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Transforming p21 *ras* protein: flexibility in the major variable region linking the catalytic and membrane-anchoring domains

Berthe M.Willumsen, Alex G.Papageorge[1], Nancy Hubbert[1], Eva Bekesi[2], Hsiang-Fu Kung[2] and Douglas R.Lowy[1]

University of Copenhagen, Institute of Microbiology, Oster Farimagsgade 2A, DK 1353 Copenhagen, Denmark, [1]Laboratory of Cellular Oncology, Building 37, Room 1B-26, National Cancer Institute, Bethesda, MD 20205, and [2]Department of Molecular Genetics, Hoffman-La Roche, Roche Research Center, Nutley, NJ 07110, USA

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The mammalian p21 *ras* proteins contain a 20-amino acid region that is highly divergent, in contrast to the strong sequence conservation that is common to other regions of these proteins. This major variable region is located near the C terminus just upstream from a conserved cysteine residue that is required for post-translational processing, membrane localization and transforming activity of the proteins. We have now used the viral oncogene (v-*ras*^{H}) of Harvey sarcoma virus to study the major variable region by deleting or duplicating parts of the gene. Reducing this region to five amino acids or increasing it to 50 amino acids has relatively little effect on the capacity of the gene to induce morphological transformation of NIH 3T3 cells. Assays of GTP binding, GTPase and autophosphorylating activities of such mutant v-*ras*^{H}-encoded proteins synthesized in bacteria indicated that the sequences that encode these biochemical activities are located upstream from the major variable region. In the context of transformation, we propose that the region of sequence heterogeneity serves principally to connect the N-terminal catalytic domain with amino acids at the C terminus that are required to anchor the protein in the membrane.

Key words: ras genes/cellular transformation/mutagenesis

Introduction

The *ras* family forms a group of closely related transforming genes that are highly conserved in evolution (Ellis *et al.*, 1982; Taparowsky *et al.*, 1983; Kataoka *et al.*, 1985; DeFeo-Jones *et al.*, 1985; Neuman-Silberberg *et al.*, 1984; Reymond *et al.*, 1984). The first members of this family to be identified were the viral (v) oncogenes v-*ras*^{H} and v-*ras*^{K} Harvey murine sarcoma virus (Ha-MuSV) and Kirsten MuSV, respectively (Ellis *et al.*, 1982). Activated forms of cellular (c) *ras* genes which efficiently induce tumorigenic transformation of NIH 3T3 cells, as do v-*ras* genes, have been isolated from tumors of many species, including humans and rodents. These genes have been activated as the result of specific point mutations in their protein-coding sequences, although elevated levels of a normal mammalian *ras* gene product can also transform NIH 3T3 cells (Taparowsky *et al.*, 1983; Shih and Weeks, 1984; Land *et al.*, 1983; Sukumar *et al.*, 1983; Yuasa *et al.*, 1984; Chang *et al.*, 1982).

The proteins encoded by *ras* genes share many structural, biochemical and functional properties. These include a similar size (189 amino acids) for the three known mammalian *ras* genes (*ras*^{H}, *ras*^{K}, *ras*^{N}) (Taparowsky *et al.*, 1983; Shih and Weeks, 1984; Dhar *et al.*, 1982; Tsuchida *et al.*, 1982; Capon *et al.*, 1983; Shimizu *et al.*, 1983), the tight binding of lipid to the mature protein, its localization on the inner surface of the plasma membrane (Shih *et al.*, 1982; Sefton *et al.*, 1982; Willingham *et al.*, 1980) and the capacity to bind guanine nucleotides as well as to hydrolyze GTP (Papageorge *et al.*, 1982; Gibbs *et al.*, 1984; McGrath *et al.*, 1984; Sweet *et al.*, 1984; Manne *et al.*, 1985).

Some p21 functions have begun to be assigned to particular domains of the protein. The point mutations that are known to enhance the transforming activity of the protein have all been localized to the N-terminal domain (at amino acids 12, 13, 59, 61 and 63) (Taparowsky *et al.*, 1983; Shih and Weeks, 1984; Sukumar *et al.*, 1983; Yuasa *et al.*, 1984; Capon *et al.*, 1983; Shimizu *et al.*, 1983; Fasano *et al.*, 1984; Tabin *et al.*, 1982); where examined, such activating mutations have been associated with a decreased GTPase activity of the protein (Gibbs *et al.*, 1984; McGrath *et al.*, 1984; Sweet *et al.*, 1984; Manne *et al.*, 1985). The extreme C terminus of the protein, specifically cysteine 186 that is found in all p21 proteins, is required for transforming activity, lipid binding and membrane localization of the protein (Willumsen *et al.*, 1984a, 1984b).

The mammalian *ras* proteins contain near their C terminus a highly divergent region of ~20 amino acids (amino acids 165-184) whose function is unknown. When the three human c-*ras* genes are compared, their almost complete lack of sequence homology within this region contrasts strikingly with their 90% homology found in the p21 coding sequences outside this segment (Taparowsky *et al.*, 1983; Shih and Weeks, 1984). This region also accounts for the major sequence heterogeneity found among the *ras* proteins of non-vertebrates (Kataoka *et al.*, 1985; DeFeo-Jones *et al.*, 1985; Neuman-Silberberg *et al.*, 1984; Reymond *et al.*, 1984; Dhar *et al.*, 1984; Powers *et al.*, 1984).

We have now carried out structure-function experiments on the sequence heterogeneity segment of the v-*ras*^{H} gene. This gene encodes a p21 protein that is identical to c-*ras*^{H} except that the viral gene carries two activating mutations in the N-terminal domain (arginine 12 and threonine 59) (Fasano *et al.*, 1984; P.E.Tambourin and D.R.Lowy, unpublished data). In addition to possessing the shared properties of activated *ras* proteins noted above, the v-*ras*^{H}-encoded protein is specifically phosphorylated at threonine 59 *in vivo* and can be autophosphorylated *in vitro* (Shih and Weeks, 1984; Papageorge *et al.*, 1982; Gibbs *et al.*, 1984). Our data indicate that most or all of the 20 amino acids within this region can be deleted or duplicated without greatly affecting the biological activity of the protein. They also genetically localize the GTP-binding domain, GTPase activity and autophosphorylating activity to sequences upstream from this variable region. These results have enabled us to formulate a model that assigns three domains to the p21 protein.

Results

In vitro transforming activity of deletion mutants

We first studied the biological consequences of deleting sequences

Table I. Transforming activity of deletion mutants

Mutant number	Amino acid structure[a]	Number of amino acids	Focus formation
Full-length			
pBW601	1–189	189	Yes[c]
Deletion mutants			
pBW1097	1–155 LIR 166–189	182	No
pBW1096	1–155 LIR 169–189	179	No
pBW1095	1–155 LIS 178–189	170	No
pBW732	1–163 PDQ 175–189	181	Yes
pBW758	1–172 PDQ 176–189	189	Yes
pBW739	1–165 PDQ 180–189	178	Yes
pBW757	1–172 PDQ 180–189	185	Yes
pBW766	1–165 PDQ 184–189	174	Yes
pBW754	1–165 PDQ 187–189	171	No
pBW756	1–172 PDQ 187–189	178	No
Control for 1–155 front end			
pBW1098	1–155 LIR 154–189	194	Yes[b]

[a]The numbers indicate the v–*ras*^{H} amino acids in front and tail ends, respectively; the letters indicate the three amino acids encoded by the oligonucleotide linker joining the front and tail ends.
[b]pBW1098 induced 5% as many foci as did the full-length v-*ras*^{H} gene; the pBW1098-induced foci were more compact and thicker than wild-type foci. Cells transformed by pBW1098 did not tend to float into the culture medium; they appeared to be more adherent to each other and formed larger colonies in soft agar than did wild-type transformants.
[c]Yes = $1-1.5 \times 10^3$ foci/μg DNA.

within, upstream from and downstream from the heterogeneous region (amino acids 165–184). We have previously noted that mutants from which amino acids 166–183 have been deleted retained their transforming activity (Table I; pBW757, 739, 766). These results suggested that a region located just upstream from the C terminus of the protein is dispensable for transformation.

We defined the left-hand boundary of this region by making mutants whose deletions began upstream from amino acid 165. A mutant from which residues 164–174 have been deleted (pBW732) retained its transforming activity. However, three mutants whose deletions began at residue 156 and extended rightward (pBW1095, 1096 and 1097) were each transformation-defective. These results suggest that the left-hand boundary of the dispensable region lies between amino acids 156 and 163.

The failure of mutants 1095, 1096 and 1097 to transform the NIH 3T3 cells was not due to an inherent defectiveness of either the N terminus (front end) or C termini (tail ends) used to generate these mutants. The tail ends in these three defective mutants have each served as tail ends for transformation-competent mutants (such as pBW1010, 1011 and 1012; see Table III); the front end used for 1095, 1096 and 1097 also served as the front end for the transformation-competent (duplication) mutant pBW1098 (Table I).

Earlier results had localized the right-hand boundary of the dispensable region to amino acids 183–185, since we had previously observed that pBW766, from which amino acids 166–183 had been deleted, transformed the NIH 3T3 cells as efficiently as did the wild-type gene and that Cys 186 was absolutely required for transformation (Willumsen *et al.*, 1984a, 1984b).

Biochemical activities of bacterially synthesized mutant proteins

We tested the biochemical activities of the p21 proteins encoded by representative mutants. In addition to analyzing mutants from which sequences in the major variable region had been deleted, we also studied mutants with changes in the C-terminal codons downstream from this region. The biochemical activities were

Table II. Biochemical activities of mutant protein

Protein	Structure[c]	GDP binding pmol/μg p21	GTPase pmol/h	Autophosphorylation	Focus formation[d]
c-*ras* p21[a]		10	20	N.A.	Yes
v-*ras* p21					
pBW601	1–189	31	<2	+++	Yes
pBW739	1–165 PDQ 180–189	12	<2	++	Yes
pBW754	1–165 PDQ 187–189	10	<2	++	Yes
pBW858[b]	1–185 S 187–189	12	<2	++	No
pBW277[b]	1–186 TP	30	<2	+++	No

[a]Manne *et al.*, (1985); c-*ras* encodes glycine at codon 12 and alanine at codon 59.
[b]Willumsen *et al.* (1984b).
[c]Structure as given in Table I.
[d]The transformation-competent v-*ras*^{H} clones induced $1-1.5 \times 10^3$ foci/μg DNA. The normal c-*ras*^{H} gene, when promoted by the Ha-MuSV LTR, induced $1-3 \times 10^2$ foci/μg DNA.

Table III. Transforming activity of duplication mutants

Mutant number	Amino acid structure[b]	Number of amino acids	Focus formation
pBW972	1–172 PDQ 172–189	193	Yes[a] (m)
pBW974	1–172 PDQ 162–189	203	Yes (m)
pBW975	1–172 PDQ 152–189	213	Yes (m)
pBW976	1–172 PDQ 146–189	219	Yes (m)
pBW1079	1–172 PDQ 139–189	226	No
pBW1080	1–172 PDQ 130–189	235	No
pBW1082	1–172 PDQ 109–189	256	No
pBW1085	1–172 PDQ 77–189	288	No
pBW1086	1–172 PDQ 57–189	308	No
pBW1087	1–172 PDQ 44–189	321	No
pBW1010	1–176 LIS 178–189	191	Yes (h)
pBW1011	1–176 LIR 169–189	200	Yes (m)
pBW1012	1–176 LIR 166–189	203	Yes (m)
pBW964	1–179 LIS 178–189	194	Yes (h)
pBW966	1–179 LIR 169–189	203	Yes (m)
pBW994	1–179 LIR 166–189	206	Yes (m)
pBW995	1–179 LIR 154–189	218	Yes (m)
pBW673	1–180 SDQ 172–189	202	Yes (l)
pBW1093	1–184 PDQ 180–189	197	Yes (h)
pBW1092	1–184 PDQ 184–189	193	Yes (h)

[a]Transforming efficiency: h = $1-1.5 \times 10^3$ foci/μg DNA; m = $0.5-5 \times 10^2$ foci/μg DNA; l = $0.5-5 \times 10^1$ foci/μg DNA.
[b]Structure as given in Table I.

determined for mutant p21 proteins synthesized in *Escherichia coli*. As seen in Table II, the GTP binding, GTPase and autophosphorylating activities were similar to those of the parental protein regardless of which sequence downstream from 165 had been deleted. This was true even for the transformation-defective mutants tested, those with lesions involving amino acids 184–189. We conclude that the amino acids responsible for these activities lie to the N-terminal side of amino acid 165.

Transforming activity and p21 synthesis by duplication mutants

Since the results obtained with the deletion mutants indicated that a net deletion of even 18 amino acids from the heterogeneous region might not significantly impair the transforming activity of v-*ras*^{H}, we also determined if insertion of additional amino acid residues in this region would interfere with the transforming activity of the protein. Our approach was to make duplication mutants by recombining tail ends of varying length with a front end that extended into the dispensable region. Duplication of v-

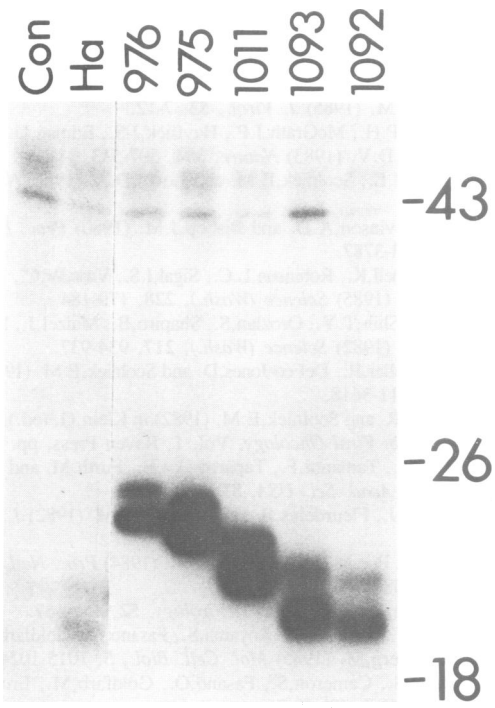

Fig. 1. Immunoprecipitation of duplication mutants. Morphologically transformed NIH 3T3 cells were labeled overnight with methionine and immunoprecipitated. Con = control cells; Ha = full-length viral p21. The numbers on the right of each panel indicate (in kd) the location of protein standards run in adjacent lanes.

ras^{H} sequences in this manner did not abolish the transforming activity of the gene (Table III). Those duplications encoding proteins that were 10–40 amino acids longer than wild-type v-*ras*^{H} generally possessed a modest (one order of magnitude) reduction in transforming efficiency. Mutants with larger duplications did not induce foci. The smaller duplication mutants that induced transformation encoded v-*ras*^{H} doublets whose migration rate corresponded with that expected for the duplication (Figure 1).

Discussion

These results indicate the existence of a relatively large region within the v-*ras*^{H} product whose size and sequence play only a minor role in the capacity of the activated p21 protein to induce cellular transformation of NIH 3T3 cells. Our data are relevant to the mammalian c-*ras*^{H} gene, which is identical to v-*ras*^{H} in this region and probably also apply to other *ras* genes. Based on results obtained with mutants carrying overlapping deletions, this dispensable region in v-*ras*^{H} is ~20 amino acids in length. The lefthand boundary of this region is located between amino acids 156 and 163. The righthand boundary is located between amino acids 183 and 185. From analysis of the GTP binding, the GTPase and autophosphorylating activities of mutant proteins synthesized in *E. coli*, it is clear that amino acids to the right of amino acid 165 do not participate directly in carrying out these biochemical functions of the protein. Similar conclusions regarding these activities have been reached independently by others (Gross *et al.*, 1985; Temeles *et al.*, 1985).

This region that is dispensable for the transforming function of the viral protein corresponds quite closely to the major region of sequence heterogeneity (amino acids 165–184) found among *ras* genes. Although the amino acids within this region are not

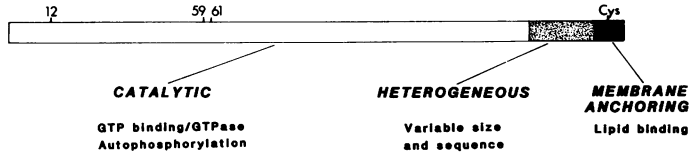

Fig. 2. Functional map of *ras* protein. As discussed in the text, activation mutations (indicated here by amino acids 12, 59 and 61) have been found in the catalytic domain. The cysteine residue required for post-translational processing and membrane localization is also shown.

conserved among different *ras* genes, its size is maintained (Taparowsky *et al.*, 1983; Shih and Weeks, 1984). The two yeast *ras* genes that have been sequenced are exceptional in being significantly longer, with coding capacities of 309 and 322 amino acids (Dhar *et al.*, 1984; Powers *et al.*, 1984). Their much larger size results almost entirely from their heterogeneous regions being 112 and 125 amino acids longer, respectively, than this region in mammalian *ras* genes.

We have found that increasing the length of the heterogeneous region of v-*ras*^{H} had only a modest effect on the transforming activity of the protein, even when the length was more than doubled. However, there does appear to be some constraint on the structure of this region, since mutants with duplications larger than 30 amino acids failed to transform the cells.

The relative unimportance of the precise sequence of this heterogeneous region to morphologic transformation has also been noted in a mammalian-*Drosophila ras* chimeric gene (Schejter and Shilo, 1985). In addition, mammalian *ras* genes (carrying their relatively short heterogeneous regions) can substitute functionally in yeast for disrupted yeast *ras* genes (Kataoka *et al.*, 1985; DeFeo-Jones *et al.*, 1985). On the other hand, our negative results obtained with very large duplications suggest that large heterogeneous regions may not function efficiently in mammalian cells. Analogous results have been obtained with a yeast *ras* gene. Although a full-length yeast *ras* gene that carried an activating mutation was non-transforming for NIH 3T3 cells, deletion of 117 amino acids from the heterogeneous region enabled this yeast gene to transform the mouse cells (DeFeo-Jones *et al.*, 1985).

The results obtained here and with mutants involving other regions of the *ras* genes have prompted us to consider a preliminary model of the p21 protein that currently contains three domains: N–catalytic domain–heterogeneous domain–membrane binding domain–C (Figure 2). We propose that the extreme C terminus of the p21 protein is necessary and sufficient to anchor the protein on the plasma membrane, in contrast to the pp60^{src} protein, which localizes to the plasma membrane via myristic acid at its N terminus (Schultz *et al.*, 1985; Buss and Sefton, 1985). In this model, the binding of lipid (probably palmitic acid) to the C-terminal domain, perhaps through Cys-186, is required for the migration of the protein from its synthesis in the cytosol to the plasma membrane. Palmitic acid binding to cysteine residues has been suggested as a common feature of membrane proteins of eucaryotes (Bolanowsky *et al.*, 1984).

The heterogeneous region may subserve a specific function in the physiological role of p21 proteins. Such a function is implied by the conservation of these sequences in c-*ras*^{H} between rodents and humans and by the conservation of its size among the different mammalian *ras* genes. However, its size and primary amino acid sequence appear to be relatively unimportant for the pathological process of cellular transformation. We suggest that in p21-mediated cell transformation the heterogeneous domain

serves primarily to connect the N-terminal catalytic domain with the C-terminal membrane-binding domain.

The freedom of the heterogeneous region to vary in size by > 40 amino acids without markedly impairing the transforming function of the protein suggests that the catalytic domain does not need to be in a precise orientation with respect to the C terminus. The requirement of membrane association for morphological transformation implies that at least some of the molecules with which the protein interacts are located at or near the membrane. Since these interactions are able to take place despite a grossly altered region between the catalytic and membrane anchor domains, we speculate either that other portions of the protein orient it in the membrane or that the interactions occur stochastically because of the fluid nature of the membranes.

Materials and methods

Construction of Ha-MuSV deletion and duplication mutants

We have previously described the generation of in-frame v-*ras*^{H} mutants by deletion and linker insertion mutagenesis of an Ha-MuSV DNA containing plasmid that can induce focal transformation of NIH 3T3 cells (Willumsen *et al.*, 1984a). In summary, the mutants are constructed by the combination of two sequenced parts of v-*ras*^{H} through a *Bcl*I linker. One segment contains an intact N-terminal domain of varying length that has been derived from a series of *Bal*31-generated deletion mutants whose v-*ras*^{H} sequences terminate at a *Bcl*I linker located within the protein-coding sequences of the gene (called 'front ends'). The second segment contains an intact C-terminal domain of varying length that was derived from an independent series of *Bal*31-generated deletion mutants whose v-*ras*^{H} sequences also terminate at a *Bcl*I linker located within the p21 coding sequences (called 'tail ends'). This procedure permits the formation of mutants from which sequences encoding specific v-*ras*^{H} amino acids have been deleted or duplicated. It should be noted that the *Bcl*I linker results in the addition of three novel amino acids within the protein at the site of the deletion or duplication. In addition to containing the Ha-MuSV transforming region (the viral long terminal repeat plus v-*ras*^{H}), the plasmids contain a thymidine kinase (tk) gene which can be used to select for mutant v-*ras*^{H} genes independently of their ability to induce cellular transformation.

Cells and DNA-mediated gene transfer

The NIH 3T3 cells, tk$^-$ NIH 3T3 cells and DNA transfection procedure have been previously described (Willumsen *et al.*, 1984a). Cells were grown in Dulbecco's modified MEM supplemented with 10% heat-inactivated fetal bovine serum and antibiotics (penicillin 100 U/ml and streptomycin 100 μg/ml). The tk$^-$ cells were maintained on 100 μg/ml BrdUrd until used. Transfection of DNA was carried out with calcium (Graham and van der Eb, 1973), using 25 μg/ml NIH 3T3 DNA as carrier. Duplicate dishes were transfected with 0.2 μg of DNA and foci were counted 11 – 14 days later. The tk$^-$ cells transfected with plasmid DNA were selected in HAT medium.

Immunoprecipitations

Cultures transfected with Ha-MuSV mutants were metabolically labeled with [^{35}S]methionine (250 μCi/ml) in methionine-free medium or with [^{3}H]palmitic acid (1 mCi/ml). Extracts of whole cells were prepared and precipitated with a p21 monoclonal antibody (Y13-238) as previously described (Papageorge *et al.*, 1982; Furth *et al.*, 1982). For cell fractionation, hypotonic swelling of the cells was followed by homogenization and low-speed centrifugation to remove nuclei. The supernatant was then fractionated into a pellet particulate fraction containing the plasma membranes and a supernatant cytosol fraction as described (Courtneidge *et al.*, 1980) and subjected to immunoprecipitation. This procedure separates the cytosol-associated pro p21 from the membrane-associated mature p21.

GDP-binding, GTPase and autophosphorylation activities

Purification of mutant protein and determination of GDP-binding activity by a filter-binding assay was carried out as described (Manne *et al.*, 1984). The ability of the proteins to release ^{32}Pi from [γ^{32}P]GTP was measured on purified proteins (Manne *et al.*, 1985). Autophosphorylation was determined by incubating the purified proteins with [γ^{32}P]GTP under the GTP binding assay conditions. The extent of phosphorylation of p21 was determined by autoradiography after SDS gel electrophoresis.

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References

Bolanowski,M.A., Earles,B.J. and Lennarz,W.J. (1984) *J. Biol. Chem.*, **259**, 4934-4940.

Buss,J. and Sefton,B.M. (1985) *J. Virol.*, **53**, 7-12.

Capon,D.J., Seeburg,P.H., McGrath,J.P., Hayflick,J.S., Edman,U., Levinson, A.D. and Goeddel,D.V. (1983) *Nature*, **304**, 507-513.

Chang,E.H., Furth,M.E., Scolnick,E.M. and Lowy,D.R. (1982) *Nature*, **297**, 479-483.

Courtneidge,S.A., Levinson,A.D. and Bishop,J.M. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 3783-3787.

DeFeo-Jones,D., Tatchell,K., Robinson,L.C., Sigal,I.S., Vass,W.C., Lowy,D.R. and Scolnick,E.M. (1985) *Science (Wash.)*, **228**, 179-184.

Dhar,R., Ellis,R.W., Shih,T.Y., Orozlan,S., Shapiro,B., Maizel,J., Lowy,D.R. and Scolnick,E.M. (1982) *Science (Wash.)*, **217**, 934-937.

Dhar,R., Nieto,A., Koller,R., DeFeo-Jones,D. and Scolnick,E.M. (1984) *Nucleic Acids Res.*, **12**, 3611-3618.

Ellis,R.W., Lowy,D.R. and Scolnick,E.M. (1982) in Klein,G. (ed.), *Oncogene Studies, Advances in Viral Oncology*, Vol. **1**, Raven Press, pp. 107-126.

Fasano,O., Aldrich,T., Tamanoi,F., Taparowsky,E., Furth,M. and Wigler,M. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 4008-4012.

Furth,M.E., Davis,L.J., Fleurdelys,B. and Scolnick,E.M. (1982) *J. Virol.*, **43**, 294-304.

Gibbs,J.B., Sigal,I.S., Poe,M. and Scolnick,E.M. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 5704-5708.

Graham,F.L. and van der Eb,A. (1973) *Virology*, **52**, 456-467.

Gross,M., Sweet,R.W., Sathe,G., Yokoyama,S., Fasano,O., Goldfarb,M., Wigler,M. and Rosenberg,M. (1985) *Mol. Cell. Biol.*, **5**, 1015-1024.

Kataoka,T., Powers,S., Cameron,S., Fasano,O., Goldfarb,M., Broach,J. and Wigler,M. (1985) *Cell*, **40**, 19-26.

Land,H., Parada,L.F. and Weinberg,R.A. (1983) *Science (Wash.)*, **222**, 771-778.

Manne,V., Yamazaki,S. and Kung,H.-F. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 6953-6957.

Manne,V., Bekesi,E. and Kung,H.-F. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 376-380.

McGrath,J.P., Capon,D.J., Goeddel,D.V. and Levinson,A.D. (1984) *Nature*, **310**, 644-649.

Neuman-Silberberg,F.S., Schejter,E., Hoffmann,F.M. and Shilo,B.-Z. (1984) *Cell*, **37**, 1027-1033.

Papageorge,A.G., Lowy,D.R. and Scolnick,E.M. (1982) *J. Virol.*, **44**, 509-519.

Powers,S., Kataoka,T., Fasano,O., Goldfarb,M., Strathers,J., Broach,J. and Wigler,M. (1984) *Cell*, **36**, 607-612.

Reymond,C.D., Gomer,R.H., Medhy,M.C. and Firtel,R.A. (1984) *Cell*, **39**, 141-148.

Schejter,E.D. and Shilo,B.-Z. (1985) *EMBO J.*, **4**, 412-417.

Schultz,A.M., Henderson,L.E., Oroszlan,S., Garber,E.A. and Hanafusa,H. (1985) *Science (Wash.)*, **227**, 427-429.

Sefton,B.M., Trowbridge,I.S., Cooper,J.A. and Scolnick,E.M. (1982) *Cell*, **31**, 465-474.

Shih,T.Y., Weeks,M.O., Gruss,P., Dhar,R., Orozlan,S. and Scolnick,E.M. (1982) *J. Virol.*, **42**, 253-261.

Shih,T.Y. and Weeks,M.O. (1984) *Cancer Invest.*, **2**, 109-123.

Shimizu,K., Birnbaum,D., Ruley,M.A., Fasano,O., Suard,Y., Edlund,L., Taparowsky,E., Goldfarb,M. and Wigler,M. (1983) *Nature*, **304**, 497-500.

Sukumar,S., Notario,V., Martin-Zanca,D. and Barbacid,M. (1983) *Nature*, **306**, 658-661.

Sweet,R.W., Yokoyama,S., Kamata,T., Feramisco,J.R., Rosenberg,M. and Gross,M. (1984) *Nature*, **311**, 273-275.

Tabin,C.J., Bradley,S.M., Bargmann,C.I., Weinberg,R.A., Papageorge,A.G., Scolnick,E.M., Dhar,R., Lowy,D.R. and Chang,E.H. (1982) *Nature*, **300**, 143-149.

Taparowsky,E., Shimizu,K., Goldfarb,M. and Wigler,M. (1983) *Cell*, **34**, 581-586.

Temeles,G.L., Gibbs,J.B., D'Alonzo,J.S., Sigal,I.S. and Scolnick,E.M. (1985) *Nature*, **313**, 700-703.

Tsuchida,N., Ryder,T. and Ohtsubo,E. (1982) *Science (Wash.)*, **217**, 937-939.

Willingham,M.C., Pastan,I., Shih,T.Y. and Scolnick,E.M. (1980) *Cell*, **19**, 1005-1014.

Willumsen,B.M., Christensen,A., Hubbert,N.L., Papageorge,A.G. and Lowy,D.R. (1984a) *Nature*, **310**, 583-586.

Willumsen,B.M., Norris,K., Papageorge,A.G., Hubbert,N.L. and Lowy,D.R. (1984b) *EMBO J.*, **3**, 2581-2585.

Yuasa, Y., Gol,R.A., Chang,A., Chiu,I.-M., Reddy,E.P., Tronick,S.R. and Aaronson,S.A. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 3670-3674.

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