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Rodent fibroblast tumours expressing human *myc* and *ras* genes: Growth, metastasis and endogenous oncogene expression

A.H. Wyllie[1], K.A. Rose[1], R.G. Morris[1], C.M. Steel[2], E. Foster[2] & D.A. Spandidos[3,4]

[1]*Department of Pathology, University Medical School, Edinburgh;* [2]*MRC Clinical and Population Cytogenetics Unit, Edinburgh;* [3]*Beatson Institute for Cancer Research, Glasgow, UK; and* [4]*Hellenic Institute Pasteur, Athens, Greece.*

Summary The effects of expression of human c-*myc* and both mutated (T24) and normal forms of human Ha-*ras*-1 were studied in an aneuploid rat fibroblast line (208F). Mutated T24 Ha-*ras* was also studied in a near-diploid cell derived from early passage Chinese hamster lung fibroblasts (CHL). In contrast to the parental fibroblasts, cells expressing any of the human oncogenes engendered rapidly growing tumours in immune-suppressed animals. Blood- and lymph-borne metastases were observed from both *ras*- and *myc*-expressing cells. In general *ras*-expressing cells were more aggressive than those expressing *myc*. In the 208F background, expression of c-*myc* was associated with an incidence of mitosis similar to that in tumours expressing T24 Ha-*ras*, but incidence of single cell death by apoptosis was higher. Quantitatively, expression of human oncogene mRNA was constant during growth *in vivo*, and similar to that sometimes observed in human neoplasms. Of 9 endogenous proto-oncogenes, 7 showed no change in expression from the parental fibroblasts, but c-*abl* and c-*fos* were strongly expressed in all cells expressing human *ras* or *myc*. Thus these tumorigenic cells, although transfected with single human oncogenes, all expressed oncogenes with both nuclear- and membrane-associated products.

There is much evidence that cellular oncogenes of the *ras* and *myc* families play a role in carcinogenesis (reviewed by Klein & Klein, 1986; Weinberg, 1985), but surprisingly little is known of their contribution to the pathology of established tumours, and in particular to features of malignancy such as growth by infiltration or metastasis. In human neuroblastomas, there is a suggestive but incomplete correlation between aggressive clinical course and amplification (with overexpression) of N-*myc* (Brodeur *et al.*, 1984; Rosen *et al.*, 1986). High levels of *ras* expression occur frequently in primary human neoplasms of many types (Slamon *et al.*, 1984; Spandidos & Agnantis, 1984; Spandidos & Kerr, 1984; De Bertoli *et al.*, 1985; Spandidos *et al.*, 1985*b*; Kurzrock *et al.*, 1986) but there is no evidence that this is necessary or sufficient for maintenance of the malignant phenotype (Gallick *et al.*, 1985; Williams *et al.*, 1985). Rodent fibroblasts in which expression of *ras* genes (with or without mutational activation) has been induced *in vitro*, do acquire metastatic ability, however, as judged by a pulmonary embolisation assay (Muschel *et al.*, 1985; Thorgeirsson *et al.*, 1985). Moreover, the metastatic phenotype appears almost immediately on expression of the inserted *ras* genes (Bradley *et al.*, 1986). *Ras*-expressing cells develop a constellation of new features. Some of these could be expected to favour autonomous growth, such as the production of tumour growth factors (Ozanne *et al.*, 1982; Anzano *et al.*, 1985; Pragnell *et al.*, 1985; Spandidos, 1985; Marshall *et al.*, 1985) and release from pre-existing control by exogenous trophic stimuli (Kasid *et al.*, 1985; Racker *et al.*, 1985; Zahn & Goldfarb, 1986), whilst some correlate strongly with invasive and metastatic ability, such as increased sialylation of surface glycoproteins (Collard *et al.*, 1985). *Ras* gene expression, however, is also associated with enhanced cellular capacity to act as a target for NK cytocidal activity (Johnson *et al.*, 1985; Trimble *et al.*, 1986) a feature likely to militate against successful metastasis (Nicolson & Poste, 1983; Hanna & Schneider, 1983). Any relationship between *ras* expression and metastatic capacity is likely to be influenced in addition by as yet unknown cellular factors since *ras* expression appears to have little to do with metastatic potential in murine cell lines of melanoma (Kris *et al.*, 1985) or epithelial origin (Muschel *et al.*, 1985). Even in fibroblasts there is evidence that the induction of metastatic potential by *ras* expression may be inhibited by products of other genes (Pozzatti *et al.*, 1986).

In this paper we observe the effect of human c-*myc* and Ha-*ras*-1 genes on early and late passage fibroblasts, inoculated subcutaneously into immune-suppressed mice. Expression of the oncogenes is ensured through their linkage to strong viral transcriptional enhancer elements (Spandidos & Wilkie, 1984; Spandidos, 1985). In particular we ask whether the tumours containing these oncogenes differ in their growth properties at the site of inoculation or in their metastatic ability, whether the abilities to metastasise by lymphatic and haematogenous routes are conferred together, or independently of each other, and whether expression of endogenous proto-oncogenes is altered.

Materials and methods

Cell lines

All cell lines were maintained in Dulbecco's modification of minimum Eagle's medium (MEM), supplemented to 10% with new-born calf serum and penicillin and streptomycin. The genesis of the cell lines has been fully described elsewhere (Spandidos & Wilkie, 1984). CHL cells derived from early passage hamster fibroblasts, whereas the 208F cell line originated from Fisher rat fibroblasts (Quade, 1979) and is aneuploid in karyotype. From the 208F cell line 3 derivative lines were obtained through insertion of mutated (T24) and non-mutated human Ha-*ras*-1 and human c-*myc* genes in high expression vectors, by the calcium phosphate transfection technique. A single derivative cell line from CHL fibroblasts was also studied, containing the mutated (T24) human Ha-*ras*-1 gene (hereafter called simply T24 Ha-*ras*). Each derivative line was expanded from a single clone. A satisfactory *myc*-expressing CHL transfectant has not been obtained. The plasmids used for transfection were pH05T1, pH06N1 and pMCGM1, containing the entire T24 Ha-*ras* gene, the entire normal human Ha-*ras*-1 proto-oncogene, and the entire human c-*myc* gene respectively (Spandidos & Wilkie, 1984; Spandidos, 1985). In pH05T1 the *ras* gene is situated adjacent to the SV40 early promoter-enhancer sequence; in pH06N1, both SV40 and Moloney virus LTR enhancers are present; whilst in pMCGM1, the Moloney virus LTR sequence is linked to the human *myc* gene. All the plasmids contain the aminoglycoside phosphotransferase

Correspondence: A.H. Wyllie.

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gene (*aph*), conferring resistance to geneticin (G418), which was used in the initial selection of the lines.

Animals

Female CBA mice of around six weeks of age were rendered incompetent immunologically by thymectomy followed by whole-body radiation and treatment with cytosine arabinoside by a modification of the method of Steel *et al.* (1978) (Hay *et al.*, 1985). CHL, 208F, and the derivative *myc* and *ras* expressing lines were suspended in Dulbecco's PBS at a concentration of $1-2 \times 10^8$ ml^{-1}, and inoculated subcutaneously in volumes of 0.1 ml, either to the left groin or the mid-dorsal region around 4 weeks after whole body irradiation. The mice were observed for tumour development and killed at times dictated by the following criteria: tumours of over 1.0 cm diameter, obvious illness, or the passage of at least four weeks with no or slow tumour development. Although these criteria are somewhat divergent, they afforded a convenient way to gather tumours of widely differing behaviour: the rapidly growing tumours appeared within 1–2 weeks and were harvested within 1–3 weeks of injection, animals with slower growing tumours could be observed for longer, and the longest periods of observation were reserved for animals in which there was no evidence of tumour development.

At autopsy, portions of the tumour, and the lungs, heart, liver, kidneys, adrenals, retro-peritoneal lymph nodes, and axillary lymph nodes were fixed in 4% neutral buffered formaldehyde, and processed through paraffin for preparation of sections stained by haematoxylin and eosin, van Gieson's stain (for collagen), or Lison's alcian blue, chlorantine fast red stain (for mast cells). Portions of the rapidly growing tumours, and some normal tissue were also snap-frozen in liquid nitrogen for RNA and protein analysis and stored at −80°C.

Analysis of RNA

RNA was extracted from pellets of at least 10^8 cultured cells, or from snap-frozen portions of tumours, by the guanidinium thiocyanate method (Chirgwin *et al.*, 1979). After repeated precipitation in ethanol, RNA was checked for integrity by observation of discrete ribosomal RNA bands on 1% agarose gels containing 6.8% formaldehyde (Maniatis *et al.*, 1982), and stained with ethidium bromide. For semi-quantitative analysis of oncogene expression a spot hybridisation assay was employed (Spandidos *et al.*, 1981). RNA (5 μg) was spotted on nitrocellulose filters and probed with oncogene DNA, labelled by nick translation with 32P-dCTP under the following hybridisation conditions: 50% formamide, 2 × SSPE, 5 × Denhardt's solution, 200 μg ml^{-1} sheared salmon DNA, 0.5% SDS at 40°C for 18–24 h. After washing to stringencies specified in the text, the filters were exposed to Fuji-Rx film.

For quantitative estimation of Ha-*ras* and c-*myc* RNA, dots containing 1, 5 and 10 μg of total cellular or tumour RNA were spotted, in triplicate, adjacent to serial dilutions, also in triplicate, of known quantities of Ha-*ras* or c-*myc* RNA, generated from the plasmids pSPHa-*ras* 2 and pMC64-10 using SP6 RNA polymerase. Filters were probed with nick translated T24 Ha-*ras* or c-*myc* sequences as described above. Following hybridisation RNA spots were cut from the nitrocellulose filter using a cork borer and the amount of hybridised probe determined by liquid scintillation. The mean value for each triplet of spots was calculated and a standard curve plotted using the values obtained for SP6-generated Ha-*ras* or c-*myc* RNA. The quantity of Ha-*ras* or c-*myc* RNA present in 1, 5 or 10 μg of total cellular or tumour RNA was determined by intrapolation on this standard curve. Values thus obtained for Ha-*ras* and c-*myc* RNA were corrected to compensate for the presence of noncoding sequences in the SP6 generated RNA. Such sequences account for approximately 50% of the Ha-*ras* RNA and 75% of the c-*myc* RNA. The resulting value was expressed as a percentage of total RNA or of poly A^+ RNA (using the assumption that 2% of total RNA is poly A^+ RNA).

Plasmids used for hybridisation probes and RNA calibration

All probes were purified linear DNA sequences, isolated from their plasmid vectors by restriction enzyme cleavage and separation on low melting temperature 1% agarose gels as described below. The ensuing list gives the designation of plasmids used for preparation of hybridisation probes, preceded by the gene sequence represented and followed in brackets by the restriction enzyme fragment selected.

Human Ha-*ras*-1: pT24-C3 (SacI 2.9 kb); human c-*myc*: pMC41-C1 (EcoRI, HindIII 8.4 kb); human Ki-*ras*-2: pSP3K (EcoRI, HindIII 0.64 kb); mouse muscle actin: pAM91(PstI, 1.1 kb); human rDNA: pHR (EcoRI, 6.7 kb) (details of these plasmids are given in Spandidos & Kerr, 1984).

v-*abl*: pSA-17 (HindIII, SacI, 1.9 kb); v-*fos*: pfos-BS (BamH1 SalI, 0.76 kb); v-*sis*: pAT/sis (PstI, 1.3 kb); v-*fes*: pfes-3 (PstI, 0.5 kb); v-*src*: psrc EcoRI-B (EcoRI, 2.95 kb) (all obtained from Dr Natalie Teich, ICRF Laboratories, Lincolns Inn Fields, London).

int-2: pint-2f (EcoRI, SacI, 0.6 kb); v-*myb*: pmyb-KS (SacI, BamH1, 1.2 kb) (from Dr Gordon Peters, ICRF, London).

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v-*erb*-B: pverb-B-DT (BamH1, 0.5 kb) (from D. Tannahill, Edinburgh University).

The plasmids pSPH-*ras* 2 and pMC64-10 were constructed by insertion into the vector SP64 of (respectively) the 2.9 kb SacI fragment of the human T24 Ha-*ras* gene (containing all four coding exons) or the 8.4 kb EcoRI/HindIII fragment of the human c-*myc* gene (containing all three coding exons). Oncogene RNA was generated by incubation with SP6 polymerase (Melton *et al.*, 1984) under the conditions recommended by the manufacturer (Bethesda Research Laboratories(BRL), Paisley, Scotland).

Immunoblotting

Monolayers of cultured cells were detached by incubation in phosphate buffered saline containing 0.02% EDTA, and lysed in 100 mM sodium chloride, 10 mM Tris pH 7.5, 0.1% SDS, 1% NP40. Portions of snap frozen tumour were treated similarly. Electrophoresis in 15% polyacrylamide gels, electrophoretic blotting on nitrocellulose, and immunostaining with Y13-259 (Furth *et al.*, 1982) were conducted as previously described (Robinson *et al.*, 1986), but using an alkaline phosphatase-streptavidin system (Blu-gene, BRL) to detect the biotinylated second antibody.

Results

Pathology of 'primary' *tumours*

A summary of the tumour development at the subcutaneous injection sites is given in Table I. Animals injected in groin and back showed similar features and are considered together.

CHL cells without human oncogenes never produced visible or palpable tumours, and on autopsy 5 weeks after inoculation no abnormality was detected at the injection site. 208F cells, without human oncogenes, although reputedly non-tumorigenic, consistently yielded small, slowly-growing nodules, not exceeding 5 mm diameter even after 5 weeks. Histologically these were low grade fibrosarcomas, with few mitotic figures. There was conspicuous collagen deposition around and between the tumour cells, and mast cells of presumed host origin were plentiful within the tumours. No necrosis was observed within these tumours, but cells at the tumour edge appeared to be infiltrating between adjacent adipocytes (Figure 1A).

Table I Pathology of fibroblast tumours expressing human oncogenes

Host cell	*Transfected* onc	*Mice injected*	*Primary tumours*	*Regressing tumours*	*Progressive tumours*	*Metastasis in lung (L)*	*Metastasis in node (N)*	*Total metastases*	*Time metastases noted (day after injection)*
Hamster fibroblast (CHL)	nil	4	0 (0%)	0 (0%)	0 (0%)	0	0	0	
	T24 H-*ras*	16	16 (100%)	5 (31.3%)	11 (68.8%)	3	3	6 (37.5%)	L: 8, 13, 13 N: 18, 25, 35
Rat fibroblast (208F)	nil	7	0 (0%)[a]	0 (0%)	0 (0%)	0	0	0	
	T24 Ha-*ras*	31	29 (93.5%)	5 (16.1%)	24 (77.4%)	6	5	8 (25.8%)	L: 8, 11, 11 } 14, 14 } 14 } N: 11, 11 } 14, 14 } 14 }
	Ha-*ras-1*	15	14 (93.3%)	2 (13.3%)	12 (80%)	0	2	2 (13.3%)	N: 12, 12
	c-*myc*	25	13 (52.0%)[b]	0 (0%)	13 (52%)	1	1	2 (8.0%)[c]	L: 22 N: 67

All bracketed figures are percentages of total injected animals. [a]As indicated in the text all animals showed small nodules at the injection site, not exceeding 0.5 cm in diameter; [b]Take rate differs significantly from rat fibroblast tumours bearing c-Ha-*ras* ($P < 0.01$) or T24 Ha-*ras* ($P < 0.0001$; Fisher's exact test); [c]Metastasis rate differs significantly from rat fibroblast tumours bearing T24 Ha-*ras* if scored at 14 days, whether all injected animals ($P < 0.01$) or only those with primary tumours ($P < 0.05$) are considered.

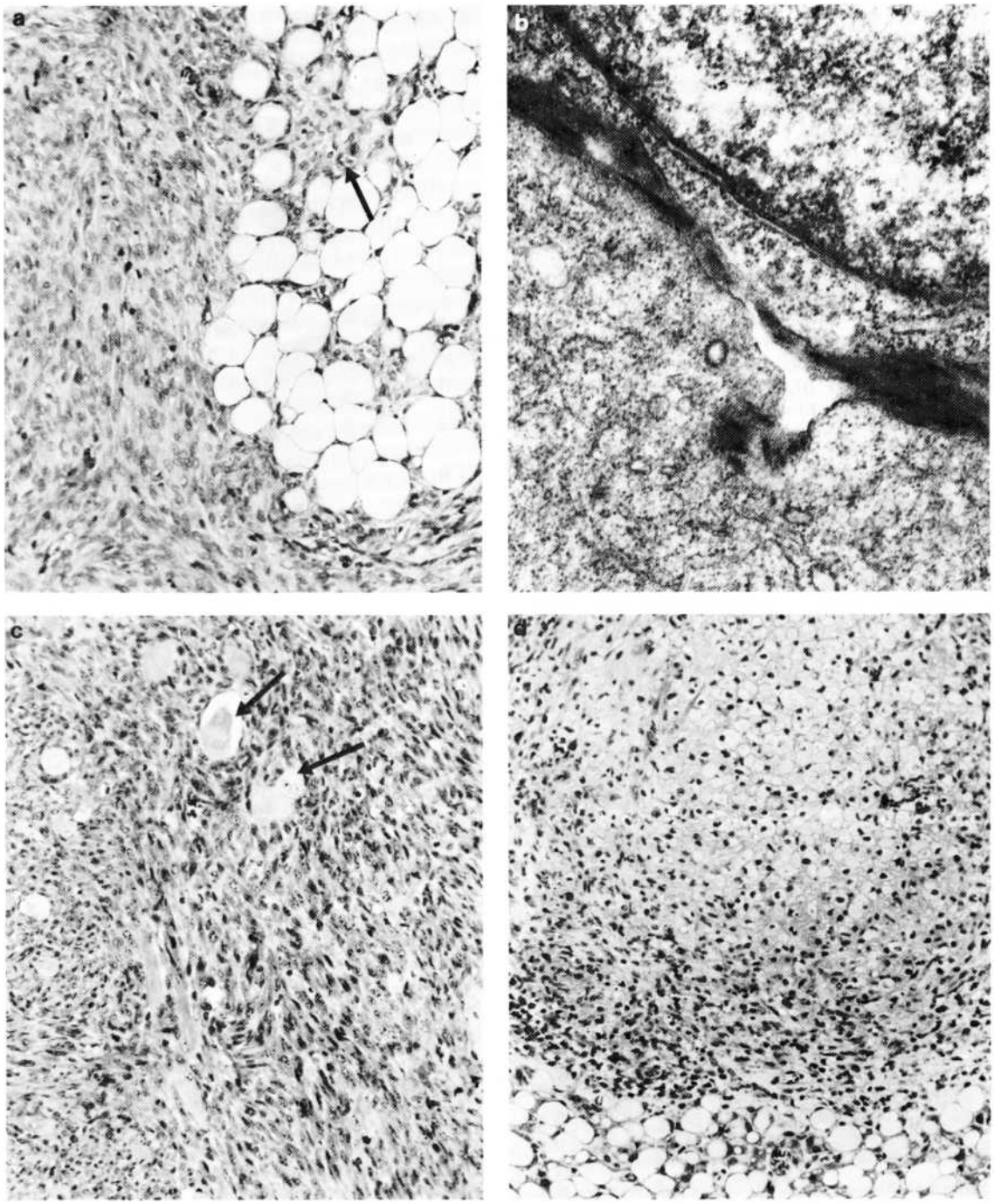

Figure 1 Growth and regression of rodent fibroblasts in immune suppressed mice. (a): An indolent s.c. nodule produced by injection of the 208F aneuploid rat line. Infiltration between adipocytes is seen (arrow). (b) Nascent collagen fibres at the membrane of CHL cells transfected with T24 Ha-*ras*. (c): Aggressive growth of 208F cells transfected with T24 Ha-*ras*. Muscle fibres in process of destruction by the tumour are seen (arrows). (d): Fat necrosis at site of regressing tumour, after injection of CHL cells transfected with T24 Ha-*ras*. (a, c, d × 160; H & E. b × 30,000; uranyl acetate and lead citrate).

CHL cells bearing the T24 Ha-*ras* gene yielded rapidly growing tumours in all animals, reaching diameters of over 1 cm within 2 weeks. The tumours infiltrated, and sometimes ulcerated, the overlying skin but were tethered to underlying connective tissue only rarely. Histologically these were aggressive fibrosarcomas with a high mitotic rate and little collagen between cells although nascent collagen bundles were identified on electron microscopy (Figure 1B). Zones of necrosis were conspicuous in the centre of the tumours but mast cells were not identified. Cells at the tumour periphery infiltrated around and within the striated muscle fibres of the panniculus carnosus, apparently destroying them (Figure 1C). Infiltration into adjacent fat and around epidermal appendages was also evident, but a mantle of mononuclear cells of uncertain histogenesis was often observed running circumferentially around the deep margin of the tumour. Several tumours showed extensive infiltration by neutrophil polymorphs, and regression was obvious in a proportion harvested between 13 and 35 days. Histologically, such clearly regressing tumours consisted of masses of fat necrosis with heavy neutrophil polymorph infiltration (Figure 1D). Despite this evidence of regression in some animals, the majority of tumour-bearing animals bore progressively enlarging tumours at the time of autopsy.

208F cells bearing T24 Ha-*ras* genes produced rapidly enlarging fibrosarcomas of similar morphology to the above (Figure 2A) and with a similar proportion showing regression. Similarly, 208F cells expressing the normal human Ha-*ras*-1 gene produced rapidly growing, potentially lethal tumours, although these showed less necrosis at similar size than the tumours bearing the mutated oncogenes. 208F cells bearing human c-*myc* also produced growing tumours, but in a significantly smaller proportion of animals than after injection of *ras*-bearing cells. In seven of the animals the tumours were aggressive fibrosarcomas (Figure 2B), but in the remaining six the morphology was intermediate between this and the indolent pattern of 208F cells alone. All these tumours were substantially larger than those produced by 208F cells alone, as observed at autopsy at closely similar times after inoculation. However, they grew less rapidly than the more aggressive variants, reached smaller diameters at the time of autopsy, and did not contain central necrotic areas.

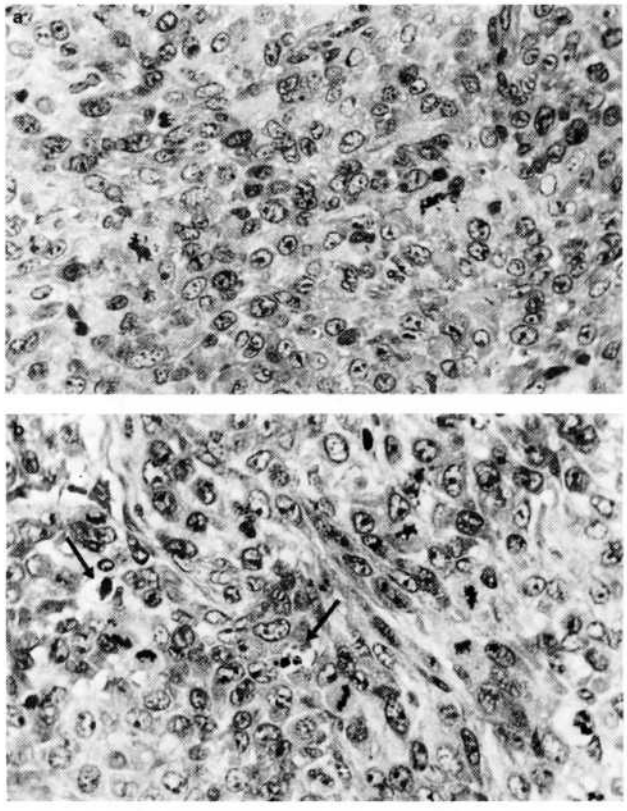

Figure 2 Aggressive fibrosarcomas at site of injection of 208F cells transfected with T24 Ha-*ras* (a) and c-*myc* (b). Numerous mitotic figures are present in both, but in (b) apoptotic bodies are also conspicuous (arrows), intimately admixed with the dividing cells. (× 500; H & E).

Oncogene expression in aggressive fibrosarcomas

Expression of c-*myc*, Ha-*ras*, and nine proto-oncogenes was assessed in examples of the aggressive fibrosarcomas, harvested within 2 weeks of inoculation (Table II and Figure 3). In tumours bearing the mutated human T24 Ha-*ras* gene, Ha-*ras* transcripts were present at levels close to those in the originally injected cells. Thus, in both CHL and 208F cells containing the T24 Ha-*ras* gene, levels of transcript were estimated at around 0.8% of total cellular mRNA, between 20- and 53-fold greater than the untransfected parent cells.

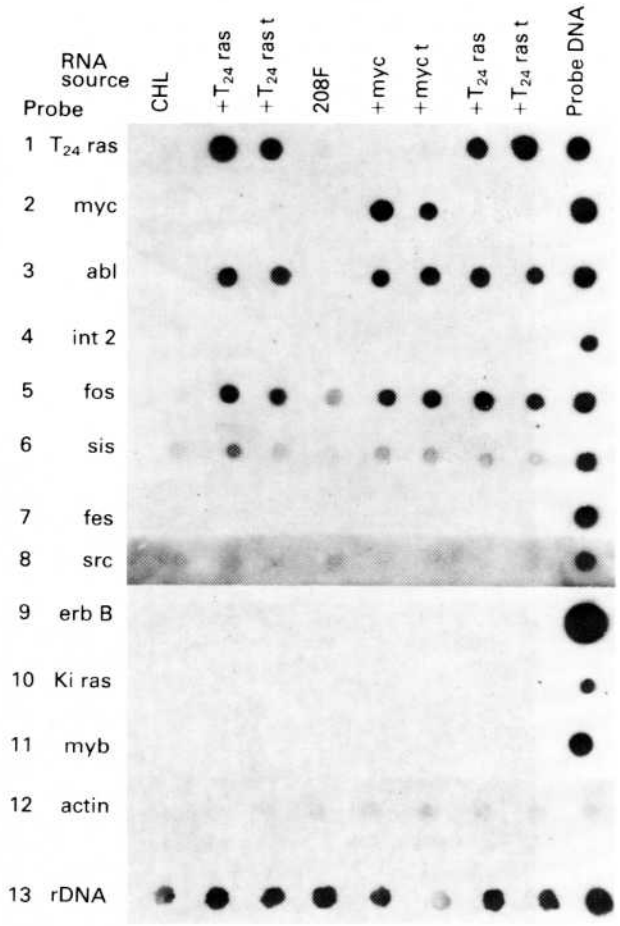

Figure 3 Hybridisation of ^{32}P probes to RNA from parental fibroblasts (CHL, 208F), their derivative cell lines *in vitro* (+T24 *ras*, +*myc*) and the tumours engendered by these (+T24 *ras t*, +*myc t*). Thirteen filters were prepared identically, by spotting 5 μg total RNA from the sources shown. The filters were also spotted with 2 ng plasmid DNA: for each filter the plasmid contained the sequence to be used as hybridisation probe. After hybridisation (as in **Materials and methods**), the filters were washed twice in 2 × SSC, 0.1% SDS at 20°C and then for a further 2 h at 65°C in 0.1 × SCC, 0.1% SDS (filters 1, 2, 10, 13) or in 1 × SSC, 0.1% SDS (filters 3–9, 11, 12). The lower stringency was chosen for these latter filters to allow for the less perfect homology between the probe and target sequences. Radiographic exposure time was adjusted so that approximately equal signals were obtained from the plasmid DNA spots, with the exception of filter 12, which was exposed for the same time as filter 13. These two filters were included to confirm that all the spots were equally loaded in terms of total RNA.