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Three different activated *ras* genes in mouse tumours; evidence for oncogene activation during progression of a mouse lymphoma

K.H.Vousden* and C.J.Marshall

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

*To whom reprint requests should be sent
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Five unrelated mouse tumours have been shown to carry activated transforming genes using the NIH/3T3 transfection assay. Three of these tumours, a T-cell lymphoma, a fibrosarcoma and a macrophage tumour, were found to carry an activated c-Ki-*ras* gene. A c-Ha-*ras* gene was shown to be activated in a myeloid leukaemia and a recently identified member of the *'ras'* gene family, N-*ras*, was found to be activated in a lung carcinoma. The T-cell lymphoma, L5178Y-ES, is a more aggressively growing metastatic variant which arose spontaneously from the parental tumour, L5178Y-E. Although DNA from both parental and variant tumours was shown to transfer a genetic marker to recipient cells equally well, only the metastatic variant carried an activated c-Ki-*ras* gene detectable by transfection. The altered growth behaviour of the L5178Y-ES cells may therefore be the result of the spontaneous activation of the c-Ki-*ras* gene after the lymphoma cells had already become tumorigenic.
Key words: mouse/oncogene/*ras*/transfection/tumour

Introduction

Activated transforming genes have been identified in a number of human and rodent tumours by transferring these genes into an established mouse cell line NIH/3T3 (for review, see Cooper, 1982). Most of the transforming genes identified so far using such transfection experiments have been shown to be related to the oncogenes carried by the Kirsten and Harvey RNA tumour viruses: viral Kirsten *ras* (v-Ki-*ras*) and viral Harvey *ras* (v-Ha-*ras*), respectively. Both the human and murine genomes contain two genes related to v-Ha-*ras*: c-Ha-*ras*-1 and c-Ha-*ras*-2, and two genes related to v-Ki-*ras*: c-Ki-*ras*-1 and c-Ki-*ras*-2 (DeFeo *et al.*, 1981; Chang *et al.*, 1982). Activated c-Ha-*ras*-1 has been identified in a human bladder carcinoma cell line and a human lung carcinoma cell line (Der *et al.*, 1982; Parada *et al.*, 1982; Santos *et al.*, 1982; Yuasa *et al.*, 1983). Chemically induced rodent skin carcinomas and mammary tumours have also been shown to have an activated c-Ha-*ras* gene (Balmain and Pragnell, 1983; Sukumar *et al.*, 1983). A much larger number of human tumours with activated c-Ki-*ras*-2 has also been identified. These tumours are mainly lung and colon carcinomas (Der *et al.*, 1982; Pulciani *et al.*, 1982; McCoy *et al.*, 1983) but activated c-Ki-*ras* has also been identified in a rhabdomyosarcoma, a bladder tumour, a gall bladder and a pancreatic carcinoma (Pulciani *et al.*, 1982), a cell line from an intermediate T-cell leukaemia (Eva *et al.*, 1983) and in chemically induced mouse fibrosarcomas (Eva and Aaronson, 1983). Another member of the '*ras*' gene family, N-*ras*, has been recently identified as activated in a number of human tumour cell lines including sarcomas, myeloid leukaemias, a neuroblastoma and a T-cell leukaemia (Marshall *et al.*, 1982; Hall *et al.*, 1983; Murray *et al.*, 1983; Shimizu *et al.*, 1983; Eva *et al.*, 1983).

To investigate further the spectrum of tissue types that contain activated transforming genes we performed transfection assays with DNA prepared from 21 mouse tumours. Five positive tumours were a methylcholanthrene-induced T-lymphoma (L5178Y-ES) and macrophage tumour (P388D1), a benzo[a]pyrene-induced fibrosarcoma (FS6M1), a spontaneous lung carcinoma (Lewis lung carcinoma LLC) and a myeloid tumour isolated from a mouse infected with Abelson murine leukaemia virus (WEHI274). In each of these tumours we have identified an activated transforming gene and have shown them all to be members of the *ras* gene family.

Results

Identification of activated 'ras' genes in five mouse tumours

High mol. wt. DNAs were isolated from 21 mouse tumours and assayed for transforming activity in a transfection assay. DNA from only five of these tumours was capable of inducing foci on NIH/3T3 cells (see Table I). Other tumour DNAs tested produced no foci in the NIH/3T3 transfection assay. These were a series of myeloid tumours (M1, WEHI3, WEHI265, J774A.1, RAW264), some spontaneous teratocarcinomas (PCC3, PCC4, LT, F9), mammary adenocarcinomas (MT1LV1p, MT1TC2p, MT1LV1Cl1sp) and a number of tumours induced by injecting NIH/3T3 cells into nude mice (NIHT1, NIHT2, NIHT3, NIHT4). The transformed cells picked from the foci differed from untransformed NIH/3T3 cells in that they grew in low serum and semisolid medium. DNA isolated from these foci was capable of transforming NIH/3T3 cells in a second round of transfection, indicating that a transferrable transforming gene had been activated in each of these tumours. Transforming activity of the LLC lung carcinoma DNA has previously been reported by Shih *et al.* (1981).

To compare the five transforming genes, DNA was isolated from the transformed foci, digested with various restriction enzymes and then assayed for transforming activity. Table II shows that the transforming activity of DNA from L5178Y-ES, FS6M1 and P388D1 was destroyed by digestion with *Eco*RI and *Hind*III, whilst *Bam*HI and *Sal*I leaves the transforming activity intact. In contrast, the transforming activity of DNA from LLC was destroyed by digestion with *Eco*RI, *Bam*HI and *Hind*III but unaffected by digestion with *Sal*I. The transforming activity of the fifth tumour WEHI274 was destroyed by digestion with *Bam*HI and *Hind*III but left intact by digestion with *Eco*RI and *Sal*I. We also found that digestion with *Bgl*II, *Pvu*II, *Kpn*I and *Sst*I destroyed the transforming activity of the WEHI274 DNA but *Xho*I digestion had no effect. These results indicated that the transforming genes identified in the lung carcinoma LLC and the myeloid leukaemia WEHI274 are dif-

Table I. Transforming efficiencies of DNA from mouse tumours and tumor cell lines

Donor DNA	Source of DNA	Efficiency of APRT transfer (azoserine-resistant colonies/μg DNA)	Efficiency of primary transfection[a] (no. foci/μg DNA)	Efficiency of secondary transfection[b] (no. foci/μg DNA)
L5178Y-E	Tumour	N.D.	<0.001 (0/21)	–
(T-lymphoma)	Cell line	0.28	<0.001 (0/22)	–
Parental line	Cell line	0.63	<0.001 (0/20)	–
L5178Y-ES	Tumour	N.D.	0.07 (15/11)	0.11, 0.11, 0.15
'Metastatic	Cell line	0.28	0.05 (18/17)	0.06
variant'	Cell line	0.23	0.02 (2/6)	N.D.
P388D1	Cell line	N.D.	0.04 (5/6)	0.11, 0.18
(Macrophage tumour)	Cell line	N.D.	0.04 (5/6)	N.D.
FS6M1	Tumour	N.D.	0.05 (18/17)	0.08, 0.13, 0.15
(Fibrosarcoma)	Tumour	N.D.	0.03 (3/6)	N.D.
	Cell line	N.D.	0.04 (5/6)	0.08, 0.17
LLC (Lung carcinoma)	Tumour	N.D.	0.25 (15/3)	0.23, 0.14, 0.34
WEHI274	Cell line	N.D.	0.02 (2/6)	0.20, 0.12
(Myeloid leukaemia)			0.01 (1/6)	
Salmon sperm (negative control)	–	N.D.	<0.001 (0/40)	–
No DNA (negative control)	–	<0.001	–	–

[a]Number of foci scored/number of plates examined is shown in parenthesis.
[b]Each value represents the transforming efficiency of a different primary focus.
N.D. Not determined.

Table II. Effect of restriction enzyme digestion on the transforming activities of the DNAs of transfectants

Restriction enzyme	No. of foci/μg DNA				
	L5178Y-ES	P388D1	FS6M1	LLC	WEHI274
Undigested	0.12	0.08	0.18	0.22	0.09
*Eco*RI	<0.001	<0.001	<0.001	<0.001	0.04
*Bam*HI	0.06	0.04	0.22	<0.001	<0.001
*Hind*III	<0.001	<0.001	<0.001	<0.001	<0.001
*Sal*I	0.04	0.02	0.10	0.14	0.13

ferent from each other and from that found in the other three tumours.

To determine whether the transforming genes detected by transfection of NIH/3T3 cells were activated '*ras*' genes, DNA from transfectants was analysed on Southern blots. If restriction sites closely linked to the transferred gene were destroyed by restriction digestion before transfection or during the transfection process then DNA prepared from transformed foci would contain novel restriction fragments hybridising to *ras* gene probes. It can be seen in Figure 1 that DNAs prepared from P388D1, L5178-ES and FS6M1 transfectants contained novel fragments hybridising to a v-Ki-*ras* probe. All secondary foci induced by the DNA from these transfectants were shown to contain the same novel fragment as the primary transfectant from which they were derived, demonstrating that this sequence was serially passaged during subsequent rounds of transfections. Furthermore it is apparent that in many of the transfectants the intensity of the bands hybridising to the v-Ki-*ras* probe is greater than that in the untransformed NIH/3T3 cells or in the tumour lines, suggesting that these sequences are amplified in these transfectants. Amplification of the transferred sequences appears to be a frequent occurrence in the transfected cells. DNA from 14 of the 18 secondary foci examined showed at least a 2-fold amplification of the bands hybridising to the v-Ki-*ras* probe. When DNA from the P388D1, L5178Y-ES and FS6M1 transfectants was hybridised to v-Ha-*ras* or N-*ras*, no evidence that the transfectants contained additional sequences hybridising to these probes was obtained (data not shown). These results therefore demonstrate that the transforming gene in P388D1, L5178Y-ES and FS6M1 is a cellular homologue of v-Ki-*ras*.

Figure 2 shows that a transformant derived from LLC DNA has novel bands hybridising to a human N-*ras* probe. Similar Southern blots probed with v-Ha-*ras* or v-Ki-*ras* showed only normal endogenous mouse sequences, with no additional bands (data not shown). This is the first demonstration of an activated N-*ras* gene in a mouse tumour.

DNA from transfectants from the fifth tumour, WEHI274, were also examined on Southern blots using the three known *ras* gene sequences as probes. In this case Figure 3 shows that novel bands were seen when the v-Ha-*ras* probe was used. The activated transforming gene identified by transfection of the tumour DNA is therefore shown to be c-Ha-*ras*. This tumour arose in a mouse which had been infected with Abelson leukaemia virus (Ab MuLV) (Harris *et al.*, 1979). We therefore analysed DNA from both WEHI274 and from transfectants for the presence of v-*abl* (Figure 4) but were unable to detect any integrated v-*abl* sequences. However, the v-*abl* sequences of ANN-1, an NIH/3T3 cell line transformed with Ab MuLV (Scher and Siegler, 1975) and of transfectants derived from ANN-1 DNA were readily detected. If, therefore, WEHI274 had been transformed *in vivo* by Ab MuLV, it appears to have lost the exogenous v-*abl* sequences whilst acquiring an activated c-Ha-*ras* gene.

Activated c-K-ras in a 'metastatic' T-lymphoma, but not in the parental line

Table I shows that the DNA prepared from the 'metastatic' variant of the T-lymphoma, L5178Y-ES, efficiently induced

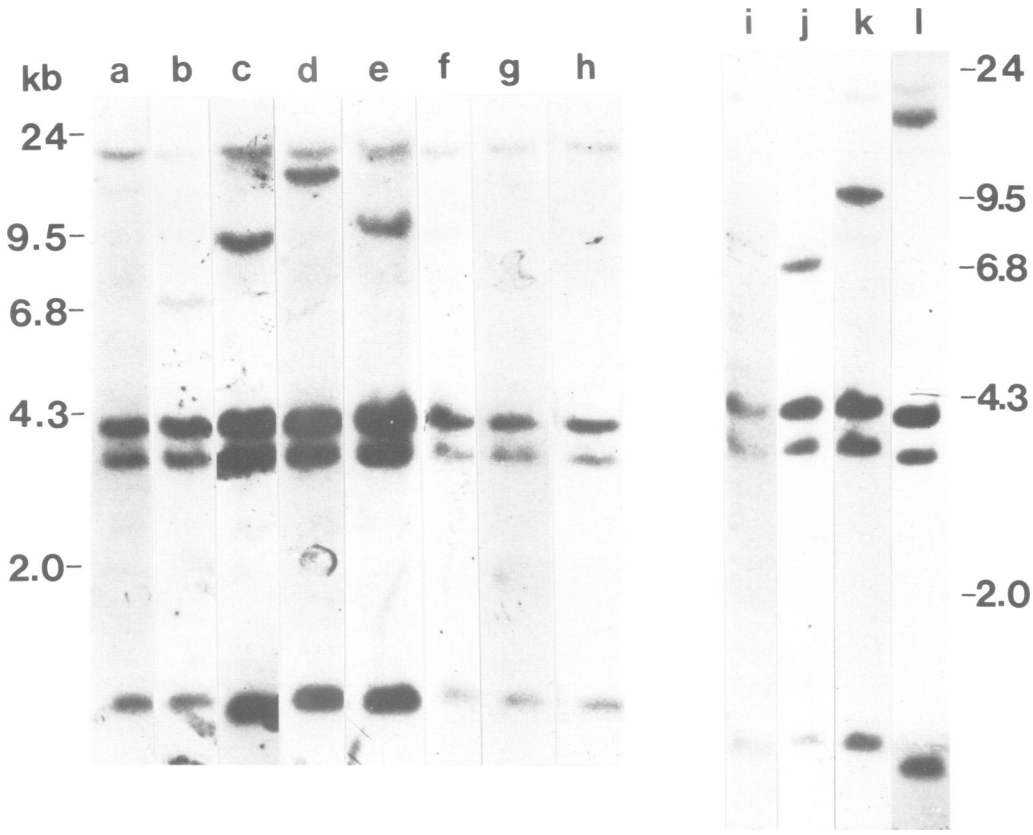

Fig. 1. Presence of novel fragments hybridising to a v-Ki-*ras* probe HiHi3 (Ellis *et al.*, 1981) in transformants derived from L5178Y-ES, P388D1 and FS6M1. DNAs were digested with *Hind*III. **Lanes a** and **i**, NIH/3T3; **b**, secondary transfectant derived from L5178Y-ES; **c**, secondary transfectant derived from P388D1; **d** and **e**, two independently derived secondary transfectants from FS6M1; **f**, L5178Y-ES parental tumour; **g**, P388D1 parental tumour line; **h**, FS6M1 parental tumour line; **j**, primary transfectant derived from L5178Y-ES; **k**, primary transfectant derived from P388D1; **l**, primary transfectant derived from FS6M1.

foci of morphologically transformed NIH/3T3 cells whereas no foci were obtained using DNA prepared from the non-metastatic parental line L5178Y-E. The difference in transforming activity between the two tumour lines was observed with DNA obtained directly from tumour cells or from cells grown in tissue culture. As a control to check the integrity of the DNA preparations, we compared the frequence of adenosine phosphoribosyl transferase (APRT) gene transfer using these DNAs (Wigler *et al.*, 1979). Table I shows that DNA preparations from L5178Y-E cells that were incapable of inducing foci on NIH/3T3 cells transferred the APRT gene to LMTK$^-$ APRT$^-$ HPRT$^-$ L cells as efficiently as the DNA prepared from L5178Y-ES. This demonstrates that L5178Y-E DNA is capable of transferring a genetic marker. These two variants arose some 15 years ago and we were able to obtain frozen tumour stocks which were within 10 passages of the divergence of L5178Y-E and L5178Y-ES. The same result was obtained using DNA from these tumours, so the activation of the transforming gene in L5178Y-ES appears to be related to the acquisition of a more aggressive tumour phenotype.

Discussion

Results from many groups have shown that with human tumour DNA the NIH/3T3 transfection assay shows a marked preference for detecting *ras* genes. Here we demonstrate that using DNA from mouse tumours in transfection experiments, again *ras* genes are detected. There seems to be no obvious tissue specificity in the activation of these genes since we have detected activated c-Ki-*ras* in a fibrosarcoma, a macrophage tumour and a T-lymphoma.

A myeloid tumour from an Ab MuLV-infected mouse was shown to contain an activated c-Ha-*ras* gene but no v-*abl* sequences. To our knowledge this is the first example of c-Ha-*ras* activation in a myeloid tumour. The loss of v-*abl* sequences from lymphoid tumours induced by Ab MuLV has been documented previously (see Rosenberg, 1982) and we have evidence that other myeloid tumours, such as RAW264, appearing in Ab MuLV-infected mice also lack v-*abl* (K.H.Vousden, unpublished results). The lack of viral sequences and the presence of an activated c-Ha-*ras*-1 transforming gene suggest that the v-*abl* oncogene is required only for the initial stages of tumour development assuming that the myeloid tumours were indeed induced by Ab MuLV. Lane *et al.* (1982) have described two Ab MuLV-induced lymphoid tumours that contain a non-*ras* transforming gene as well as the v-*abl* sequences. Such tumours may represent an intermediate stage in tumour development where both transforming genes co-exist in one cell. Subsequent selection may then result in cells which have lost the v-*abl* sequences outgrowing the rest of the population, perhaps because the v-*abl* protein is sometimes toxic (Ziegler *et al.*, 1981).

We have also shown that only the 'metastatic' variant L5178Y-ES derived from a T-lymphoma L5178Y-E contains an activated c-Ki-*ras* oncogene. This result has two implications; firstly it demonstrates that some other gene(s) undetectable by NIH/3T3 transfections must be responsible for the

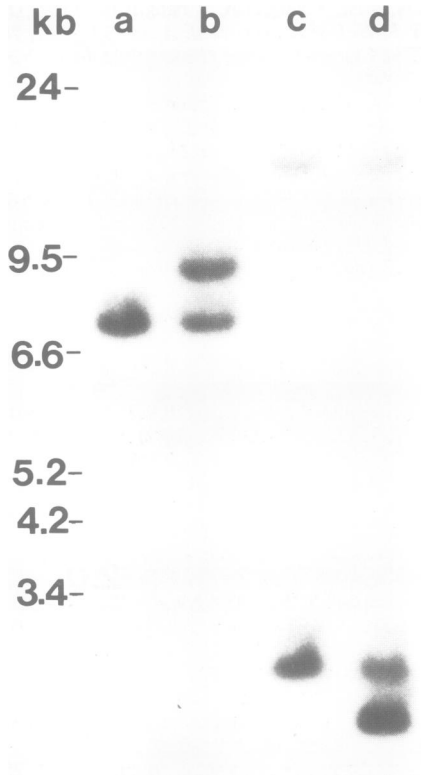

Fig. 2. Presence of a novel fragment hybridising to a human N-*ras* probe from pAT8.8 (Hall *et al.*, 1983) in a transformant derived from Lewis lung carcinoma. **Lanes a** and **c**, NIH/3T3; **lanes b** and **d**, primary transfectant derived from LLC. DNAs run in **lanes a** and **b** digested with *Eco*RI; DNAs run in **lanes c** and **d** digested with *Pvu*II.

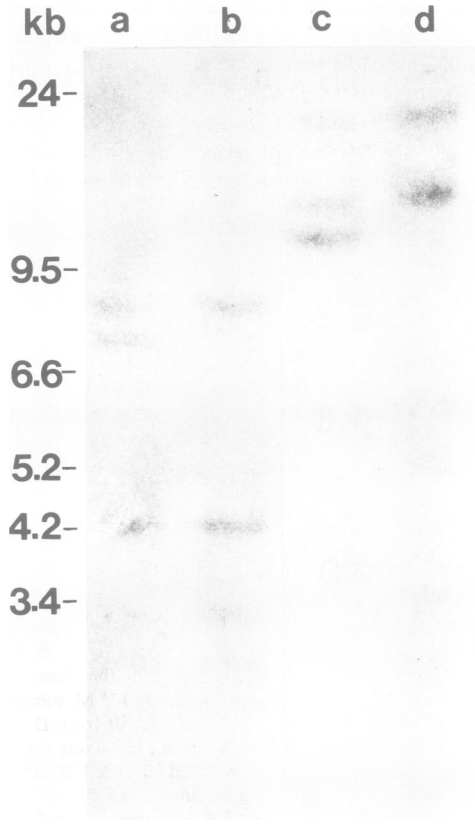

Fig. 3. Presence of a novel fragment hybridising to a v-Ha-*ras* probe from BS9 (Ellis *et al.*, 1980) in a transformant derived from WEHI274. **Lanes a** and **c**, secondary transfectant derived from WEHI274; **lanes b** and **d**, NIH/3T3. DNAs run in **lanes a** and **b** digested with *Hind*III; DNAs run in **lanes c** and **d** digested with *Pst*I.

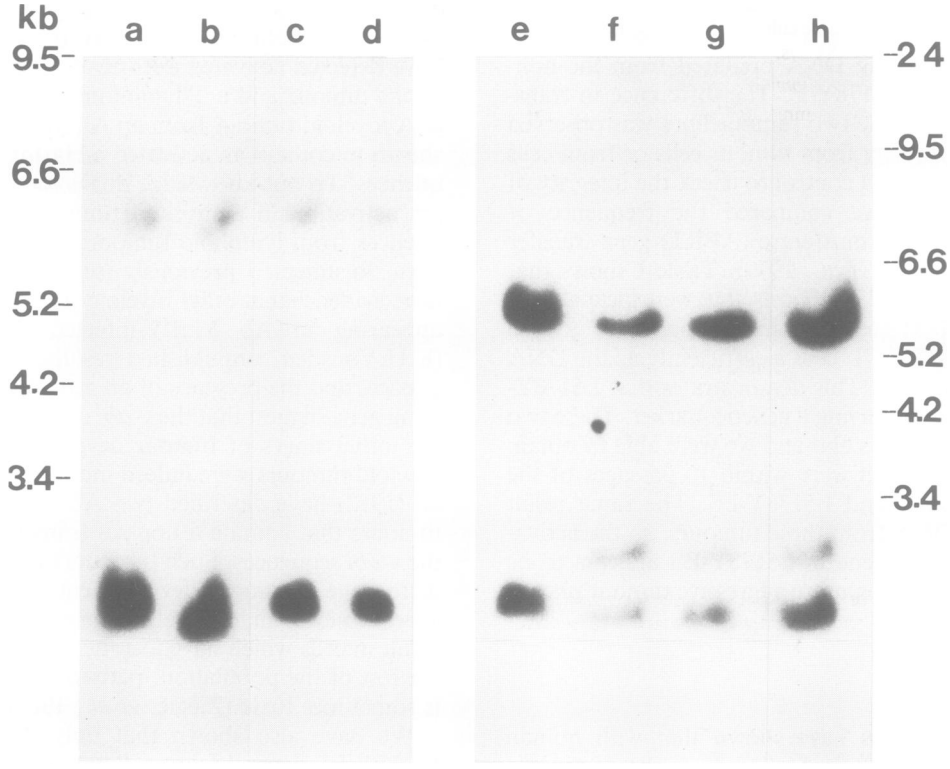

Fig. 4. Absence of exogenous v-*abl* sequences in WEHI274 and transformants derived from WEHI274. DNAs were digested with *Sst*I and the filter probed with the v-*abl* insert from pSA-17 (Dale and Ozanne, 1981). **Lanes b** and **e**, NIH/3T3; **lane a**, WEHI274 parental tumour line; **lanes c** and **d**, secondary transfectants derived from WEHI274; **lane f**, ANN-1; **lanes g** and **h**, primary transfectants derived from ANN-1.

malignancy of L5178Y-E cells; secondly it suggests that the altered growth behaviour of L5178Y-ES cells may be a consequence of the activation of a *ras* gene. The activation of this gene has occurred in a cell which is already tumorigenic. Such an observation provides a potential mechanism to explain the phenomenon of tumour progression (Foulds, 1969). The phenotype of tumours is rarely static and there is a general tendency to evolve cells with a more aggressive anaplastic phenotype. We suggest that such cells could arise as the result of the random activation of cellular *ras* genes after the cells had become tumorigenic. Such cells with additional activated oncogenes would have altered properties and would therefore be subject to the powerful selective forces operating in tumours (Poste and Fidler, 1980). The cellular oncogenes, and in particular the *ras* genes, may therefore play a role not only in the conversion of normal into tumour cells (Land *et al.*, 1983; Newbold *et al.*, 1983; Ruley, 1983) but also in tumour progression.

Materials and methods

Cells and cell culture

L5178Y-E and L5178Y-ES cells and tumours (Parr, 1972) and FS6M1 cells and tumours (Mantovani, 1978) were kindly provided by Dr. S. Eccles. These cells were cultured in RPMI 1640 medium with 10% foetal calf serum. L5178Y-ES cells required the addition of 5×10^{-5} M mercaptoethanol. P388D1 cell lines (Dawe and Potter, 1957) were a gift from Dr. Y. Gillespie and Mrs. I.M. Williams, WEHI274 (Harris *et al.*, 1979) was also provided by Dr. Y. Gillespie. These cell lines were cultured in RPMI 1640 medium with 5% foetal calf serum. The Lewis lung carcinoma (LLC) tumour was a gift from Dr. T. Stephens. NIH/3T3 clone D4 cells were cultured in Dulbecco's modified Eagles medium (DMEM) with 10% newborn calf serum.

Transfection assays

In each transfection using high mol. wt. DNA, 20 μg of DNA was applied to 60 mm culture dishes seeded 24 h previously with 3×10^5 NIH/3T3 clone D4 cells in the form of a calcium phosphate co-precipitate (Shih *et al.*, 1979). In transfections using restriction enzyme-digested DNAs, 10 μg of completely digested DNA and 20 μg high mol. wt. mouse carrier DNA were added to each plate of NIH/3T3 cells. The cells were cultured in DMEM with 5% calf serum and foci were scored after 16–18 days.

Restriction enzyme digestion and size fractionation

DNAs were digested under conditions recommended by the manufacturer using a 10-fold excess of enzyme. Digestion was monitored using a parallel control digestion containing an aliquot of the DNA and enzyme mixture with 1 μg of λ DNA.

Southern blots

Digested DNAs were electrophoresed in 0.7% agarose gels and transferred to nitrocellulose paper essentially as described by Southern (1975). Hybridisation was carried out at 42°C in a buffer containing 50% formamide and 10^6 c.p.m./ml nick-translated probe.

The probes used were the v-Ki-*ras* probe HiHi3 (Ellis *et al.*, 1981), the v-Ha-*ras* insert from BS9 (Ellis *et al.*, 1980), the human N-*ras* insert from pAT8.8 (Hall *et al.*, 1983) and the v-*abl* insert from pSA-17 (Dale and Ozanne, 1981). Blots probed with v-Ki-*ras* and v-Ha-*ras* were washed in 0.6 x SSC/0.1% SDS at 60°C; blots probed with N-*ras* were washed in 0.6 x SSC/0.1% SDS at 45°C and blots probed wth v-*abl* were washed in 0.1 x SSC/0.1% SDS at 68°C. Autoradiography was carried out using an intensifying screen at −70°C for 2 weeks.

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