Genet. Res., Camb. (1989), **54**, *pp.* 149–153 *With 2 text-figures Printed in Great Britain*

Origin of rare Ha-*ras* alleles: relationship of VTR length to a 5′ polymorphic *Xho* I site

GLENN D. BAXTER[1], NICHOLAS K. HAYWARD[1], RUSSELL J. COLLINS[2] AND MARTIN F. LAVIN[1]*

[1] *Queensland Institute of Medical Research, Herston, Brisbane 4006. Australia*
[2] *Division of Immunology, Department of Pathology, Royal Brisbane Hospital, Brisbane, 4006*

(*Received 7 November 1988 and in revised form 21 February 1989*)

Summary

Amongst the four common Ha-*ras* alleles in both controls and cancer patients, we detected the presence of a polymorphic *Xho* I site associated specifically with the 6·6 and 7·7 kb *Bam* HI fragments but absent from the 7·1 and 8·2 kb alleles, as recently reported by others. We have extended this study and report here, the consistent appearance of this *Xho* I site in unusual alleles close in size to the two common alleles of 6·6 and 7·7 kb, in control lymphoblastoid DNA samples in a variety of tumor DNAs. Unusual alleles grouped around the 7·1 and 8·2 kb common alleles on the other hand, did not possess the *Xho* I site. The consistent presence of the *Xho* I site polymorphism, in the unusual Ha-*ras* alleles surrounding the 6·6 and 7·7 kb common alleles and its absence in alleles around the 7·1 and 8·2 kb common alleles, suggests that the unusual ones are derived from the corresponding common alleles to which they are closest in size.

1. Introduction

The existence of discrete transforming sequences in the genomes of some tumours was established by transformation of cell lines by transfection of DNA isolated from a variety of tumours (Krontiris & Cooper, 1981; Shih *et al.* 1981; Perucho *et al.* 1981). The majority of oncogenes isolated by this method are related to a small family of retroviral *onc* genes, designated *ras* (Der *et al.* 1982; Eva *et al.* 1983; Shimizu *et al.* 1983). The human cellular *ras* gene family consists of three proto-oncogenes, c-Harvey (Ha)-*ras*, c-Kirsten (K)-*ras* and N-*ras*. Activated *ras* genes, isolated from some tumours, contain somatic mutations at specific sites (Tabin *et al.* 1982; Reddy *et al.* 1982; Capon *et al.* 1983). Activation resulting in overexpression may also be involved in transformation. Increased levels of p21, the product of the *ras* gene, have been observed in a wide range of tumours (Spandidos & Kerr, 1984; Tanaka *et al.* 1986; Hand *et al.* 1987). Moreover in experimental systems, increased levels of p21 can promote the morphological and tumourigenic transformation of NIH 3T3 cells (Chang *et al.* 1982; Stacey & Kung, 1984).

Recent analyses of sequences involved with control of expression of the c-Ha-*ras*-1 gene demonstrate the presence of a promoter region immediately upstream of the untranslated exon-1 (Ishii *et al.* 1985; Damante *et al.* 1987). In addition to this regulatory domain, it appears that a region located approximately 1·5 kb downstream from the 3′ terminus of the Ha-*ras* coding sequences may act as an enhancer (Cohen *et al.* 1987; Rabinowe & Krontiris, 1987). This region called the variable tandem repeat (VTR) consists of a 28 bp consensus sequence and changes in the number of these repeat units is the basis for a *Bam* HI restriction fragment length polymorphism (RFLP) (Capon *et al.* 1983), which gives rise to four common alleles of diverse sizes which appear to be inherited in a Mendelian fashion (Krontiris *et al.* 1985). However in addition to these common alleles, a number of unusual alleles exist. There is considerable debate as to whether the frequency of these rare forms of the Ha-*ras* gene is higher in cancer patients than in unaffected controls. A number of groups have reported significant associations (Krontiris *et al.* 1985, 1986; Lidereau *et al.* 1986; Hayward *et al.* 1988; Carter *et al.* 1988), whereas several groups have not found an increased frequency of rare alleles in cancer patients (Heighway *et al.* 1986; Thein *et al.* 1986; Ceccherini-Nelli *et al.* 1987; Ishikawa *et al.* 1987; Gerhard *et al.* 1987; Radice *et al.* 1987).

Recently an *Xho* I site polymorphism, located in the

* Corresponding author.

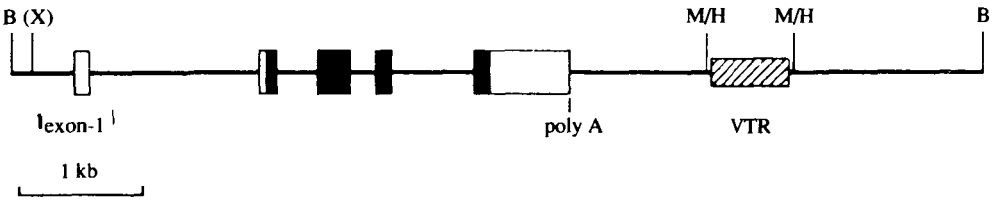

Fig. 1. Map of the human c-Ha-*ras*-1 gene contained in the 6·6 kb *Bam* HI fragment of the plasmid pEJ. Open boxes represent untranslated exons and closed boxes represent coding regions. The polymorphic *Xho* I site is located approximately 200 bp downstream from the 5′ *Bam* HI site. The variable tandem repeat region depicted as a hatched box, is located approximately 1·5 kb downstream from the coding sequences and is flanked by *Msp* I/*Hpa* II sites. B, *Bam* HI; H, *Hpa* II; M, *Msp* I; X, *Xho* I.

5′ flanking region of the Ha-*ras* gene was reported (Chandler *et al.* 1987). The presence of this polymorphism is tightly linked to the length of the VTR, being always present in the 6·6 and 7·7 kb common *Bam* HI alleles and consistently absent in the two other common alleles (7·1 and 8·2 kb).

This study was designed to investigate the origin of unusual alleles observed in some control lymphoblastoid DNA samples and in DNAs from various malignancies which were previously used for the study of rare Ha-*ras* alleles in this laboratory (Hayward *et al.* 1988). To this end we have extended studies on the *Xho* I site polymorphism associated with the c-Ha-*ras*-1 gene, in particular to determine which of the unusual alleles grouped around the four common alleles possess the *Xho* I site.

2. Materials and methods

(i) *Samples*

The sample group consisted of 15 individuals without personal or family history of cancer in the first-degree relatives, twenty-two patients with leukaemia (14 with B-cell chronic lymphocytic leukaemia (B-CLL) and 8 with T-cell acute lymphoblastic leukaemia (T-ALL)), 5 patients with malignant melanoma and 3 Wilms' tumour patients.

Control, malignant melanoma and Wilm's tumour patients' DNA samples were isolated from Epstein–Barr virus transformed B-lymphoblastoid cell lines or tumour lines, established from these patients while leukaemia DNA samples were isolated from peripheral blood lymphocytes from patients with B-CLL or T-ALL.

(ii) *DNA extraction and Southern hybridization*

High-molecular-weight DNA was isolated using caesium chloride gradient centrifugation (Weeks *et al.* 1986). Restriction enzyme digests using 6 U of enzyme/μg DNA, were carried out in buffers supplied by the manufacturer (Amersham). DNA (10 μg) was digested overnight with *Msp* I and *Hpa* II and electrophoresed in 1·4% agarose gels. Samples were also digested overnight with *Bam* HI or for 6 h with *Bam* HI followed by addition of *Xho* I and further incubation overnight and electrophoresed in 0·65% agarose gels. DNA was transferred to Hybond-N nylon membranes (Amersham) by the method of Southern (1975). Prehybridization was carried out at 42 °C for 5 h in 50% deionized formamide, 5 × SSC, 5 × Denhardt's solution, 50 mM sodium phosphate, 0·1% SDS and 250 μg/ml denatured salmon sperm DNA. Hybridization was performed for 24 h in the same solution containing 2×10^6 cpm of ^{32}P-labelled probe/ml of hybridization solution. The plasmid, pEJ, containing the human c-Ha-*ras*-1 genomic sequence in a 6·6 kb *Bam* HI fragment (Shih & Weinberg, 1982) was used for the analysis of Ha-*ras* associated polymorphisms (Fig. 1). After hybridization, filters were rinsed twice in 2 × SSC, 0·1% SDS at room temperature and washed for 10 min in 1 × SSC, 0·1% SDS at room temperature followed by a wash in 0·2 × SSC, 0·1% SDS at 65 °C for approximately 30 min until background counts were removed. Filters where then air dried, wrapped in Glad Wrap and exposed to Kodak XRP-5 X-ray film using intensifying screens at −70 °C.

3. Results

(i) Xho I site polymorphism

Fifteen control DNA samples and 30 DNA samples from cancer patients (22 leukaemia, 3 Wilms' tumour and 5 melanoma) were examined for the presence of an *Xho* I site 5′ to the Ha-*ras* coding region. DNA samples were digested with *Bam* HI alone or double digested with *Bam* HI and *Xho* I, electrophoresed, blotted and hybridized with the plasmid pEJ. The presence of the *Xho* I site was detected by a shift in mobility of the Ha-*ras* allele due to a decrease in size by approximately 200 bp after digestion with *Bam* HI/*Xho* I compared to *Bam* HI alone (Fig. 1). In both the control and tumour samples examined, the presence of the *Xho* I site was restricted to the common *Bam* HI alleles of 6·6 and 7·7 kb (Fig. 2) which correspond to *Msp* I/*Hpa* II alleles of 1·0 and 2·05 kb respectively (Table 1). The *Xho* I site was not present in the other two common alleles of 7·1 and 8·2 kb in size, which contained *Msp* I/*Hpa* II fragment lengths

https://doi.org/10.1017/S0016672300028524 Published online by Cambridge University Press

Table 1. *Comparison of c-Ha-ras-1 allele sizes with the presence of a 5′ Xho I site polymorphism in control and tumour DNA samples*

Msp I/*Hpa* II fragment size (kb)	*Bam* HI fragment size (kb)	Number of alleles examined		*Xho* I site
		Control	Tumour[a]	
1·0[b]	6·6	15	32	+
1·12	6·7	—	2	+
1·15	6·7	—	2	+
1·3	6·9	—	1	−
1·33	6·9	—	1	−
1·5[b]	7·1	6	8	−
1·68	7·2	—	1	−
1·80	7·4	—	1	+
1·87	7·5	—	1	+
2·05[b]	7·7	3	4	+
2·2	7·8	—	2	+
2·32	7·9	—	1	−
2·47	8·0	1	—	−
2·65[b]	8·2	5	2	−
2·8	8·4	—	1	−
2·87	8·4	—	1	−

Common and associated unusual alleles are grouped together.
[a] The tumour group comprised 22 leukaemia, 3 Wilms' tumour and 5 melanoma samples.
[b] Common allele.

of 1·5 and 2·65 kb respectively. All of the unusual alleles examined showed a pattern similar to the common allele to which they were closest in size (Fig. 2, Table 1). Alleles with VTR regions (*Msp* I/*Hpa* II fragment lengths) of 1·12 and 1·15 kb in size possessed the *Xho* I site as did the common allele with a VTR length of 1·0 kb. Unusual alleles with a VTR range of 1·3 to 1·68 kb, that were clustered around the common allele with a 1·5 kb VTR, did not have an *Xho* I site. Unusual alleles with 1·80, 1·87 and 2·2 kb VTRs, which were close in size to the common allele with a 2·05 kb VTR, possessed the *Xho* I site, while the unusual alleles with 2·47, 2·8 and 2·87 kb VTRs, close in size to the common allele with the 2·65 kb VTR, lacked this site.

One of the samples, derived from a melanoma patient, showed loss in intensity of the upper 7·1 kb allele compared with the lower 6·7 kb allele (Fig. 2, lane 3) indicating partial loss of this allele.

4. Discussion

Variation in the number of a 28 bp tandemly repeated, consensus sequence within the VTR region has been primarily used to distinguish Ha-*ras* alleles (Capon *et al.* 1983; Krontiris *et al.* 1985). More recently, Chandler *et al.* (1987) observed a new polymorphism in the 5′ flanking region of the Ha-*ras* gene which shows strong linkage disequilibrium with the length of the VTR region. This polymorphism which is determined by the presence or absence of an *Xho* I site was found to result from change in nucleotide sequence and not from methylation of an internal cytosine residue which is possible at this site. Analysis of DNA from different human tissues revealed the presence of an *Xho* I site in Ha-*ras* alleles containing 1·0 or 2·05 kb VTRs (6·6 and 7·7 kb *Bam* HI fragments) whereas this site was absent in alleles containing 1·5 and 2·65 kb VTRs (7·1 and 8·2 kb *Bam* HI fragments). We have extended these results to show that the unusual alleles, clustered around the four common alleles in DNA from patients with leukaemia and other solid tumours or in unaffected controls, appear to be related to the common alleles to which they are closest in size, with respect to the presence or absence of the *Xho* I site. This resembles the presence of a *Taq* I site polymorphism within the VTR region that has been found to occur consistently in the 2·65 kb common allele and also in rare alleles around this

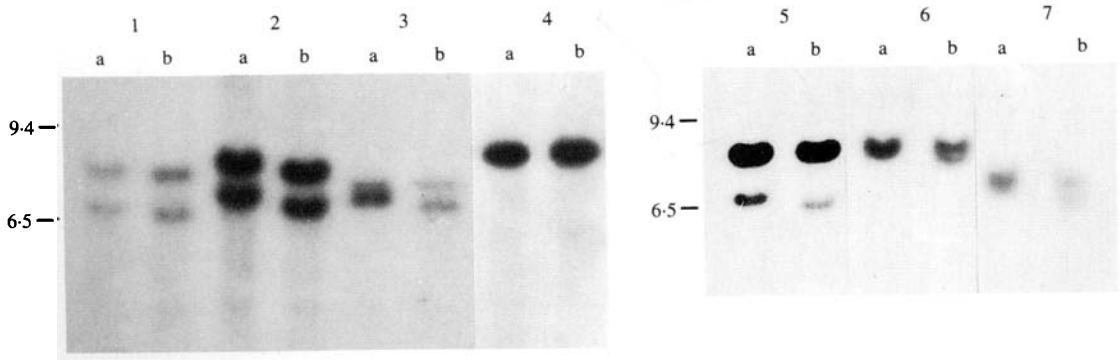

Fig. 2. *Xho* I site polymorphism in c-Ha-*ras*-1 alleles in samples from controls and leukaemia patients. DNA samples were digested with *Bam* HI (a) or *Bam* HI/*Xho* I (b) electrophoresed in 0·65 % agarose gels, transferred to nylon membranes and probed with the plasmid, pEJ. The sizes (kb) of the common alleles are shown on the left. Samples showed the following *Bam* HI allelic fragments: sample 1, 7·7/6·6 kb (control B-cell line); sample 2, 7·7/6·7 kb (melanoma patient B-cell line); sample 3, 7·1/6·7 kb (melanoma cell line); sample 4, 8·2/8·2 kb (melanoma cell line); sample 5, 7·9/6·6 kb (melanoma cell line); sample 6, 8·2/7·8 kb (Wilms' B-cell line); sample 7, 7·1/6·6 kb (Wilms' B-cell line).

https://doi.org/10.1017/S0016672300028524 Published online by Cambridge University Press

fragment size (Radice *et al.* 1987). This *Taq* I site was not observed in any of the other three common alleles or the rare alleles clustered around them.

Chandler *et al.* (1987) have proposed that the mutational event at the *Xho* I site is linked to duplication events involving the 28 bp sequence within the VTR by which the four common alleles arose from a single ancestral gene with the *Xho* I site being either differentially lost or gained in two of the four alleles during this process. Unusual allelic variants of Ha-*ras* have subsequently arisen by small increases or decreases in the number of the 28 bp tandem repeats. A recent report by Jeffreys *et al.* (1988), which demonstrated that the spontaneous mutation rate at extremely variable human minisatellite regions is as high as 5% per gamete, provides support for the existence of mechanisms capable of generating these changes. Our results provide evidence that the presence or absence of the *Xho* I site has been faithfully transmitted during the generation of these unusual alleles. A mechanism such as this could also account for the observed association of the *Taq* I sites internal to the VTR with only the 2·65 kb common allele and its variants reported by Radice *et al.* (1987).

Amongst the informative heterozygotes in this study, only one sample, derived from a malignant melanoma patient, displayed decreased intensity of an allelic band demonstrating partial loss of this Ha-*ras* allele. The generation of homozygosity has been observed in other malignancies such as breast, colon and lung but rarely in leukaemia (Krontiris *et al.* 1985; Yokota *et al.* 1986).

Some evidence exists that the length of the VTR region may be important in the regulation of expression of the Ha-*ras* gene. Subclones of EJ-*ras* lacking the VTR region show reduced expression (Krontiris *et al.* 1985). Ishii *et al.* (1986) provided evidence that the VTR is an enhancer of the Ha-*ras* gene and that different conformations of this region vary in enhancing activity. While no direct evidence is available, it is possible that cancer susceptibility may be linked to certain numbers of 28 bp repeats, with these rare alleles specifically causing enhanced expression of the Ha-*ras* gene and consequent cellular transformation. The results obtained here employing linkage to an *Xho* I site suggest that rare alleles appear to arise specifically from individual common alleles by relatively small changes in the number of 28 bp repeats.

We wish to thank Dr Esther Chang for providing the pEJ clone and the Department of Pathology, Royal Brisbane Hospital, for blood and tumour samples. This work was supported by grants from the Australian National Health and Medical Research Council, the Queensland Cancer Fund and the University of Queensland Cancer Research Fund.

References

Capon, D. J., Chen, E. Y., Levinson, A. D., Seeburg, P. H. & Goeddel, D. V. (1983). Complete nucleotide sequences of the T24 human bladder carcinoma oncogene and its normal homologue. *Nature* **302**, 33–37.

Carter, G., Worwood, M. & Jacobs, A. (1988). The Ha-*ras* polymorphism in sporadic and familial myelodysplasia. *Blood* **70**, Suppl. 1, 276a.

Ceccherini-Nelli, L., De Re, V., Veil, A., Molaro, G., Zilli, L., Clemente, L. & Boiocchi, M. (1987). Ha-*ras* restriction fragment length polymorphism and susceptibility to colon adenocarcinoma. *British Journal of Cancer* **56**, 1–5.

Chandler, L. A., Ghazi, H., Jones, P. A., Boukamp, P. & Fusenig, N. E. (1987). Allele specific methylation of the human c-Ha-*ras*-1 gene. *Cell* **50**, 711–717.

Chang, E. H., Furth, M., Scolnick, E. & Lowy, D. (1982). Tumorigenic transformaton of mammalian cells induced by a normal human gene homologous to the oncogene of Harvey murine sarcoma virus. *Nature* **297**, 479–483.

Cohen, J. B., Walter, M. V. & Levinson, A. D. (1987). A repetitive sequence element 3′ of the human c-Ha-*ras*-1 gene has enhancer activity. *Journal of Cellular Physiology*, Suppl. **5**, 75–81.

Damante, G., Filetti, S. & Rapoport, B. (1987). Nucleotide sequence and characterization of the 5′ flanking region of the rat Ha-*ras* proto-oncogene. *Proceedings of the National Academy of Science, U.S.A.* **84**, 774–778.

Der, C. J., Krontiris, T. G. & Cooper, G. M. (1982). Transforming genes of human bladder and lung carcinoma cell lines are homologous to the *ras* genes of Harvey and Kirsten sarcoma viruses. *Proceedings of the National Academy of Science, U.S.A.* **79**, 3637–3640.

Eva, A., Tronick, S. R., Gol, R. A., Pierce, J. H. & Aaronson, S. A. (1983). Transforming genes of human hemopoietic tumors: frequent detection of *ras* related oncogenes whose activation appears to be independent of tumor phenotype. *Proceedings of the National Academy of Science, U.S.A.* **80**, 4926–4938.

Gerhard, D. S., Dracopoli, N. C., Bale, S. J., Houghton, A. N., Watkins, P., Payne, C. E., Green, M. H. & Housman, D. E. (1987). Evidence against Ha-*ras*-1 involvement in sporadic and familial melanoma. *Nature* **325**, 73–75.

Hand, P. H., Vilasi, V., Thor, A., Ohuchi, N. & Schlom, J. (1987). Quantitation of Harvey *ras* p21 enhanced expression in human breast and colon carcinomas. *Journal of the National Cancer Institute* **79**, 59–65.

Hayward, N. K., Keegan, R., Nancarrow, D. J., Little, M. H., Smith, P. J., Gardiner, R. A., Seymour, G. J., Kidson, C. & Lavin, M. F. (1988). c-Ha-*ras*-1 alleles in bladder cancer, Wilms' tumour and malignant melanoma. *Human Genetics* **78**, 115–120.

Heighway, J., Thatcher, N., Cerny, T. & Haselton, P. S. (1986). Genetic predisposition to human lung cancer. *British Journal of Cancer* **53**, 453–457.

Ishii, S., Merlino, G. T. & Pastan, I. (1985). Promoter region of the human Harvey *ras* proto-oncogene: similarity to the EGF receptor promoter. *Science* **230**, 1378–1382.

Ishii, S., Nagase, T. & Imamoto, F. (1986). *Second Annual Meeting on Oncogenes*, pp. 111. Hood College, Frederic, Maryland.

Ishikawa, J., Maeda, S., Takahashi, R., Kamidono, S. & Sugiyama, T. (1987). Lack of correlation between Ha-*ras* alleles and urothelial cancer in Japan. *International Journal of Cancer* **40**, 474–478.

Jeffreys, A. J., Royle, N. J., Wilson, V. & Wong, Z. (1988). Spontaneous mutation rates to new length alleles at

tandem repetitive hypervariable loci in human DNA. *Nature* **332**, 278–281.

Krontiris, T. G. & Cooper, G. M. (1981). Transforming activity of human tumor DNAs. *Proceedings of the National Academy of Science, U.S.A.* **78**, 1181–1184.

Krontiris, T. G., DiMartino, N. A., Colb, M. & Parkinson, D. R. (1985). Unique allelic restriction fragments of the human Ha-*ras* locus in leukocyte and tumor DNAs of cancer patients. *Nature* **313**, 369–374.

Krontiris, T. G., DiMartino, N. A., Colb, M., Mitcheson, H. D. & Parkinson, D. R. (1986). Human restriction fragment length polymorphisms and cancer risk assessment. *Journal of Cellular Biochemistry* **30**, 319–329.

Lidereau, R., Escot, C., Theillet, C., Champeme, M. H., Brunet, M., Gest, J. & Callahan, R. (1986). High frequency of rare alleles of the human c-Ha-*ras*-1 proto-oncogene in breast cancer patients. *Journal of the National Cancer Institute* **77**, 697–701.

Perucho, M., Goldfarb, M., Shimizu, K., Lama, C., Fogh, J. & Wigler, M. (1981). Human tumor derived cell lines contain common and different transforming genes. *Cell* **27**, 467–476.

Rabinowe, S. N. & Krontiris, T. G. (1987). Enhancement of human c-Ha-*ras*-1 transcription by the downstream variable tandem repeat (VTR). *Blood* **68**, Suppl. 1, 262a.

Radice, P., Pierotti, M. A., Borrello, M. G., Illeni, M. T., Rovini, D. & Della Porta, G. (1987). HRAS1 proto-oncogene polymorphisms in human malignant melanoma: *Taq* I defined alleles significantly associated with the disease. *Oncogene* **2**, 91–95.

Reddy, E. P., Reynolds, R. K., Santos, E. & Barbacid, M. (1982). A point mutation is responsible for the acquisition of transforming properties by the T24 human bladder carcinoma oncogene. *Nature* **300**, 149–152.

Shih, C., Padhy, L. C., Murray, M. & Weinberg, R. A. (1981). Transforming genes of carcinomas and neuroblastomas introduced into mouse fibroblasts. *Nature* **290**, 261–264.

Shih, C. & Weinberg, R. A. (1982). Isolation of a transforming sequence from a human bladder carcinoma cell line. *Cell* **29**, 161–169.

Shimizu, K., Birnbaum, D., Ruley, M. A., Fasano, O., Suard, Y., Edlund, L., Taparowsky, E., Goldfarb, M. & Wigler, M. (1983). Structure of the Ki-*ras* gene of the human lung carcinoma cell line Calu-1. *Nature* **304**, 497–500.

Southern, E. M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology* **98**, 503–517.

Spandidos, D. A. & Ker, I. B. (1984). Elevated expression of the human *ras* oncongene family in premalignant and malignant tumors of the colorectum. *British Journal of Cancer* **49**, 681–688.

Stacey, D. W. & Kung, H. F. (1984). Transformation of NIH 3T3 cells by microinjection of Ha-*ras* p21 protein. *Nature* **310**, 508–511.

Tabin, C. J., Bradley, S. M., Bargmann, C. I., Weinberg, R. A., Papageorge, A. G., Scolnick, E. M., Dhar, R., Lowy, D. R. & Chang, E. H. (1982). Mechanism of activation of a human oncogene. *Nature* **300**, 143–149.

Tanaka, T., Slamon, D. J., Battifora, H. & Cline, M. J. (1986). Expression of p21 *ras* oncoproteins in human cancers. *Cancer Research* **46**, 1465–1470.

Thein, S. L., Oscier, D. G., Flint, J. & Wainscoat, J. S. (1986). Ha-*ras* hypervariable alleles in myelodysplasia. *Nature* **321**, 84–85.

Weeks, D. P., Beerman, N. & Griffith, O. M. (1986). A small scale, five hour procedure for isolating multiple samples of CsCl purified DNA: application to isolations from mammalian, insect, higher plant, algal, yeast, and bacterial sources. *Analytical Biochemistry* **152**, 376–385.

Yokota, J., Tsunetsugu-Yokota, Y., Battifora, H., Le Fevre, C. & Cline, M. J. (1986). Alterations of *myc*, *myb*, and Ha-*ras* proto-oncogenes in cancers are frequent and show clinical correlation. *Science* **231**, 261–265.