The EMBO Journal vol.4 no.2 pp.407–412, 1985

# Characterization of functional domains of p21 *ras* by use of chimeric genes

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Communicated by D.Baltimore

**Comparison of the predicted amino acid sequences of different members of the *ras* family in vertebrates has shown that the N-terminal 120 residues are highly conserved while the C terminus is variable. To test the possible role of the variable residues in cell transformation, chimeras were constructed containing the N-terminal 111 amino acids of the human Ha-*ras* EJ oncogene and the C terminus of two *Drosophila ras* genes. We show that one of these constructs which has only 20 conserved residues between positions 121 and 189, can transform rat-1 cells, and the transformed cells are capable of inducing lethal tumors in rats. The second construct containing the C terminus of another *Drosophila ras* gene exhibits a transforming capacity as well, but only after linkage to a viral transcriptional promoter. These results show that the majority of residues within the C terminus can be replaced without abolishing the transforming potential of p21 *ras*.**

*Key words:* *ras* oncogene/p21 *ras*/chimeras/*Drosophila* oncogenes/evolution

## Introduction

DNA transfections have consistently identified the involvement of the *ras* oncogenes in a variety of tumor types (Land *et al.*, 1983). The mutations in the genes that were activated *in vivo* have always been localized to codons 12 or 61 (Tabin *et al.*, 1982; Reddy *et al.*, 1982; Taparowsky *et al.*, 1983; Yuasa *et al.*, 1983). *In vitro* mutagenesis of the region coding for residues 1–111 showed that mutations in other positions (codons 13, 59 and 63) could also activate the Ha-*ras* gene (Fasano *et al.*, 1984). The specific effect of these mutations on the structure and function of the *ras* gene product, p21 *ras*, and the reason for their clustering at the N terminus is not known.

The *ras* genes comprise a family of three members in vertebrates: Ha-*ras*, Ki-*ras* and N-*ras*. The predicted protein products of the three genes are remarkably similar in their structure. The N-terminal 120 residues are highly conserved, while the C terminus is variable between amino acid residues 121 and 132 and 165 and 184 (termed the minor and major variable regions, respectively) (Dhar *et al.*, 1982; Tsuchida *et al.*, 1982; Shimizu *et al.*, 1983; McGrath *et al.*, 1983). The extreme four residues at the C terminus are conserved and were shown to be the region where the protein binds to the plasma membrane (Willumsen *et al.*, 1984). The variable sequences at the C terminus appear to be important for the distinct function of each of the *ras* proteins, since they were shown to be highly conserved in the rat and human homologs of Ha-*ras* and Ki-*ras* (Shimizu *et al.*, 1983). The variable residues may thus specify the unique role of each protein in the family, while the N terminus shared by all members fulfills a common function.

The pattern of sequence conservation in the *ras* proteins became more apparent when *ras* homologs from lower eukaryotes were isolated and sequenced. The N-terminal residues were shown to be highly conserved in two *Drosophila* (Neuman-Silberberg *et al.*, 1984), and two yeast *ras* genes (Powers *et al.*, 1984; Dhar *et al.*, 1984). The C termini of the fly and yeast homologs exhibit a variability pattern generally similar to that of the vertebrate gene products. The *Drosophila* C termini are co-linear with the vertebrate homologs while those of yeast are longer.

To test the possible role of the variable domains in the C terminus of p21 *ras* proteins, we have constructed chimeras containing the N terminus of the human Ha-*ras* EJ oncogene with a mutation in codon 12 (Capon *et al.*, 1983), and the C terminus of two *Drosophila ras* genes. We show that both of these constructs can transform rat-1 cells. These results show that the variable regions of vertebrate p21-*ras* can be replaced without abolishing the transformation potential of the protein and imply that the functions performed by these regions are not essential for transforming activity.

## Results

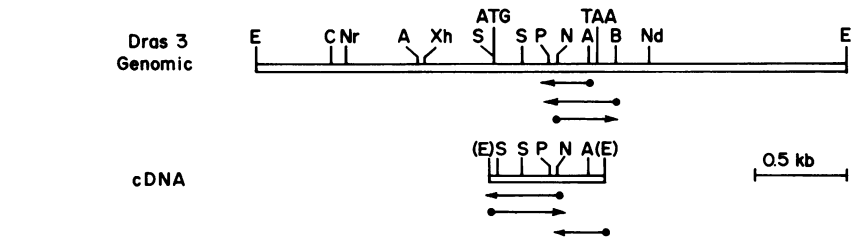
### *Dras3 genomic and cDNA clones*

Hybridization of the rat Ha-*ras* probe to *Drosophila* genomic blots has identified three homologous bands (Shilo and Weinberg, 1981). The three genes have been cloned and mapped to positions 85D, 64B and 62B on polytene chromosomes (Neuman-Silberberg *et al.*, 1984). The nucleotide sequence of the first two genes has been previously reported (Neuman-Silberberg *et al.*, 1984). We present here further structural characterization and the nucleotide sequence of the third gene termed D*ras*3, mapping to position 62B.

The genomic clone of D*ras*3 was used to isolate 10 cDNA clones by screening $10^5$ phages of an embryonic cDNA library. The largest cDNA clone (650 bp) was mapped with restriction enzymes and shown to be co-linear with the genomic clone (Figure 1), demonstrating the lack of introns in this gene. The nucleotide sequence of D*ras*3 was determined using both genomic and cDNA clones (Figure 1). Alignment of the sequences of the genomic and cDNA clones at the 3′ end shows that the 3′ non-coding sequence of the cDNA is only 40 bp long, followed by a poly(A) tail. This sequence does not have the consensus polyadenylation signal (Proudfoot and Brownlee, 1976), but the sequence ACTAAA, which is located 14 bp prior to the poly(A) tail addition site, may serve that role. The predicted protein of D*ras*3 contains 182 amino acids and has a mol. wt. of 21.0 kd.

### *Alignment of Dras3 with the human Ha-ras*

The predicted amino acid sequence of D*ras*3 was aligned with the human Ha-*ras* sequence (Figure 2). The overall homology

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-59                TAGAATTTAGCACAAAACAAGAGATATACGAAGGATATACAAACAGCACAGCAGCCAGC
    met arg glu tyr lys ile val val leu gly ser gly gly val gly lys ser ala leu  19
  1 ATG CGT GAG TAC AAA ATC GTG GTC CTC GGA AGC GGC GGC GTG GGC AAA TCC GCG CTG
    SphI

    thr val gln phe val gln cys ile phe val glu lys tyr asp pro thr ile glu asp  38
 58 ACG GTC CAG TTT GTC CAG TGC ATC TTC GTG GAG AAG TAC GAT CCC ACC ATC GAG GAC

    ser tyr arg lys gln val lys val asn glu arg gln cys met leu glu ile val asn  57
115 AGC TAC AGG AAG CAG GTG AAG GTG AAC GAA CGC CAG TGC ATG CTG GAG ATC GTG AAC
                                                 SphI

    thr ala gly thr glu gln phe thr ala met arg asn leu tyr met lys asn gly ser  76
172 ACG GCG GGT ACG GAG CAG TTC ACG GCC ATG CGG AAT TTG TAC ATG AAG AAC GGC AGC

    asp ser cys trp ser thr arg ser arg arg asn arg arg leu thr ile cys arg thr  95
229 GAT TCG TGC TGG TCT ACT CGA TCA CGG CGC AAT CGA CGT TTA ACG ATC TGC AGA ACG
                                                          PstI

    arg glu gln ile leu arg val lys asp thr asp asp val pro met val leu val gly 114
286 CGC GAG CAG ATA CTC CGG GTG AAG GAC ACA GAC GAT GTG CCC ATG GTG CTC GTG GGC
                                                    NcoI

    asn lys cys asp leu glu glu glu arg val val gly lys glu leu gly lys asn leu 133
343 AAC AAG TGC GAC CTC GAA GAG GAG CGC GTC GTC GGC AAG GAG CTG GGC AAG AAC TTG

    ala thr gln phe asn cys ala phe met glu thr ser ala lys ala lys val asn val 152
400 GCC ACC CAG TTC AAC TGC GCC TTC ATG GAG ACC TCA GCC AAA GCC AAA GTG AAT GTG

    asn asp ile phe tyr asp trp ser gly arg ser thr arg ser arg pro arg arg asn 171
457 AAC GAT ATT TTC TAC GAC TGG TCC GGC AGA TCA ACA AGA AGT CGC CCG AGA AGA AAC
                                                       AvaI

    arg arg ser arg lys val pro cys val leu leu ter                         182
514 AGA AGA AGC CGA AAA GTT CCA TGT GTT CTG CTA TAA GACTTCCAAAAACCAACAAAACTAAAA
571 AACAAAAAAAAAATATATAGCAGAGGAGGAGGAGAGAGGAGCAAAAC
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**Fig. 1.** Restriction map, nucleotide sequence and predicted amino acid sequence of D*ras*3. Restriction maps of both the genomic and cDNA clones are shown. DNA sequencing strategy is shown below the maps, with dots marking end-labeling sites. In the detailed sequence, nucleotide numbers designated in relation to the initiation codon are marked on the left, while amino acid positions are marked to the right. The arrow at the 3′ end represents the position where the sequences of the genomic and cDNA clones diverge. The cDNA clone has an additional seven A residues followed by the *Eco*RI linker. Selected restriction sites are underlined. Abbreviations: E, *Eco*RI; Nd, *Nde*I; B, *Bam*HI; A, *Ava*I; N, *Nco*I; P, *Pst*I; S, *Sph*I; Xh, *Xho*I; Nr, *Nru*I; C, *Cla*I; H, *Hind*III; Pv, *Pvu*II; ATG, initiation codon; TAA, termination codon. The *Eco*RI sites of the cDNA clone, in parenthesis, represent artificial *Eco*RI linkers added during construction of the cDNA library.

is 48%. Generally, the N terminus is more conserved than the C terminus. Residues 12, 13 and 59 which were shown to be involved in the oncogenic activation of the gene are conserved, but residues 61 and 63 are not. The variability at the C terminus extends beyond the minor and major variable regions. The C terminus of D*ras*3 is rich in arginines, similar to the variable region of Ki-*ras* exon 4B (Shimizu *et al.*, 1983; McGrath *et al.*, 1983), and to the *Drosophila* D*ras*1 and D*ras*2 genes (Neuman-Silberberg *et al.*, 1984) which were shown to be rich in basic amino acids. The D*ras*3 putative protein terminates with cysteine followed by three non-charged residues, a feature common to all *ras* homologs isolated so far.

### *Construction of chimeric genes*

The distinction among vertebrate *ras* proteins is made by virtue of the sequence variability in regions of the C terminus. These regions are highly conserved among vertebrate species for each class of the *ras* proteins (Shimizu *et al.*, 1983) and thus appear to be essential for the normal function of the protein. However, they may be dispensable for the transforming activity of the protein once the gene has been activated by mutations in codons 12 or 61. To test this possibility we have constructed chimeric genes containing the 5′ activated portion of the human EJ oncogene and the 3′ variable region of the *Drosophila* D*ras*1 or D*ras*3 genes (Figure 3). The two parts were ligated through a conserved *Nco*I site which is located within codon 111. The chimeric genes have the promoter and the first 111 codons from the EJ gene. For the 3′ end, the appropriate *Nco*I-*Bam*HI fragment of the D*ras*1 cDNA clone was used (Neuman-Silberberg *et al.*, 1984), and the SV40 early polyadenylation signal was added. In the case of D*ras*3, the 3′ end *Nco*I-*Bam*HI fragment of the genomic clone was used. Although the fragment extends beyond the point for termination of transcription, the SV40 polyadenylation signal was added as well, since this gene does not have

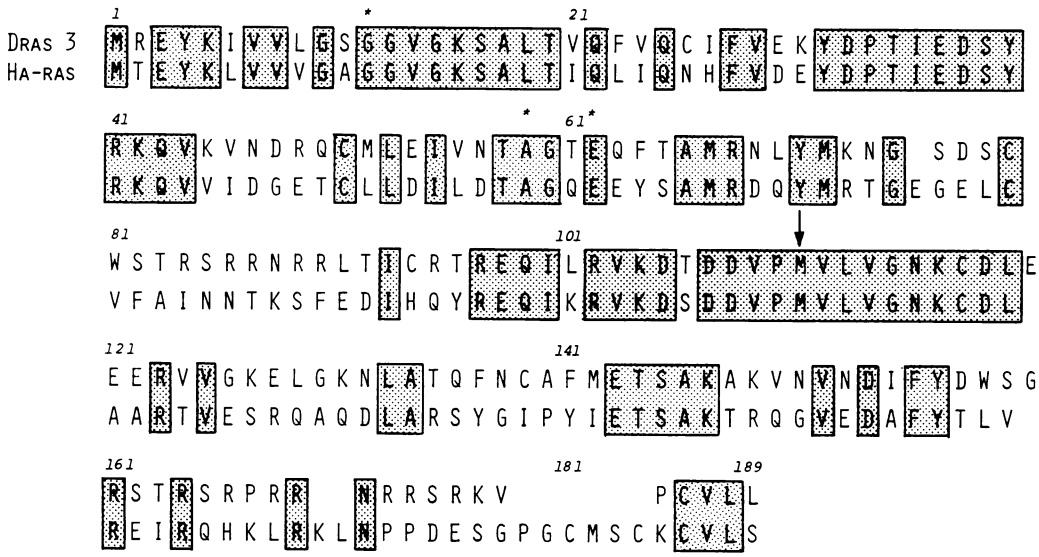


**Fig. 2.** Alignment of the predicted amino acid sequences of D*ras*3 with the human Ha-*ras*. The human sequence is drawn on the bottom line and the homologous residues are boxed. Numbering of residues follows the human sequence. Positions 12, 59 and 61 are marked by asterisks. The position of the *Nco*I site used for constructing the chimeric genes is marked by an arrow.

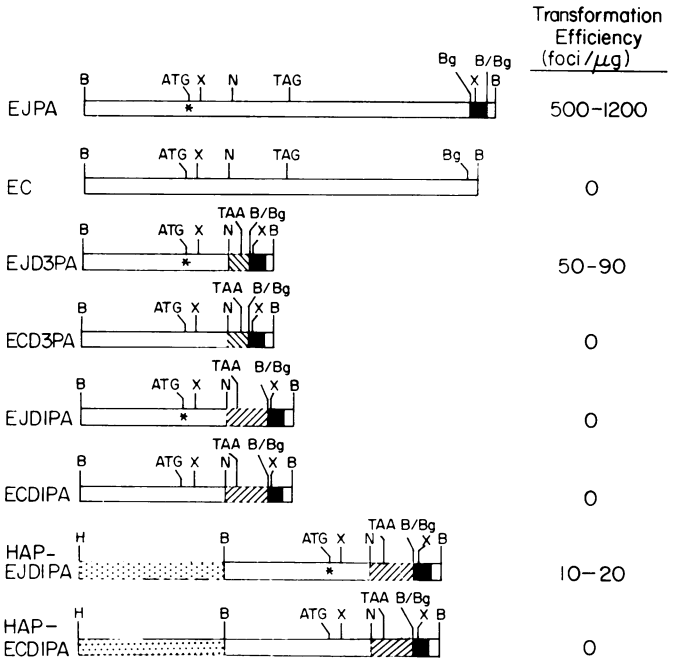


**Fig. 3.** Structure and transforming efficiency of the chimeric constructs. Construct names describe their structural content: the human *ras* sequences (EJ or EC) are represented by open boxes, the *Drosophila ras* sequences (D1 or D3) by hatched boxes, the viral promoter sequences by dotted boxes and the SV40 polyadenylation signal (PA) by black boxes. Asterisks within the human sequence indicate a mutation in codon 12. Abbreviations: B, *Bam*HI; X, *Xba*I; N, *Nco*I; Bg, *Bgl*II; H, *Hind*III; ATG, initiation codon; TAG or TAA, termination codons; B/Bg, ligation of *Bam*HI-*Bgl*II sites.

the consensus sequence for polyadenylation. An additional construct contained the forementioned EJ-D*ras*1 chimera linked to the promoter of Harvey sarcoma virus.

### *Biological activity of the chimeras*

The different constructs were transfected onto rat-1 cells (Figure 3). Transfection of the EJ positive control resulted in the appearance of foci after 11–14 days (1200 foci/μg DNA). The EJ-D*ras*3 chimera reproducibly induced the appearance of ~100 foci/μg DNA. Although these foci were clearly visible after 14–17 days, their cells were somewhat less refractile and criss-crossed than the control EJ foci. Transformation by the chimera was indeed caused by the mutation in codon 12 of the EJ gene and not by the juxtaposition of the two foreign sequences, since the DNA of a similar chimera with the 5′ end of EC (the normal counterpart of EJ) and the 3′ end of D*ras*3 did not demonstrate transforming activity (Figure 3).

The EJ-D*ras*1 chimera did not exhibit transforming activity over a wide range of plasmid DNA amounts tested (0.1–2.5 μg/transfection). To ensure that adequate amounts of the chimeric product were being produced, the construct was placed under the transcriptional control of the viral promoter. Indeed, transfection with the modified construct resulted in the induction of foci with an efficiency comparable with the D*ras*3 chimera. Again, no transforming activity was obtained from the control chimera which contained the normal human sequence.

The reasons for the reduced efficiency of transformation of the chimeras in comparison with the EJ genes are not known. One explanation may be a reduced stability of the chimeric gene products. In the case of the EJ-D*ras*1 chimera, the addition of a strong transcriptional promoter may have compensated partially for these effects by allowing an overproduction of the transcript.

### *Characterization of the foci*

Representative foci induced by the EJ-D*ras*3 construct were grown and characterized. A southern blot analysis of their DNA is shown on Figure 4. A probe containing the 5′ *Bam*HI-*Nco*I fragment of EJ hybridizes with a set of bands in the DNA of both the control EJ foci and the foci induced by the chimeric gene. Noticeable is the presence of a common 0.9-kb *Xba*I fragment in all the chimeric foci resulting from an internal *Xba*I fragment in the constructs. As shown in the rat-1 DNA lane, the human probe did not react with the rat c-Ha-*ras* genes under the stringent hybridization conditions used. Hybridization of a similar blot with a probe prepared

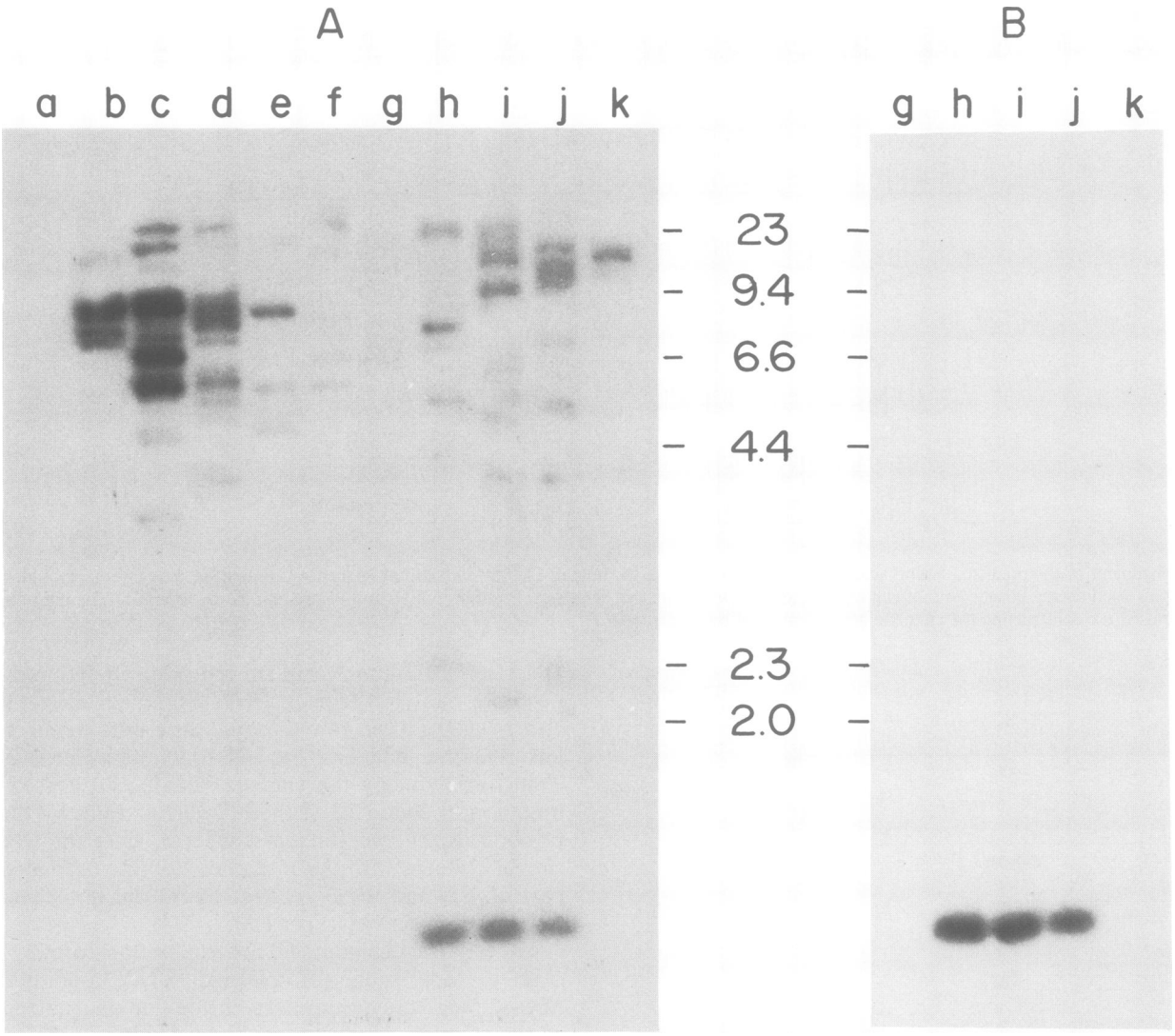


**Fig. 4.** Southern blot analysis of foci-derived DNAs. **Panel A**, hybridization with a probe prepared from the purified 5′ end 2.4-kb *Bam*HI-*Nco*I fragment of the human EJ gene. **Panel B**, hybridization with a probe prepared from the purified 1.5-kb *Nru*I-*Bam*HI fragment of the *Drosophila* D*ras*3 genomic clone. Lane assignments: **a**, rat-1 DNA; **b, c, d**, DNA from foci termed D2, D7 and D9, respectively, which were derived from the transfection of the pEJD3PA chimeric plasmid; **e,f**, DNA of foci termed B1 and B2, respectively, derived from transfection of pEJPA; **g**, rat-1 DNA; **h, i, j**, D2, D7 and D9 DNAs, respectively; **k**, B1 DNA. **Lanes a–f** were digested with *Eco*RI and **lanes g–k** with *Xba*I. Mol. wt. size markers are shown in kb.

from the *Drosophila* D*ras*3 gene detected bands only in the DNA of the foci induced by the chimera. Again, the presence of a common 0.9-kb *Xba*I fragment is visible.

Characterization of the foci induced by the EJ-D*ras*1 chimera linked to the viral promoter was performed by immunoprecipitation. The *ras* monoclonal antibody Y13-259 was applied to lysates of [$^{35}$S]methionine-labeled foci cells. A specific band which migrated slightly faster than control p21 was observed following polyacrylamide gel electrophoresis. The intensity of this band was equivalent to that of p21 immunoprecipitated in a similar fashion from cells transformed by the intact EJPA construct (data not shown).

*Tumorigenicity of the foci*

To test the tumorigenicity of the foci induced by the EJ-D*ras*3 chimeric plasmids, foci-derived cells were injected s.c. into 9 week old male Fisher rats (2 x $10^7$ cells per rat). Ten days after injection of the EJ transformed cells tumors appeared in 4/4 rats. The rate of the appearance of tumors after injection of cells from three foci induced by the chimeric plasmid was somewhat slower, but after 2–3 weeks tumors had appeared in 4/4 animals in all three cases. Rats bearing tumors from the focus induced by the EJ gene died within 3–4 weeks after injection of the cells, while rats bearing tumors from the foci induced by the chimeric gene died within 6–10 weeks after injection of the cells. The non-transfected rat-1 cells were also tumorigenic when injected into rats, but these tumors appeared long after the emergence of tumors in rats injected with focus-derived cells. The tumorigenicity results are summarized in Table I.

**Table I.** Tumorigenicity of foci-derived cells

| Injected cells | Tumors | Time of appearance (days) | Lethality (weeks) |
|---|---|---|---|
| B1 (EJ focus) | 4/4 | 10 | 3–4 |
| D2 (EJ-D*ras*3 focus) | 4/4 | 21 | 9–10 |
| D7 (EJ-D*ras*3 focus) | 4/4 | 14 | 8–9 |
| D9 (EJ-D*ras*3 focus) | 4/4 | 14 | 6–8 |
| Rat-1 | 4/4 | 60 | 14–16 |

## Discussion

The observation that the EJ-D*ras* chimeric plasmids can induce the appearance of foci which are tumorigenic provides new insights into the structure-function relationship of the p21 *ras* proteins. The C termini of the *Drosophila ras* homologs used, especially in the EJ-D*ras*3 chimera, are not similar to any of the known variable sequences of the *ras* genes in vertebrates and are unlikely to replace the normal function of the original C terminus in the rat-1 cells. The chimeras thus represent activated human *ras* oncogenes which have been extensively modified at their 3′ end. Alteration of 49 amino acid residues between positions 121 and 189 did not abolish the transforming activity or the tumorigenic properties of the protein. We can therefore conclude that the variable sequences at the C terminus are not essential for the transforming activity once a mutation has been introduced into codon 12. The transformation potential appears to reside in the conserved residues, most of which cluster in the N terminus.

It has recently been suggested that the p21 *ras* proteins are analogous to the family of G proteins (Gilman, 1984). Similar to G-proteins, *ras* proteins are membrane-associated, bind GTP (Shih *et al.*, 1980) and contain an intrinsic GTPase activity (McGrath *et al.*, 1984; Sweet *et al.*, 1984). This analogy implies that, like G-proteins, *ras* proteins may be involved in signal transduction from the membrane into the cell. Agents causing constitutive activation of G-proteins (e.g., cholera toxin or non-hydrolysable GTP analogs) act by disrupting the GTPase activity. Remarkably, the mutation in codon 12 of Ha-*ras* also results in a drastic reduction of GTPase activity (McGrath *et al.*, 1984; Sweet *et al.*, 1984). This finding suggests that once activated by a mutation, p21 *ras* functions continuously, in the absence of the normal activation signals and independent of the GTPase regulatory control.

In light of this model for oncogenic activation, the results presented here imply that the variable sequences in the C terminus of p21 *ras* proteins are not involved in the constitutive function of the activated protein. This activity is mostly localized to the N terminus. However, the variable residues may well be essential for the normal function of the proteins. In view of the possible role of *ras* proteins as signal transducers, these regions may function in recognition of specific stimuli which differ for each of the *ras* protein types. Oncogenic activation of p21 *ras* may allow it to function irrespective of the normal triggering processes.

## Materials and methods

*Cloning and sequencing of Dras3*

Isolation of the genomic clone from a Charon 4A library of *Drosophila melanogaster* DNA and its subsequent subcloning into a plasmid vector have been described (Neuman-Silberberg *et al.*, 1984). The 3.2-kb cloned genomic *Eco*RI fragment is smaller than the authentic 4.5-kb *Eco*RI genomic fragment since it was obtained as a junction fragment from a library prepared from sheared *Drosophila* DNA. The cDNA clone was isolated from a λgt-10 cDNA phage library prepared from *Drosophila* embryonic poly(A) RNA (provided by M.Goldschmidt-Clermont and D.Hogness), using a 1.9-kb *Bam*HI-*Eco*RI fragment which contained the entire coding region of the genomic clone as a probe. The isolated clones were subcloned into the *Eco*RI site of the plasmid vector pUC12. Sequencing was performed by the method of Maxam and Gilbert (1980) using reverse transcriptase to end label the fragments.

*Construction of chimeras*

The pEJPA construct was obtained by ligating *Bgl*II-digested alkaline phosphate-treated pEJ (a plasmid in which the EJ gene is cloned into the *Bam*HI site of pBR322) to a 300-bp *Bgl*II-*Bam*HI fragment containing a polylinker linked to the early polyadenylation signal of SV40. The polylinker contains a *Xba*I site which, together with the unique *Xba*I site in the EJ gene, served to indicate the nature and orientation of the EJPA and subsequent constructs. The pEJD1PA and pEJD3PA constructs were obtained by ligating *Nco*I and *Bam*HI-digested, alkaline phosphatase-treated pEJPA with the gel-purified 3′ end *Nco*I-*Bam*HI fragments of EF1 (the D*ras*1 cDNA clone) or of the D*ras*3 genomic clone, respectively. The pECD1PA and pECD3PA constructs were obtained by ligating *Nco*I-*Bam*HI digested, alkaline phosphatase-treated pEC with the 3′ end gel-purified *Nco*I-*Bam*HI fragments of pEJD1PA and pEJD3PA, respectively. The constructs linked to the viral promoter were obtained by ligating *Bam*HI-digested pEJD1PA and pECD1PA to *Bam*HI-digested, alkaline phosphatase-treated pHAP which contains the *Hind*III-*Bam*HI fragment of the Harvey murine sarcoma virus *ras* gene promoter (Ellis *et al.*, 1980), cloned in pUC12.

*DNA transfections*

DNA transfections were performed by the calcium phosphate precipitation method (Graham and Van der Eb, 1973). The chimeric plasmid DNA (typically 0.3 μg DNA) was co-precipitated with 40 μg of sheared mouse liver carrier DNA and transfected onto 2 x $10^6$ rat-1 recipient cells. The transfections were scored for foci 3 weeks later. Each transfection was repeated at least twice.

*Southern blotting*

5 μg of cellular DNA were digested with restriction enzymes, electrophoresed through 1% agarose gels and transferred to nitrocellulose filters. Hybridizations were done at 68°C overnight with 5 x SSCPE, 1 x Denhardt's solution, 0.1 mg/ml denatured salmon sperm DNA, 10% dextran sulphate and 5 x $10^5$ c.p.m./ml of nick-translated probe with a specific activity of ~1 x $10^8$ c.p.m./μg of DNA. Following hybridization, the filters were washed for several hours with 0.3 x SSC and 0.1% SDS at 68°C.

## Acknowledgements

We thank R.A.Weinberg for the EJ and EC clones, M.Goldschmidt-Clermont and D.Hogness for the cDNA library, and V.Rotter for help in animal injections. This work was supported by a grant from the U.S.–Israel Binational Science Foundation and the Charles H.Revson Foundation Chair to B.S.

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*Received on 19 November 1984*