoplastic disease occurring in women in Saudi Arabia, accounting for 5.7% of all cancers observed in females[1]. The reason for this high incidence is unknown and does not appear to be related to goitre. Thyroid cancer is therefore of particular interest in this region and, as oncogenes have gained considerable notoriety over the past few years for their association with neoplastic disease[2–4], we decided to investigate which of the proto-oncogenes are activated in thyroid tumour tissue. In particular, we were interested in determining whether we could detect the presence of a dominant transforming gene in any of the thyroid tumour DNA samples by transfecting tumour DNA into murine 3T3 cells.

The development of the technique for DNA transfer into animal cells[5,6] has led to the discovery of cellular transforming genes (in human tumour cells) because of their ability to induce foci of morphologically distinct transformed murine 3T3 cells[7]. A number of new oncogenes have been discovered by this method, including *N-RAS*[8], *MET*[9] and *MAS*[10]. Furthermore, this method has also allowed the transforming potential of known oncogenes to be demonstrated[7]. To increase the sensitivity of the 3T3 transfection technique, cotransfection with a selectable marker has also been used in conjunction with tests of tumourigenicity of the transfected 3T3 cells in nude mice[11]. It is a modification of this latter approach that we have used in attempting to detect the presence of dominant transforming genes in thyroid tumours.

Materials and methods. Each tumour was snap-frozen in liquid nitrogen immediately after surgical removal, and then stored at −70 °C until required. The frozen tumour tissue was ground under liquid nitrogen and high-molecular-weight DNA was prepared either from this material, or from 3T3 cell pellets, by using the proteinase K method[12].

Murine 3T3 fibroblasts were routinely grown in Dulbecco's modified Eagles medium, supplemented with 10% (v/v) newborn calf serum, in a humidified CO_2 incubator at 37 °C. During propagation of the cultures, the cells were always harvested before they became confluent. In each transfection experiment 2×10^5 cells were seeded into each 90-mm petri dish, and left overnight before transfection. Usually 20 dishes were used for each test.

20 µg high molecular weight DNA was coprecipitated with 1 µg pSVNeo^R. After one day, the cells were washed and transformed cells selected in medium containing 800 µg/ml geneticin (Sigma). The appearance of morphologically distinct foci was scored after 14 days. Foci were picked using cloning rings and the cells grown up for DNA preparation; the latter then being used in a second round of transfection. In total three rounds of transfection were performed.

The nude mice colony was kept under sterile conditions and bred by back-cross to the Swiss Balb-C strain. 10^6 transfected 3T3 cells were injected subcutaneously at each site of inoculation. Formation of primary tumours was scored at 18 days after inoculation.

20 µg DNA from each third-round transfectant sample was digested to completion with EcoRI, size fractionated on a 1% (w/v) agarose gel and blotted onto Hybond nylon filters (Amersham). Conditions of prehybridization, hybridization and washing were according to the protocols in the Amersham handbook. The filters were exposed to Kodak XAR or XRP film with intensifying screens at −70 °C for 1–5 days to visualize the hybridization fragments.

The human Alu repeat sequence probe was Blur 8 (Amersham), which was cut out of its plasmid by Bam HI, and then purified by gel electrophoresis. This probe was labelled with ^{32}P-dCTP by nick translation. The oncogene probes (*H-RAS, K-RAS, N-RAS, ERB-B, ERB-B2, FOS, FMS, MYC, N-MYC, MOS, SIS*) were all human oncogene sequences inserted into pSP65 (Amersham amprobes). Each of these was labelled with ^{32}P-UTP by riboprobe synthesis. Under the experimental conditions used the *H-RAS* probe hybridized to normal and activated human *H-RAS* but not to other *RAS* genes nor to mouse *H-RAS*.

Results. To enhance the efficiency of identifying genuine DNA-induced foci, these experiments involved cotransfection with a selectable marker (pSVNeo®), which endowed resistance to geneticin toxicity. The transformation of murine 3T3 fibroblasts by transfection with human thyroid tumour DNA was therefore scored by the appearance of morphologically distinct foci of cells in the presence of geneticin. Initially, we used DNA prepared from anaplastic, follicular and papillary carcinomas. Examples of foci of transformed 3T3 cells, observed after the first round transfections, are shown in figure 1. Foci induced by follicular or papillary carcinoma DNA (fig. 1, c and d) were very similar, being discreet, multilayered and often apparently containing unidentified inclusions. The anaplastic carcinoma (TA-01) DNA-induced foci,

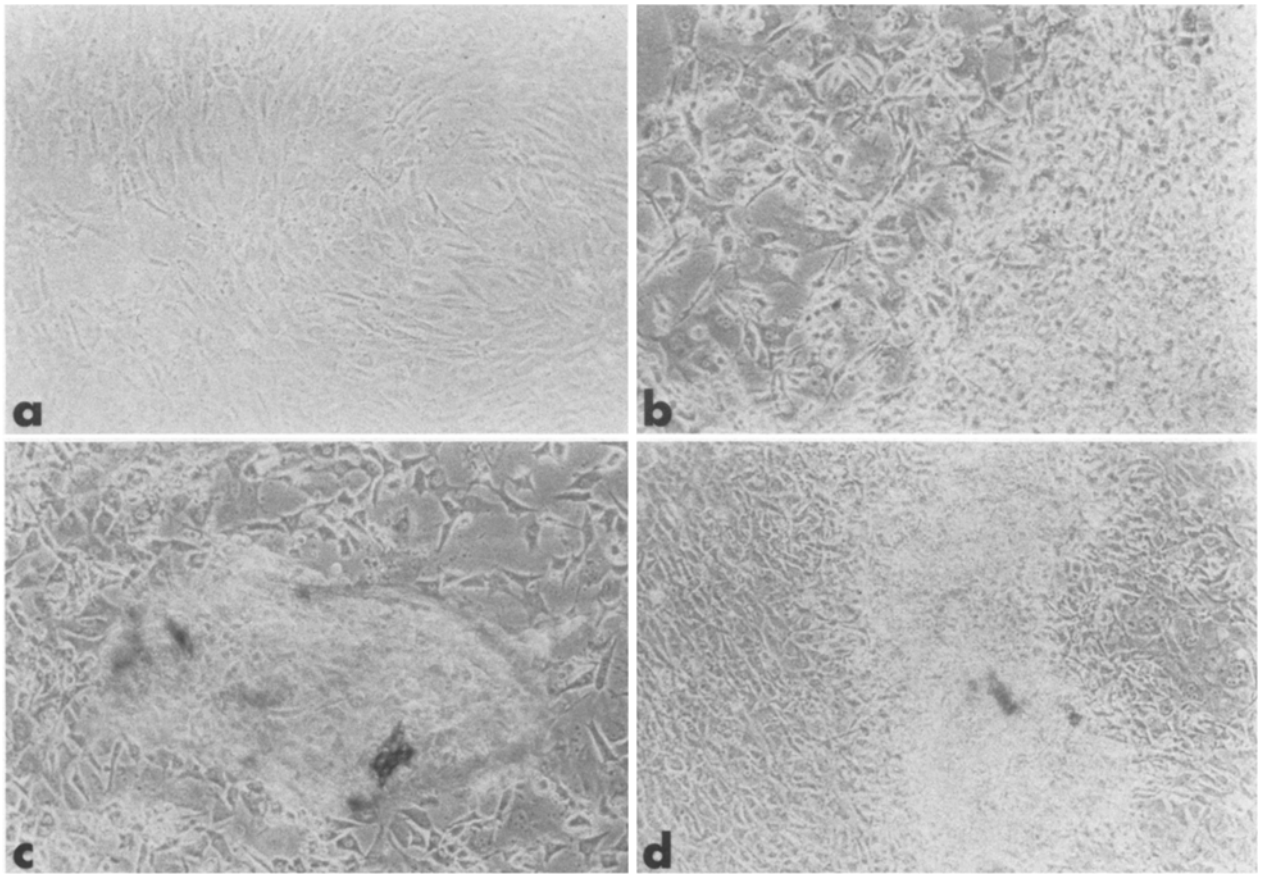

Figure 1. Morphology of 3T3 cells cotransfected with human DNA and pSVNeo® (First round transfection). *a* cells transfected with normal leukocyte DNA; *b* cells transfected with anaplastic carcinoma DNA; *c* cells transfected with follicular carcinoma DNA; *d* cells transfected with papillary carcinoma DNA.

Table 1. Frequency of focus appearance in mouse 3T3 fibroblast cultures after transfection with human thyroid tumour DNA

Tumour type	Transformation frequency (foci/dish) 1st round	2nd round	3rd round
Anaplastic	0.20±0.09	7.10±1.64	1.60±0.45
Follicular	0.20±0.11	5.53±0.88	2.63±0.71
Papillary	5.55±3	3.95±0.65	2.40±0.8
Control (normal human leukocytes)	0.0	0.20±0.13	0.30±0.2

however, were much larger and had the appearance of a very densely packed monolayer of cells (fig. 1 b). However, afer the second round of transfection the morphologies of all foci appeared to be similar and resembled the first round anaplastic foci. The transformation efficiencies during each round of transfection are summarized in table 1. During the period of this study the mouse 3T3 cultures gradually exhibited an increasing number of spontaneously transformed cells. The numbers of these, however, were still comparatively small compared to the test cultures in the second and third rounds of transfection (table 1).

To determine whether the transformed 3T3 cells that we had identified were also tumourigenic, we inoculated the cells into nude mice. After 18 days the mice were scored for tumour formation. These data are summarised in table 2. It is clear that all of the foci of transformed cells, that we have identified, are capable of producing tumours in nude mice. The normal human DNA control also demonstrates that the pSVNeo® plasmid in no way contributes to such tumour formation. Although these tumours are clearly well developed, we could find no evidence of metastatic spread to internal organs (table 2), even in those mice that were kept for 6 weeks prior to sacrifice. We also examined the histology of the primary tumours, but there appeared to be no discernable differences between the tumours formed from the three types of third-round transfectant cells. All tumours appeared as an encapsulated densely packed mass of morphologically similar cells with blood vessels within the tumour body (data not shown).

Although spontaneously transformed 3T3 cells are not thought to form tumours in nude mice, it was important to demonstrate that the third-round transfectant cells contain human DNA sequences. Therefore, a Southern blot was prepared from the third-round transfectant DNA samples and hybridized to a human specific repetitive DNA sequence probe (Blur 8). Figure 2 shows the result of this experiment. The Blur 8 probe clearly hybridizes to human (fig. 2, lane 1) and not to 3T3 DNA (Fig. 2, lane 7). There is also evidence of hybridization to

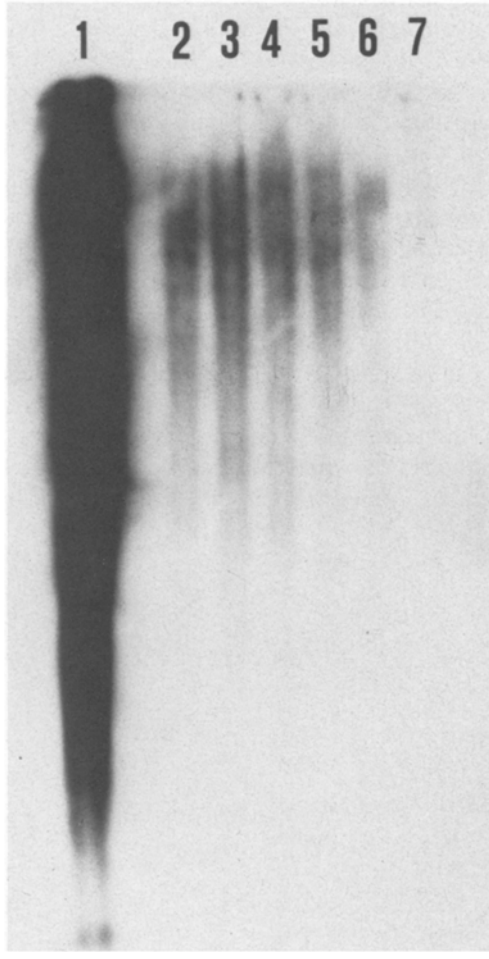

Figure 2. Southern blot analysis of human sequences present in third-round 3T3 transfectant DNA samples. Filters were probed with a ^{32}P-dCTP labelled Blur-8 probe. DNA samples used in this experiment were isolated from the following cells: lane (1) human leucocytes, (2) anaplastic transfectant, (3) follicular transfectant clone A, (4) papillary transfectant, (5) follicular transfectant clone B, (6) 3T3 transfected with activated human *H-RAS*, (7) 3T3.

Table 2. Tumour formation in nude mice after inoculation with transfected 3T3 cells

Type of DNA transfected into 3T3 cells	Number of inoculation sites	Number of primary tumours	Number of metastases
Normal human + pSVNeo®	6	0	0
Anaplastic thyroid carcinoma + pSVNeo® (third round transfectant)	6	6	0
Follicular thyroid carcinoma + pSVNeo® (third round transfectant)	6	6	0
Papillary thyroid carcinoma + pSVNeo® (third round transfectant)	6	6	0

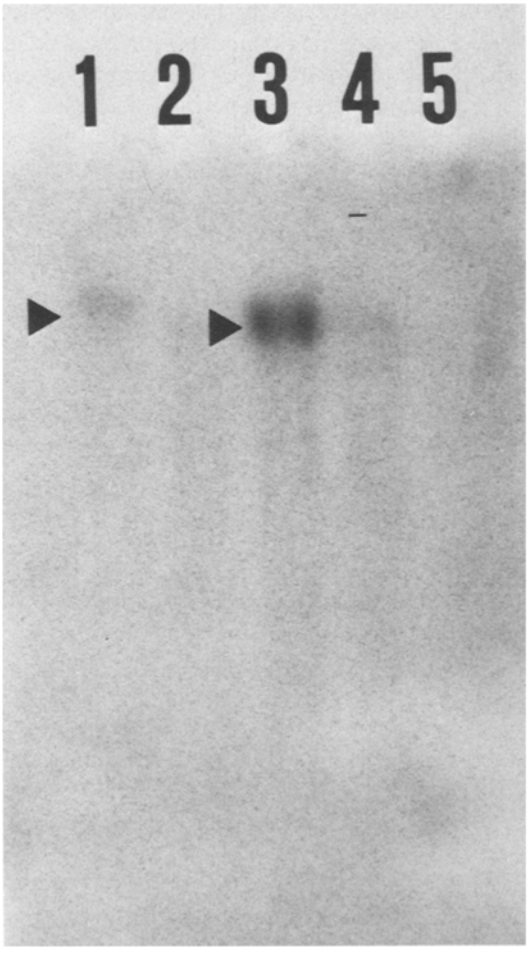

Figure 3. Southern blot analysis of the *H-RAS* oncogene in third-round transfectant DNA samples. Filters were probed with a ^{32}P-UTP labelled *H-RAS* riboprobe. DNA samples used in this experiment were isolated from the following cells: lanes (1) human leucocytes, (2) follicular transfectant, (3) anaplastic transfectant, (4) papillary transfectant, (5) 3T3.

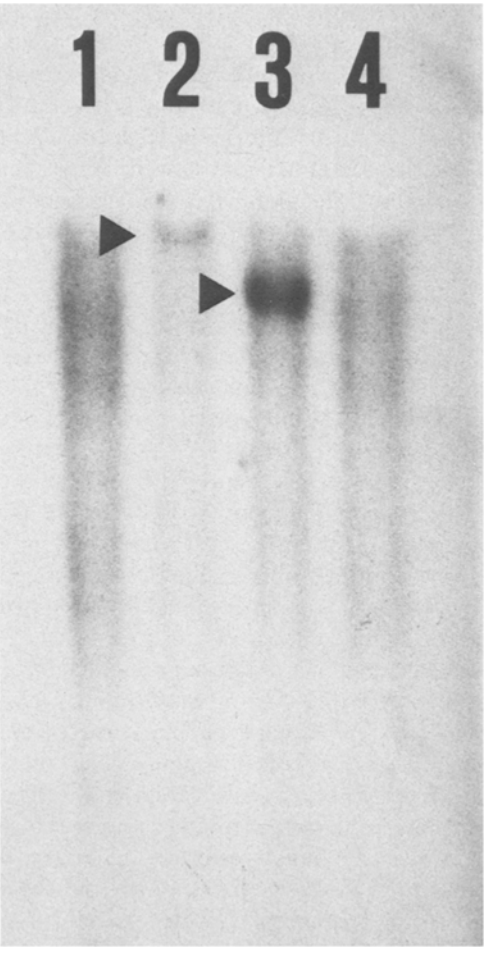

Figure 4. Southern blot analysis of the *H-RAS* oncogene in anaplastic carcinoma first-found transfectant DNA samples. Filters were probed with a ^{32}P-UTP labelled *H-RAS* riboprobe. DNA samples used in this experiment were isolated from the following anaplastic DNA-transformed 3T3 cell cultures: lanes (1) TA-03 transfectant, (2) TA-04 transfectant, (3) TA-05 transfectant, (4) 3T3 cells.

DNA prepared from 3T3 cells that had been transfected with a plasmid containing the activated human *H-RAS* gene (fig. 2, lane 6), but most important, there are clearly human DNA sequences present in all of the third-round transfectant DNA samples (fig. 2, lanes 2–5).

To determine whether any of the human DNA sequences that we have detected in the third-round transfectants represent known oncogenes, we prepared Southern blots of the transfectant DNA samples and hybridized them to a range of oncogene probes. As all of the transfectant DNA samples contained pSVNeo®, it was important to prevent any possible contamination of our probes with ^{32}P-labelled plasmid sequences, and therefore we used oncogene riboprobes for these hybridizations. Figure 3 shows the results of probing the Southern blots with a human *H-RAS* riboprobe. The *H-RAS* probe hybridized to the human DNA (lane 1) but not to the mouse 3T3 DNA (lane 5). It is clear, however, that human *H-RAS* is also present in the anaplastic carcinoma (TA-01) transfectant culture (fig. 3, lane 3). There is no evidence for the presence of human *H-RAS* in the other transfectant cultures. We have so far been unable to detect the presence of any other human oncogenes in the latter cultures (data not shown). As human *H-RAS* was observed to be present in the third-round anaplastic transfectants, we decided to screen other thyroid anaplastic tumours for the presence of an activated *H-RAS* oncogene. Four tumours were used in this study and they were classed as anaplastic arising from a papillary carcinoma background (TA-02), anaplastic arising from a follicular carcinoma background (TA-03), anaplastic carcinoma, poorly differentiated (TA-04) and anaplastic arising from a papillary carcinoma background (TA-05). We transfected 3T3 cells with high-molecular-weight DNA prepared from these tumours, as described in the Materials and Methods section. The cells were selected for resistance to geneticin toxicity but then, without any further rounds of transfection, injected into nude mice. Three of the four first-round transfectant cultures formed tumours (TA-03, TA-04, TA-05). DNA was then prepared from these

tumours and analysed by Southern blotting. Figure 4 shows the hybridization of a human *H-RAS* riboprobe to such a blot. There is no evidence of hybridization to the 3T3 DNA (fig. 4, lane 4) or to the TA-03 transfectant (fig. 4, lane 1), but hybridization is clearly visible with TA-04 and TA-05 transfectants (fig. 4, lanes 2 and 3).

Discussion. Very little is known of oncogene activation in human thyroid cancer and those observations which have been reported are not altogether consistent. The amplification of *H-RAS* has been observed in a thyroid carcinoma [13] but comparisons of the levels of RAS p21 protein in normal and neoplastic thyroids have produced conflicting results [14, 15]. Enhanced expression of both *MYC* and *FOS* has been reported in a number of thyroid tumour samples [16, 17]. However, as the expression of these genes is cell cycle dependent, this apparent enhanced expression may not be a causative factor in thyroid cell transformation, but rather may reflect the increased level of cell proliferation in tumours compared to normal thyroid tissue [18]. It should also be noted, however, that genomic changes have been detected in the *MYC* gene in a number of thyroid tumour samples [13, 19]. Also, enhanced expression of *ERB B* and *ERB B2* has been reported in thyroid papillary carcinomas and adenomas respectively [16], and abnormal expression of MOS observed in a medullary carcinoma [20]. Therefore, at present no clear conclusion can be drawn as to whether specific oncogene activations are necessary for the development of particular forms of thyroid cancer.

One interesting recent report, which used the 3T3 transfection system to detect transforming oncogenes in papillary carcinomas, claimed to have identified a new oncogene [21]. This was not a *RAS* gene, but was apparently present in every one of the papillary DNA transfectants studied. We have so far not been able to identify the transforming gene in our papillary transfectant, nor been able to determine whether it is the same as that isolated by Fusco et al [21]. It appears that *RAS* proteins are present in both normal and neoplastic thyroids [22], and activation of the *RAS* genes has been observed in both papillary and follicular thyroid carcinomas [23] but not so far in the anaplastic tumours. The present study however has demonstrated the activation of *H-RAS* in two anaplastic tumours which had arisen from a background of papillary carcinoma (TA-01 and TA-05), and in a third (TA-04), whose origin is uncertain.

The activation of *H-RAS* is now recognised as being commonly associated with human tumours [2–4]. Although *H-RAS* is known to code for a GTP-binding membrane protein [24] its role in carcinogenesis is unclear. In the 3T3 fibroblast system, activated *H-RAS* apparently functions as a promoter by releasing the cells from proliferation control. Studies of carcinogen-induced tumour formation in mice [25], however, have suggested that activated *H-RAS* may function as an initiator rather than a promoter of tumour growth in epithelial cells. The possibility that *H-RAS* activation initiates tumour formation of the thyroid, and the present limited evidence for the *H-RAS* oncogene in papillary carcinomas, might suggest that the occurrence of *H-RAS* activation predisposes a papillary carcinoma to become anaplastic. If this is true, then it could prove to be a useful prognostic indicator for the development of the highly malignant anaplastic carcinomas.

Note added in proof: Since submission of this paper we have identified the transforming oncogene in tumour TA-03 as *K-RAS*.

Acknowledgements. This work was supported by KFSH & RC Project Grant No. 86-0005.

* All correspondence to: Dr. M. H. Goyns, Department of Clinical Oncology, Royal Hallamshire Hospital, Sheffield S 10 2JF, England.

1 Mahboubi, E., Ann. Saudi Med. *7* (1987) 265.
2 Der, C. J., Clin. Chem. *33* (1987) 641.
3 Goyns, M. H., Cancer J. *1* (1986) 183.
4 Nishimura, S., and Sekiya, T., Biochem J. *243* (1987) 313.
5 Graham, F. L., and Van der Eb, A., Virology *52* (1973) 456.
6 Jainchill, J. L., Aaronson, S. A., and Todaro, G. J., J. Virol. *4* (1969) 549.
7 Land, H., Parada, L. F., and Weinberg, R. A., Science *222* (1983) 771.
8 Shimizu, K., Goldfarb, M., Suard, Y., Perucho, M., Li, Y., Kamata, T., Ferimisco, J., Stavnezer, E., Fogh, J., and Wigler, M., Proc. natl Acad. Sci. *80* (1983) 2112.
9 Copper, C. S., Park, M., Blair, D. G., Tainsky, M. A., Huebner, K., Croce, C. M., and Vande Woude, G. F., Nature *311* (1984) 29.
10 Young, D., Waitches, G., Birchmeier, C., Fasano, O., and Wigler, M., Cell *45* (1986) 711.
11 Fasano, O., Birnbaum, D., Edlung, L., Fogh, J., and Wigler, M., Molec cell. Biol *4* (1984) 1695.
12 Gross-Bellard, M., Oudet, P., and Chambon, P., Eur. J. Biochem. *36* (1973) 32.
13 Pluzhikova, G. F., Serova, O. M., Knyazev, P G., Fedorov, S. N., Novikov, L. B., Valdina, E. A., Moiseenko, V. M., Semiglazov, V. F., Wagner, R. I., and Seitz, J. F., USSR Eksp. Onk. *9* (1987) 15.
14 Johnson, T. L., Lloyd, R. V., and Thor, A., Am. J. Path. *127* (1987) 60.
15 Mizukami, Y., Nonomura, A., Hashimoto, T., Terahata, S., Matsubara, F., Michigishi, T., and Noguchi, M., Cancer *61* (1988) 873.
16 Aasland, R., Lillehaug, J. R., Male, R., Josendal, O., Varhaug, J. E., and Kleppe, K., Br. J. Cancer *57* (1988) 157.
17 Terrier, P., Sheng, Z.-M., Schlumberger, M., Tubiana, M., Caillou, B., Travagli, J. P., Fragu, P., Parmentier, C., and Riou, G., Br. J. Cancer *57* (1988) 43.
18 Yamashita, S., Ong, J., Fagin, J. A., and Melmed, S., J. clin. Endocr. Metab. *63* (1986) 1170.
19 Del Senno, L., Deglo Uberti, E., Bernardi, F., Marchetti, G., Perrotta, C., Gambari, R., Barbieri, R., Buzzoni, D., Pansini, G., and Conconi, F., Cancer Detec. Preven. *10* (1987) 159.
20 Parker, M. H., Seid, J. M., Stringer, B. M., Ingemansson, S., Woodhouse, N., and Goyns, M. H., Cancer Lett. *43* (1988) 185.
21 Fusco, A., Grieco, M., Santoro, M., Berlingieri, M. T., Pilotti, S., Pierotti, M. A., Della Porta, G., and Vecchio, G., Nature *328* (1987) 170.
22 Yoshida, K., Hamatani, K., Koide, H., Ikeda, H., Nakamura, N., Akiyama, M., Tsuchiyama, H., Nakayama, E., and Shiku, H., Cancer Res. *48* (1988) 5503.
23 Lemoine, N. R., Mayall, E. S., Wyllie, F. S., Farr, C. J., Hughes, D., Padua, R. A., Thurston, V., Williams, E. D., and Wynford-Thomas, D., Cancer Res. *48* (1988) 4459.
24 DeVos, A. M., Tong, L., Milburn, M. V., Matias, P. M., Jancarik, J., Noguchi, S., Nishimura, S., Miura, K., Ohtsuka, E., and Kim, S. H., Science *239* (1988) 888.
25 Quintanilla, M., Brown, K., Ramsden, M., and Balmain, A., Nature *322* (1986) 78.

0014-4754/89/040372-05$1.50 + 0.20/0