ANTICANCER RESEARCH 7: 991-996 (1987)

Oncogene Activation in Malignant Transformation: A Study of H-*Ras* in Human Breast Cancer

DEMETRIOS A. SPANDIDOS

The Beatson Institute for Cancer Research, Garscube Estate, Switchback Road, Bearsden, Glasgow, G61 1BD, Scotland, UK

ABSTRACT Two types of oncogene activation can occur in tumors. Quantitative changes have been demonstrated in a variety of animal and human tumors using molecular hybridization and immunohistochemical analyses. Qualitative changes in oncogene expression have also been found in a variety of tumors. In this study the existence of point mutations and other genetic changes in the H-ras1 gene in human breast tumors is demonstrated using oligonucleotide hybridization analysis. The role of quantitative and qualitative changes in oncogene expression in the multistep process of cancer is evaluated in view of these new findings.

INTRODUCTION

Functional analysis of transforming genes in vitro has indicated that only a limited range of mutations result in activation of these genes (1). Point mutations of ras gene family coding sequences have been detected both early and late in the development of cancer (2,3). Since these mutations affect the transforming properties of ras genes as monitored by the NIH3T3 cell focus assay, it is assumed that they play an important role in cancer. Moreover, it has been demonstrated that overexpression of oncogenes may in some cases contribute significantly to the development of the malignant phenotype. Thus, overexpression of normal ras can trigger immortalization (4) or tumorigenic conversion (4,5) of cells in vitro. Using molecular hybridization (6-8) or immunohistochemical methods (9,10) a variety of human benign and malignant tumors were found to express oncogene transcripts or products at higher levels than comparable normal tissues. Moreover, in some tumors or tumor derived cell lines multiple events affecting both quantitative and qualitative aspects of oncogene expression have taken place (11,12).

Although a single gene has been demonstrated to be capable of directly triggering malignant conversion of normal cells both in vitro (4) and in vivo (13) it has been shown in some cases at least that chromosomal instability is a secondary result (4,14) and that transcriptional activation of endogenous oncogenes in the transformed cells may be be induced (15).

In the present study the mutational activation of the H-ras1 gene in breast tumors has been examined using hybridization with synthetic oligonucleotide probes and this is discussed in relation to previous data on the expression of H-ras1 gene in these tumors and clinical studies.

MATERIALS AND METHODS

All biopsy material was obtained from female patients who were treated for breast cancer at the Hellenic Anticancer Institute, Athens, Greece. The isolation of DNA involved the disruption of cells in guanidine-HCl and centrifugation in a CsCl gradient as previously described (7,16). The DNA was further treated with 50 µg/ml proteinase K overnight, extracted with phenol/chloroform, ethanol precipitated and dissolved in 1mM Tris HCl, 0.1mM EDTA pH 8.0 before digestion with restriction enzymes.

The 20 mer oligonucleotides specific for the normal and T24 mutant H-ras1 genes were purchased from the MRC Institute of Virology, Glasgow. The oligonucleotides were end-labelled using γ-^{32}P-ATP (5000 Ci/mmol, Amersham, UK) and T4 polynucleotide kinase.

Key Words: Oncogene activation, malignant transformation, H-*ras*, human breast cancer.

This paper was presented at the 1st International Workshop *New Perspectives in Cancer Research*, 15-18 October 1987, Chalkis, Greece.

0250-7005/87 $2.00+.40

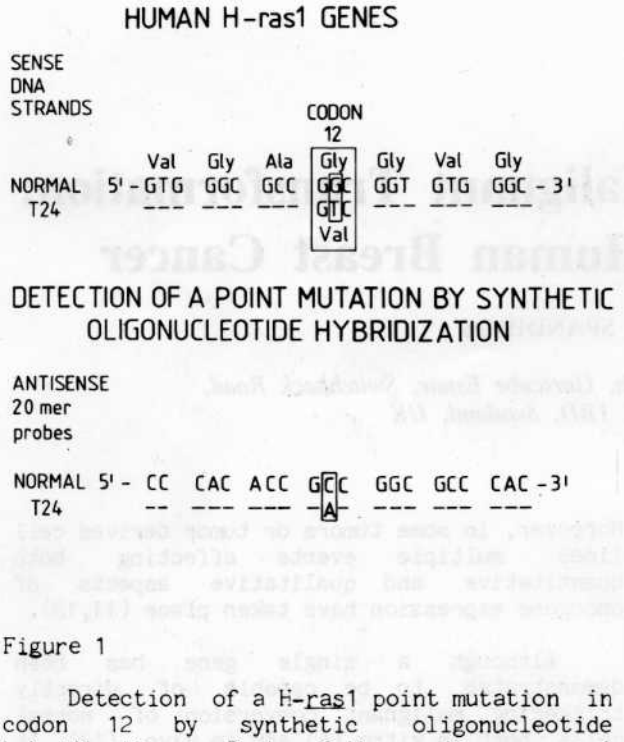

Figure 1

Detection of a H-ras1 point mutation in codon 12 by synthetic oligonucleotide hybridization. Part of the coding exon 1 around codon 12 of the normal or T24 mutant H-ras1 gene is shown in the upper part and the antisense 20 mer probes used in this study in the lower part of the figure.

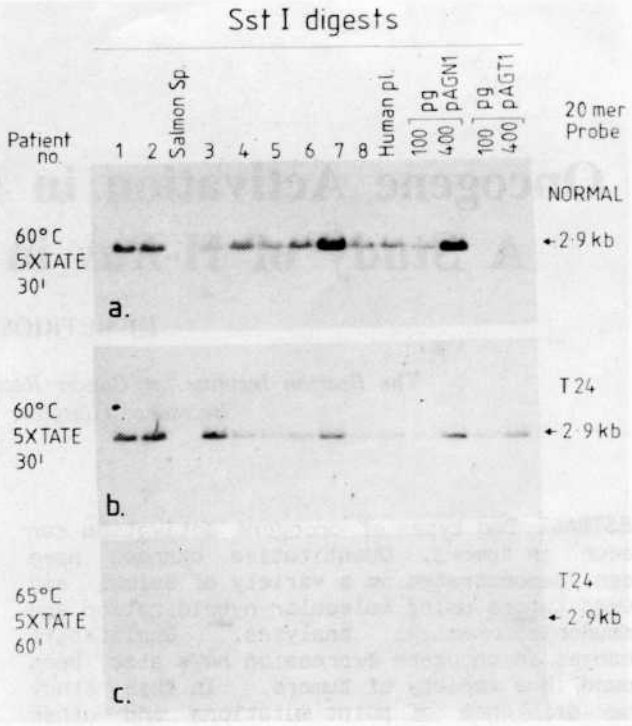

Figure 2

Detection of normal and T24 mutant c-H-ras1 gene in breast tumors by gel hybridization assay. Autoradiographs of dried gels containing DNA isolated from breast tumors are shown. The DNA was digested with SstI and hybridized to the ^{32}P-labelled synthetic oligomer probe indicated. The normal c-H-ras1 codon 12 oligomer recognizing glycine (gly) in (a) and the T24 mutant specific oligomer recognizing valine (val) in b and c. The 2.9 Kb SstI fragment contains the exon coding for amino acid 12 of the c-H-ras1 gene. Conditions for washing the probe are described in Materials and Methods and are indicated at the side of the autoradiographs.

Agarose gel electrophoresis and direct-gel hybridization were carried out as follows. The DNA digests were electrophoresed in a 0.5 cm thick 0.8% agarose gel. The DNA was denatured *in situ* in 0.5 M NaOH, 1.0 M NaCl (2 x 20 min at room temperature) and neutralized in 0.5 M Tris HCl pH 7.5, 1.0 M NaCl (2 x 20 min at room temperature). Gels were dried on to Whatman 3 MM paper. The dried gel was wetted with distilled water to remove the Whatman 3 MM paper and sealed in a plastic bag for hybridization. Hybridization was performed in 10 ml 5 x SSPE buffer (1 x SSPE = 10 mM sodium phosphate pH 7.0, 0.18 M NaCl and 1 mM EDTA) containing 0.3% sodium dodecylsulfate (SDS) and 10 µg/ml salmon sperm DNA at 55°C for 16 h. Hybridized gels were washed as follows: 1. For the normal oligonucleotide antisense probe: twice at RT with 5 x TATE (5.0 M tetramethyl-ammonium chloride) containing 0.1% SDS for 30 min each wash and once at 60°C with the same buffer for 30 min. 2. For the T24 mutant oligonucleotide antisense probe; twice at RT with 5 x TATE containing 0.1% SDS, once at 60°C for 30 min and once at 65°C for 60 min in the above buffer.

RESULTS

DNA from twenty-four human breast tumors was analyzed with oligunucleotide probes in the present study. An RNA analysis of the expression of the H-ras gene in the same tumors was previously carried out (7). The 20 mer oligunucleotide probes used in these studies are depicted in Fig. 1. The results of *in situ* hybridization with oligonucleotide probes are shown in Figures 2, 3 and 4. As positive controls digests of 100 and 400 pg DNA of plasmids pAGN1 and pAGT1 were used (4).

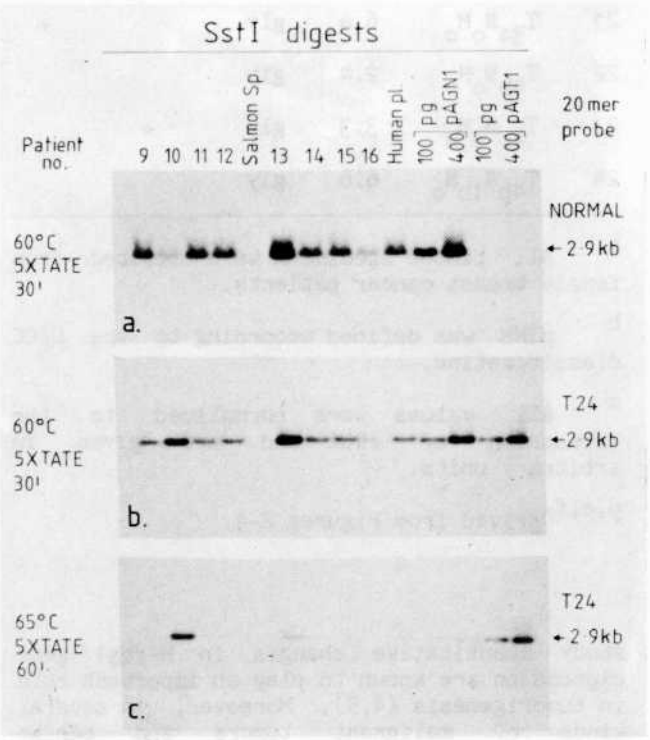

Figure 3

Detection of normal and mutant c-H-ras1 gene in breast tumors by gel hybridization assay. For details see legend of Fig. 2.

These amounts of plasmid DNA are equivalent to approximately 1 and 4 copies of the H-ras1 gene. Plasmid pAGN1 carries the normal and plasmid pAGT1 the T24 mutant H-ras1 gene. Additional controls were the EJ bladder carcinoma cell line carrying only the mutant T24 H-ras1 gene, the human placental DNA carrying two normal H-ras1 alleles and the salmon sperm DNA as negative control. The increased stringency of hybridization at 65°C was required to prevent the non-specific binding of the T24 mutant probe to the normal H-ras1 gene.

As shown in Figures 2-4, only two tumor DNAs (patients Nos 3 and 10) in 24 analyzed turned out to be carrying a mutant H-ras1 gene together with the normal allele. Moreover, amplification of the normal allele (patients Nos 1, 2, 4, 7, 9, 13, 23) or partial loss of one of the normal alleles (patients Nos 16, 21) as judged from the intensity of the 2.9 kb SstI bands, and confirmed by scanning, may have occurred. None of the adjacent normal tissue DNAs had any of these changes (data not shown).

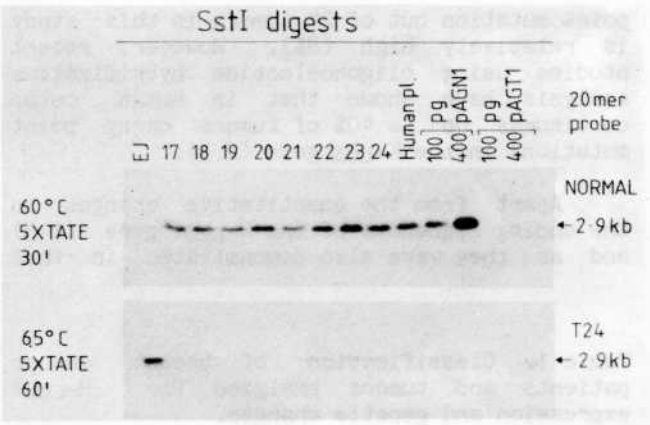

Figure 4

Detection of normal and mutant c-H-ras1 gene in breast tumors by gel hybridization assay. For details see legend of Fig. 2.

A comparison of the data described here on the DNA changes with the data we have reported earlier on the expression of the H-ras and some other properties of the tumors (7,17) is shown in Table 1. These data suggests that no obvious correlation exists between the parameters examined here that is tumor stage, oncogene expression and mutation.

DISCUSSION

Recent results have suggested that genotype analysis of the c-H-ras1 locus in combination with other clinical parameters may be of prognostic value in assessing the potential for cancer. Thus, studies have shown that 50% of the breast tumor patients were found to have rare H-ras1 genotypes (18). Moreover, in parallel studies, although one allele appeared to be lost in 27% of the tumors from heterozygous patients, there was no preference for retention of common alleles over the rare alleles (19).

In the present study we have found a single point mutation in the H-ras1 gene in two human breast tumors and possible H-ras1 amplification or losses in other breast tumors. Although point mutations in the human H-ras1 gene have been previously described in some established cell lines derived from breast tumors (20), this is the first demonstration of the presence of the glycine to valine mutation found in a human breast primary tumor. Since ras gene point mutations have rarely been detected in human breast carcinomas the detection of 2 cases of H-ras

point mutation out of 24 tumors in this study is relatively high (8%). However, recent studies using oligonucleotide hybridization analysis have shown that in human colon carcinomas up to 40% of tumors carry point mutations in the K-ras gene (2,3).

Apart from the quantitative changes in the coding sequences of the H-ras1 gene (1-3) and as they were also demonstrated in this study quantitative changes in H-ras1 gene expression are known to play an important role in tumorigenesis (4,5). Moreover, in several kinds of malignant tumors and benign conditions including human breast tissues the levels of H-ras1 transcripts (6-8) or ras p21 protein (9,10) have been found to be elevated when compared to the normal tissue. Transcriptional regulatory sequences have been found at both the 5' (21) and 3' (22) ends of the H-ras1 gene. A core enhancer consensus sequence TGTGG is also present in the complementary strand of the variable tandem repeat (VTR) at the 3' end of the H-ras1 gene (Fig.5) although this was missed on previous inspection of the sequence because of the arbitrary boundaries chosen to depict the repeated element (22). Moreover, several Sp1 potential binding sites GGGGCG or their inverted complement CGCCCC are located in the 5' positive regulatory element contained within the 0.8 kb SstI DNA fragment (21).

Table 1. Classification of breast cancer patients and tumors analyzed for H-ras1 expression and genetic changes.

Patient No.[a]	pTNM[b]	H-ras1 expression[c]	aa at codon 12[d]	H-ras1 amplification[e]	loss[f]
1	$T_{1a}N_{1a}M_o$	12	gly	+	
2	$T_{1a}N_{1b}M_o$	13	gly	+	
3	$T_{1a}N_{1b}M_o$	5.3	gly,val		
4	$T_{1a}N_{1b}M_o$	9.3	gly	+	
5	$T_{2a}N_oM_o$	1.9	gly		
6	$T_{2a}N_{1a}M_o$	24	gly		
7	$T_{2a}N_{1a}M_o$	3.0	gly	+	
8	$T_{2a}N_{1a}M_o$	3.7	gly		
9	$T_{2a}N_{1a}M_o$	9.1	gly	+	
10	$T_{2a}N_{1b}M_o$	14	gly,val		
11	$T_{2a}N_{1b}M_o$	16	gly		
12	$T_{2a}N_{1b}M_o$	28	gly		
13	$T_{2a}N_{1b}M_o$	7.3	gly	+	
14	$T_{2a}N_{1b}M_o$	7.8	gly		
15	$T_{2a}N_{1b}M_o$	14	gly		
16	$T_{2a}N_{1b}M_o$	8.6	gly		+
17	$T_{2a}N_{1b}M_o$	8.5	gly		
18	$T_{2a}N_2M_o$	15	gly		
19	$T_{3a}N_oM_o$	13	gly		
20	$T_3N_oM_o$	2.3	gly		
21	$T_{3a}N_oM_o$	6.6	gly		+
22	$T_{4b}N_oM_o$	2.4	gly		
23	$T_{4b}N_oM_o$	3.3	gly	+	
24	$T_{4b}N_{1b}M_o$	6.6	gly		

[a] All tissue specimens were obtained from female breast cancer patients.

[b] pTNM was defined according to the UICC classification.

[c] All values were normalized to the expression of rRNA and are given in arbitrary units.

[d,e,f] Derived from Figures 2-4.

The fact that a large majority of human breast tumors express at high levels the H-ras1 gene may be that H-ras gene expression is regulated by other factors than strictly mutations. It has been suggested that hormone-dependency of mammary carcinomas may correlate with quantitative changes in normal p21 protein (23). This proposal is supported by the finding that the level of p21 in DMBA-induced mammary carcinomas in the rat was sharply decreased in tumors regressing upon hormone withdrawal (24).

Our results obtained by analyzing

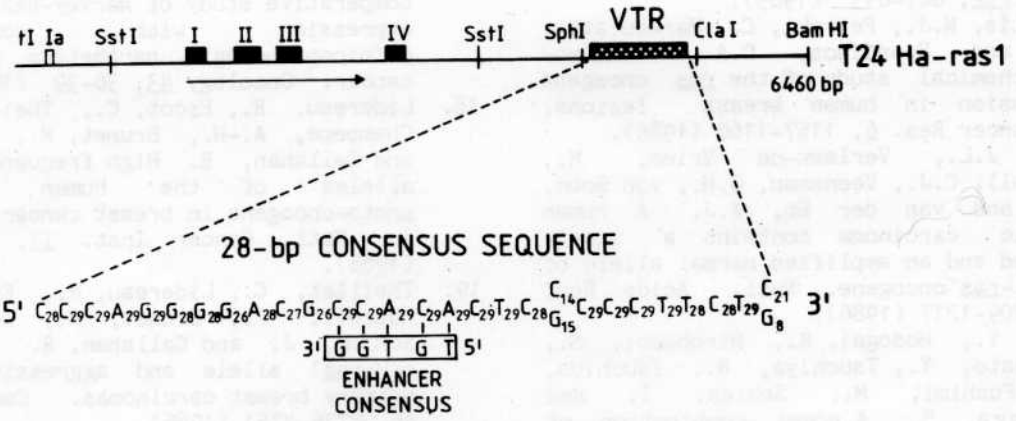

Figure 5

Organization of the human T24 H-ras1 gene, the variable tandem repeat (VTR) region and its enhancer consensus sequence. The coding sequences are represented by black boxes, the non-coding 5'-sequences by an open box and the VTR sequences by a crosshatched box. The direction of transcription is indicated by an arrow.

structural mutations on the H-ras1 gene and expression of this gene studied by molecular hybridization analysis or immunohistochemical methods suggest that there is no correlation between these two events and between tumor stage and H-ras1 activation.

We consider that qualitative and quantitative changes in ras gene expression may be independent events selected during initiation and progression of malignant tumors in vivo. Further studies will be required to assess the relative importance of these changes.

ACKNOWLEDGEMENTS

I would like to thank Dr. N.J. Agnantis of the Hellenic Anticancer Institute, Athens, Greece, for the human breast tissue material used in these studies, Lesley Holmes for end labelling the oligonucleotides and James Neil for critical reading of the manuscript. The Beatson Institute is supported by the Cancer Research Campaign of U.K.

REFERENCES

1. Barbacid, M. ras genes. Ann. Rev. Biochem. (in press).
2. Bos, J.L., Fearon, E.R., Hamilton, S.R., Barlaam-de Vries, M., van Boom, J.H., van der Eb, A. and Vogelstein, M. Prevalence of ras gene mutations in human colorectal cancers. Nature 327, 293-297 (1987).
3. Forrester, K., Almoguera, C., Han, K., Grizzle, W.E. and Perucho, M. Detection of high incidence of K-ras oncogenes during human colon tumorigenesis. Nature 327, 298-303 (1987).
4. Spandidos, D.A. and Wilkie, N.M. Malignant transformation of early passage rodent cells by a single mutated human oncogene. Nature 310, 469-475 (1984).
5. Chang, E.H., Furth, M.E., Scolnick, E.M. and Lowy, D.R. Tumorigenic transformation of mammalian cells induced by a normal gene homologous to the oncogene of Harvey murine sarcoma virus. Nature 297, 479-483 (1982).
6. Spandidos, D.A. and Kerr, I.B. Elevated expression of the human ras oncogene family in premalignant and malignant tumours of the colorectum. Br. J. Cancer 49, 681-688 (1984).
7. Spandidos, D.A. and Agnantis, N.J. Human malignant tumors of the breast as compared to their respective normal tissue have elevated expression of the Harvey ras oncogene. Anticancer Res. 4, 269-272 (1984).
8. Spandidos, D.A., Lamothe, A. and Field, J.N. Multiple transcriptional activation of cellular oncogenes in the human head and neck solid tumors. Anticancer Res. 5, 221-224 (1985).
9. Williams, A.R.W., Piris, J., Spandidos, D.A. and Wyllie, A.H. Immunohistochemical detection of the ras oncogene p21 product in an experimental tumor and in human colorectal neoplasms. Br. J.

Cancer 52, 687-693 (1985).
10. Agnantis, N.J., Petraki, C., Markoulatos, P. and Spandidos, D.A. Immunohistochemical study of the ras oncogene expression in human breast lesions. Anticancer Res. 6, 1157-1160 (1986).
11. Bos, J.L., Verlaan-de Vries, M., Marshall, C.J., Veeneman, G.H., van Boom, J.H. and van der Eb, A.J. A human gastric carcinoma contains a single mutated and an amplified normal allele of the Ki-ras oncogene. Nucl. Acids Res. 14, 1209-1217 (1986).
12. Taya, Y., Hosogai, K., Hirohashi, S., Shimosato, Y., Tsuchiya, R., Tsuchida, N., Fushimi, M., Sekiga, T. and Nishimura, S. A novel combination of K-ras and myc amplification accompanied by point mutational activation of K-ras in a human lung cancer. EMBO J. 3, 2943-2946 (1984).
13. Quaife, C.J., Pinkert, C.A., Ornitz, D.M., Palmiter, R.D. and Brinster, R.L. Pancreatic neoplasia induced by ras expression in acinar cells of transgenic mice. Cell 48, 1023-1034 (1987).
14. Spandidos, D.A., Freshney, M. and Wilkie, N.M. Heterogeneity of cell lines derived after transformation of early passage rodent cells by the Ha-ras1 human oncogene. Anticancer Res. 5, 387-392 (1985).
15. Wyllie, A.H., Rose, K.A., Morris, R.G., Steel, C.M., Foster, E. and Spandidos, D.A. Rodent fibroblast tumors expressing human myc and ras genes: growth, metastasis and endogenous oncogene expression. Br. J. Cancer (in press).
16. Spandidos, D.A. and Wilkie, N.M. Expression of exogenous DNA in mammalian cells. In: In Vitro Transcription and Translation - A Practical Approach (Hames, B.D. and Higgins, S.J., eds.). IRL Press, Oxford, pp. 1-48 (1984).
17. Agnantis, N.J., Parissi, P., Anagnostakis, D. and Spandidos, D.A. Comparative study of Harvey-RAS oncogene expression with conventional clinicopathologic parameters of breast cancer. Oncology 43, 36-39 (1986).
18. Lidereau, R., Escot, C., Theillet, C., Champeme, A.-H., Brunet, M., Gest, J. and Callahan, R. High frequency of rare alleles of the human c-Ha-ras1 proto-oncogene in breast cancer patients. J. Natl. Cancer Inst. 77, 697-701 (1986).
19. Theillet, C., Lidereau, R., Escot, C., Hutzell, P., Brunet, M., Gest, J., Schlom, J. and Callahan, R. Loss of a c-H-ras1 allele and aggressive human primary breast carcinomas. Cancer Res. 46, 4776-4781 (1986).
20. Kraus, M.H., Yuasa, Y. and Aaronson, S.A. A position 12-activated H-ras oncogene in all HS578T mammary carcinosarcoma cells but not normal mammary cells of the same patient. Proc. Natl. Acad. Sci. USA 81, 5384-5389 (1984).
21. Spandidos, D.A. and Riggio, M. Promoter and enhancer like activity at the 5' end of normal and T24 Haras1 genes. FEBS Letters 203, 169-174 (1986).
22. Spandidos, D.A. and Holmes, L. Transcriptional enhancer activity in the variable tandem repeat DNA sequence downstream of the human Ha-ras1 gene. FEBS Letters 218, 41-46 (1987).
23. De Bortoli, M.E., Abou-Issa, H., Haley, B.E. and Cho-Chung, Y.S. Amplified expression of p21 ras protein in hormone-dependent mammary carcinomas of humans and rodents. Bioch. Bioph. Res. Comm. 127, 699-706 (1985).
24. Huang, F.S. and Cho-Chung. Hormone-regulated expression of cellular ras^{H} oncogene in mammary carcinomas in rats. Biochem. Biophys. Res. Commun. 123, 141-147 (1984).

Received August 7, 1987
Accepted September 4, 1987