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Summary

raf oncogene is a serine, threonine-specific protein kinase and was first identified as a transforming gene of murine oncogenic virus, 3611-MSV. Several cases of activated c-raf oncogenes were reported using DNA-mediated transfection experiments. All of them, including the rat activated c-raf which was reported by me, were shown to be suffered from rearrangements leading to the replacement of the N-terminal half of the product with other peptide sequences. I postulated that there is a sequence in the N-terminal half of the c-raf product which inhibits the enzyme from being activated. Losing the "suppressing sequence" by replacement was supposed to be the primary cause of the activation. To test this hypothesis, I constructed a series of c-raf cDNA mutants lacking different parts of the coding region. These mutants were subcloned in expression vectors and were transfected to NIH 3T3 cells to check the transforming activities. Mutants lacking one of three conserved regions in the N-terminal half were demonstrated to be activated. The possible mechanism of this activation and the relationship with the kinase activity are discussed.

Introduction

The molecular mechanism of human carcinogenesis is one of the most enthusiastic research fields partly because cancer is currently the leading cause of mortality in the civilized countries. The most important breakthrough appeared in 1981-1982, when the genes responsible for the transformation in vitro were identified by DNA-mediated transfection experiments (Tabin et al., 1982; Reddy et al., 1982). More importantly, the transforming genes of these human cancer cells turned out to be the mutated versions of human endogenous and presumably important genes.

So far, more than forty transforming genes have been identified by retroviral transduction or DNA-mediated transfection experiments (Weinberg et al., 1985). Many of them were found to encode protein kinases that phosphorylate serine, threonine or tyrosine residues. In contrast with the fact that most of the phosphorylated amino acid residues in the cells are serine or threonine, many transforming genes with protein kinase activity are tyrosine-specific. Some of the tyrosine-specific protein kinase oncogene have hydrophobic transmembrane regions in their mid-portions. They are supposed to be exposed to the outer surface of the cells. Several examples among them were demonstrated to be the receptors of growth factors (Downward et al., 1984; Sherr et al., 1985). The other group of tyrosine kinase oncogenes does not have transmembrane region and is supposed to mediate signal transduction from the cell membrane to the inner parts. Recently lck gene was shown to be coupled with CD4 and CD8 antigen in T cells and mediate the signal from the cell surface (Veillette et al., 1988). However, most of these tyrosine kinase oncogene remain to be investigated about their physiological functions.

In contrast with the tyrosine-specific kinase oncogenes, the number of the serine, threonine-specific kinase oncogenes is so small; so far only the mos and raf oncogenes are known. The c-mos oncogene was recently identified as a component of proteins regulating the meiosis in Xenopus laevis oocyte (Sagata et al., 1988). On the other hand, the function of c-raf gene in vivo remains unclear. The raf gene was first identified as the transforming gene of murine retrovirus 3611-MSV (Rapp et al.,1983). It was shown to be one of the oncogenes that have serine, threonine-specific protein kinase activity (Moelling et al., 1984). Its product was identified in the

cytoplasm (Molders et al., 1985) and it is supposed to transduce an intra-cellular signal from the cytoplasmic membrane to the nucleus (Smith et al., 1986).

The c-raf oncogene was found to be activated in several transformants of NIH3T3 cells transfected with DNAs from human cancers and some experimental animal models of cancer (Fukui et al., 1985; Shimizu et al., 1985; Ishikawa et al., 1986a; Stanton & Cooper, 1987; Tahira et al., 1987). In all cases in which the structural changes of the activated c-raf were studied, the activated forms were found to have a rearrangement that resulted in production of a fusion protein with the product of another gene (Fukui et al., 1987; Ishikawa et al., 1987). The fused proteins consisted of the carboxyl-terminal half of the c-raf product and the amino-terminal half of other gene products. No change in the carboxyl-terminal half, which contains the consensus sequence for the kinase domain including the ATP binding site, has been found in the activated c-raf. The amino-terminal halves of activated c-raf's were found to be derived from various genes. In v-raf, this sequence was derived from the retroviral gag sequence (Bonner et al., 1985), but in most cases the origin of the amino-termini of activated c-raf's could not be identified and was found to differ from case to case. Recently, amino-terminus of rat activated c-raf (Ishikawa et al., 1987a) was found to be identical to the tpr gene which also caused met activation (Kings et al., 1988). From the findings mentioned above, I proposed that loss of the amino-terminal half of the c-raf product was important for its activation, and speculated that the amino-terminal half of the c-raf product contains a sequence that prevents it from being activated.

To gain some insight into the structure-function relationship of the amino-terminal half of the raf product, I cloned a cDNA of rat A-raf, which is a putative serine, threonine-specific protein kinase and is highly homologous to rat c-raf (Ishikawa et al., 1987b). The two rat cDNAs showed high homology in the carboxyl-terminal half, the kinase domain. In addition to this, three highly conserved regions were also identified in their amino-terminal halves (Ishikawa et al., 1986b; 1987b). In the second of these three homologous regions, I identified a cysteine-rich sequence that is highly homologous with a sequence of protein kinase C (Ishikawa et al., 1989b). These conserved regions are also present in human c-raf-1 (Bonner et al., 1986) and human A-raf-1

(Beck et al., 1987). Because these three regions are conserved between different genes and among different species, I speculated that they play their functions independently as domains or subdomains of the raf product.

To prove the presence of the putative activation-suppressiing sequence in the raf peptide sequence, it was mandatory to construct a series of deletion mutants which lack several parts of the sequence and to examine the transforming activity of each deletion mutant. However, protein is generally known to form a highly ordered three-dimensional structure. It is possible that even a small mutation can disrupt the protein's function by perturbing the whole ordered structure. To limit the functional change accompanied by the mutations definitely to the function accomplished by the deleted sequence, I wished to construct the deletion mutants by removing the domain segments. The three putative domains identified by the comparison between c-raf and A-raf were the candidates for the segments to be deleted. Secondly, I wished not to change the translational efficiency of the mutated mRNAs by introducing mutations. Generally the translation efficiency is known to be subject to the sequence around the translation start site. I therefore made the sequence around the start site unchanged in each mutants. To meet the restriction mentioned above, I constructed a mutant library of c-raf cDNA by linker insertion. Using these linker-insertion mutants, I then obtained deletion mutants that lacked conserved regions in the amino-terminal half. In this way I detected a sequence that prevents c-raf protein from being activated (Ishikawa et al., 1988).

Materials and Methods

In vitro mutagenesis of c-raf cDNA

A mutant library was constructed based on the random linker insertion method developed by Heffron et al. (1987). A rat c-raf cDNA of 2.5kb which contains full length of the coding region (Ishikawa et al., 1987a) was subcloned into pGEM2 (Promega) to obtain pGR3. The supercoiled form of pGR3 was separated on cessium chloride density gradient. A sample of 200μg of the supercoiled form was treated with 40ng/μl of DNAase I (Worthington) in 2ml reaction mixture

consisting of 100mM sodium acetate (pH 5.0), 5mM $MgSO_4$ and 34 ng/ml ethidium bromide (Parker et al., 1977) for 20min at 37°C. About one fourth of the plasmid was converted to the nicked form by this digestion, as judged by agarose gel electrophoresis. The reaction was terminated by heating at 65°C for 10min followed by phenol/chloroform extraction and ethanol precipitation.

DNA was dissolved in 8ml of TE (10mM Tris, 1mM EDTA) and 8.4g of cessium chloride and 400 µg of 20mg/ml ethidium bromide were added. This solution was centrifuged in a vertical rotor (Beckman VTi65) at 65krpm and 20°C for 3.5h. The upper band, consisting of the nicked form and possibly the linear form, was collected. A total of 24 µg of DNA was obtained and dissolved in 300µg of reaction mixture consisting 1mM zinc chloride. S1 nuclease (Pharmacia) was added at a concentration of 27 units/ml and the sample was incubated at 37°C for 30 min.

About one third of the DNA was linearized by this treatment. The sample was then extracted with phenol/chloroform and precipitated from 70% ethanol. The DNA was treated with Klenow fragment to blunt the ends. A sample of 5µg of unphosphorylated synthetic XhoI linker d(CCTCGAGG) (Toyobo) was phosphorylated with T4 polynucleotide kinase and [γ^{32}P]ATP and then incubated further with cold ATP. This reaction mixture was directly ligated to the plasmid DNA with the aid of T4 DNA ligase. DNA was then digested with 240 units of XhoI (Boehringer) for 12h. Aliquots were subjected to electrophoresis and autoradiographed. The labeled linker was successfully ligated with the linearized form of the plasmid but not with other forms. Total sample was electrophoresed overnight in 0.7% agarose gel containing ethidium bromide at 30 volt. The linearized DNA was electroeluted from the gel and extracted twice with phenol and once with phenol/chloroform. The DNA was then precipitated from 70% ethanol by centrifugation in a Beckman TLA100.2 rotor at 50krpm for 15 min and dissolved in 20µl of 0.1x TE. A total of 2.4µg DNA was obtained. DNA was self-ligated by treatment with T4 DNA ligase at a DNA concentration of 100ng/µl. An aliquot was used for transformation of E.coli AG1 (Stratagene). About 1.8×10^4 ampicillin-resistant colonies/µg DNA were obtained.

Restriction mapping of inserted XhoI linker

Five hundred independent clones from the linker-inserted mutant library were cultured on a small scale and each plasmid DNA was extracted. Insertion of XhoI linker and its location was examined by digestion with restriction enzymes.

Determination of the nucleotide sequence around the inserted linker

The nucleotide sequences around the linker in mutants in which the inserted linker was in the coding region of the amino-terminal half of the c-raf product were examined. Several hexadecamer nucleotide sequences from the coding sequence of rat c-raf cDNA were synthesized in an Applied Biosystems 380A DNA synthesizer. By use of these oligonucleotides as primers, the nucleotide sequences around the linker were determined by the dideoxy-termination method (Sanger et al., 1977).

Construction of deletion mutants

Information on the nucleotides of the inserted region was analyzed using the computer program RLIMP (Random Linker Insertion Management Program). RLIMP, which runs on NEC PC-9801, a personal computer, include the function to determine the combination of mutants that would produce in-frame deletions.

Transfection of plasmid DNA

NIH3T3 cells were transfected with plasmids by the calcium phosphate precipitation method as described (Wigler et al., 1977) using salmon sperm DNA as carrier. Transformed foci were scored after 12 to 18 days. In the case of the pEV142-based plasmid, induction of metallothionein promoter was performed by adding $ZnCl_2$ at the concentration described in the text immediately after transfection.

For examining the expression of the transfected gene, plasmids were co-transfected with pRSVneo plasmid (1/200 of total DNA) and cultured 4 weeks in the presence of 400μg/ml G418.

Northern blot analysis

G418 selected colonies were harvested and total RNAs were extracted by the method of Chomczynski & Saccchi (1987). RNAs were separated in 1% agarose-formalin gel by electrophoresis and transferred onto nylon membrane as described (Goldberg, 1980). Hybridization was carried out at 42°C in 50% formamide, 0.65M NaCl, 0.1M sodium PIPES (pH 6.8) 5 x Denhardt's solution, 0.1% SDS, 5mM EDTA, 10% dextran sulfate, salmon sperm DNA (100μg/ml) and [32P]labeled v-raf XB probe (Rapp et al., 1983). Filters were washed four times for 20 min each with washing solution containing 2 x SSC, 0.2% sodium pyrophosphate and 0.1% SDS at 50°C.

Results

Construction of a random-linker-insertion mutant library of c-raf cDNA

I constructed a deletion mutant library of c-raf cDNA by the method of random linker insertion mutation (Heffron et al., 1978) as described in Materials and methods. Using this method, I obtained a library of mutants which have a single XhoI linker at a random site of the plasmid containing whole coding region of rat c-raf cDNA.

The library was consisted of 10^4 independent clones. The original plasmid pGR3 has no XhoI site, so insertion of the linker could easily be confirmed by digestion with XhoI. Five hundred clones of the original library were examined for the presence of the linker after amplification of each plasmid DNA in E. coli AG1. About 60% of the clones were found to have the inserted linker. Figure 1a shows the positions of the inserted linker in about 300 clones. The linker was inserted into almost all parts of the plasmid except the regions of the replication origin and ampicillin resistance gene. This result reflects the fact that these two regions are essential for the plasmid. In about 180 clones, the linker was inserted into the coding region of c-raf cDNA, and in 69 it was inserted into the 5'-terminl half, which should contain the putative activation-suppressing sequence. The nucleotide sequences around the point of insertion were analyzed in 42

clones in which the linker was in the 5' region. Figure 1b shows the precise positions of the inserted linker in these 42 clones.

Construction of deletion mutants

A previous study (Ishikawa et al., 1987b) revealed that three regions, named A,B and C, are conserved in the amino-terminal halves of the rat c-raf and A-raf products deduced from the sequences of their cDNA (Figure 2). Assuming that these conserved regions have some functional role, I constructed a set of mutants with deletions of one, two or all three of these regions A,B and C.

By combining two appropriate mutants with the linker at position X and Y, respectively , I could obtain mutants with deletions of the sequence between X and Y. By appropriate choice of X and Y, the resultant deletion mutant has an in-frame deletion. The location of the linker of each mutant was input into a personal computer, and calculation indicated the possibility of constructing 270 mutants with an in-frame deletion. Eight of these were constructed first (Figure 2). These constructs (C1,3,5,6,7,9,10 and 11) were confirmed by nucleotide sequencing to have the predicted, in-frame deletion. The inserts of these constructs were recloned into a retroviral vector pLJ, which has the LTR of murine leukemia virus to express the cloned cDNA (Piwnica-Worms et al., 1986).

Transforming activity of deletion mutants

The deletion mutants in pLJ (pLJC1,3,5,6,7,9,10 and 11) were transfected into NIH3T3 cells to test their transforming activity. Table 1 summarizes the results. pLJR3, which contains the normal rat c-raf cDNA, R3 (Ishikawa et al., 1987a), showed no transforming activity. pLJC11, which lacks 78 amino acids including region C, showed high transforming activity. Its transforming activity was comparable to that of pLJA5, a positive control which possessed A5 as an insert. cDNA of A5 was previously isolated from an NIH3T3 transformant obtained by transfecting rat hepatocellular carcinoma DNA and shown to contain the entire coding region of activated c-raf

(Ishikawa et al., 1987a). In addition, pLJC1 also showed weak but reproducible activity, whereas other clones had no activity in repeated assays. The expression vector pLJ has the polyoma small, middle and large T sequence to increase the plasmid copy number, and with this vector I could not exclude the possibility that these polyoma tumor antigens had some effect on the transforming activity of mutant raf cDNAs.

Therefore, I used another vector to confirm the results. For this, I recloned each deletion mutant into pEV142, Which has an inducible, mouse metallothionein I promoter to express the cloned cDNA (Palmiter et al., 1982). The constructs (pEVC1,3,5,7,9,10,11) were transfected into NIH3T3 cells. Without induction, only the pEVA5, which has A5 as an insert showed significant transforming activity, but when 100 mM zinc chloride was added to the medium to activate the metallothionein I promoter, pEVC11 showed as high transforming activity as pEVA5. Focus formation of pEVC11 was dose-responsive to the concentration of zinc ion in the medium (Figure 3). Northern blot analysis revealed that the levels of expression in pEVA5, pEVC1, pEVC11 and pEVR3 transfectants were increased about five-fold after induction (Figure 4). No other mutants formed any foci even after induction, except for that pEVC1 sometimes formed several foci after induction.

As shown in Figure 5, a characteristic property of the sequence deleted in C11 is that it is rich in serine and threonine, and these two amino acids constituting 38% of the total 78 amino acids.

Critical sequences for the transforming activity of C11

As mutant C11 has a relatively large deletion including region C and its adjacent sequences, we constructed four other mutants with smaller deletions. The maps of these four mutants, CM1 to CM4, are shown in Figure 5. These clones were recloned into pEV142 and transfected into NIH 3T3 cells. CM3 showed comparable transforming activity to C11, while CM2 showed slightly less activity. CM2 and CM3 lacked the first 36 and 51 codons, respectively, of the deletion in

C11. CM1 and CM4 showed no transforming activity. Thus, the region which is included in the deletion of CM2 and CM3 but not in that of CM1, seems to be important for activation.

Discussion

I have proposed the existence of a sequence in the amino-terminal half of the c-raf product that inhibits activation of this product. In the present study I demonstrated that such an activation-suppressing sequence was actually present.

My results showed that deletion of 78 amino acids just upstream of the kinase domain resulted in activation of the c-raf product. This sequence includes region C. The amino acid sequence of region C of the rat A-raf product is identical to that of the region C of the rat c-raf product. I then reduced the size of the deletion , and found that a 36 codon deletion in the CM2 mutant was sufficient to activate the c-raf product. Since CM1 showed no transforming activity, the sequence between nucleotide 772 and 822, encoding 17 amino acids, or/and the sequence between 716 and 732 seems to be important for suppressing the transforming activity. This region of 17 amino acids contains 6 serine and 2 threonine residues (Figure 5). Moreover, in this region, the sequence of 6 amino acids RSTSTP, corresponding to the left half of the C-region, is wholly conserved in the products of the rat and human A-raf, and human c-raf. It is also conserved in that of *Drosophila* D-raf except that two threonine residues are replaced by other amino acids (Nishida et al., 1988). Assuming that there is a common sequence for suppressing of all raf family genes, the left part of region C seems to be the most likely candidate for such a role. The sequence from 716 to 732 which is not conserved in the raf family seems to be less important. Of course I can not exclude the possible importance of flanking sequence(s) to the left part of region C for the suppression of transforming activity. Since CM2 showed less activity than CM3 or CM11, the sequence in the right half of the region C may also contribute to regulation of kinase activity.

In all but two cases in which the structure of activated c-raf is known, the region C has been shown to be deleted by rearrangement. In one of these two exceptional cases (Molders et al.,

1985), c-raf was found to be activated by insertion of Mo-MuLV LTR into intron 5, upstream of region C. However, translation was suggested to start from the first ATG present in the truncated c-raf (Schultz et al., 1988). This first ATG is present downstream of region C, and so the sequence of this region may not be translated in the protein product. In the other exceptional case (Nakatsu et al., 1986), a stretch of hydrophobic amino acids is reported to be fused with the carboxyl-terminal half of the c-raf product containing the region C to produce the activated form. Detailed information is not available about the activated product, but this highly hydrophobic sequence may prevent the activation-suppressing function of region C by influencing its tertiary structure. A-raf is also known to be activated by rearrangement with the consequent loss of a region that includes this sequence (Beck et al., 1987).

Region C is composed of a sequence of 14 amino acids with the characteristic feature that the serine and threonine residues, which constitute 50% of the total 14 amino acid residues, are clustered in two portions, the left half of region C having a cluster of 2 serine and 2 threonine residues. Regarding the total sequence of 648 amino acids, 16% are serine and threonine residues.

For exhibition of the transforming activity of pEVC11, it was necessary to activate the methallothionein I promoter by addition of zinc to the culture medium, whereas clone A5 displayed transforming activity without induction. Expression levels of mutated genes are comparable between EVC11 and EVA5 transformants before induction and about 5 times higher after induction of zinc (Figure 4). Thus, a higher level of transcript of the deletion mutant of c-raf than of A5 seemed necessary to display transforming activity. Possibly a second genetic event that results in quantitative or qualitative change of the product is necessary for high transforming activity A candidate for a second genetic event leading to quantitative change, which was brought into A5 but not into C11, may be the sequence affecting translation efficiency, because the nucleotide sequences around the initiating ATG of the c-raf gene are probably not a good target for translation (Kozak, 1986). In contrast, the activated form, A5, has a sequence which exactly matches the consensus sequence of Kozak. This may explain the phenotypic differences between A5 and C11 with the methallothionein promoter under the uninduced condition.

Mutants with large deletions including region C (C1, C5, C6) had little or no transforming activity. Since the amount of mRNA of mutated c-raf in EVC1 transfectants was very high (Figure 4), the low transforming activity of EVC1 seems to be due to problems other than mRNA transcription rate or stability. One possible mechanism is instability of the gene product due to large deletion. This is less likely, because LTR-inserted activated c-raf has transforming activity. Another is impairment of the essential function of protein kinase activity. Our result suggests a role of region B in enhancement of transforming activity. Since mutant C1, C5 and C6 which lack both regions B and C showed little transforming activities, region B which includes a cysteine rich region seems to be necessary for exhibition of transforming activity in region C deletion mutants.

All c-raf genes activated by recombination with other genes, including LTR insertion mutants, lack both region B and C but exhibit transforming activity. All of our deletion mutants constructed in this study keep normal c-raf sequence up to nucleotide 195 including initiation codon at nucleotide 42, while this original sequence is replaced by other sequences in all activated c-raf genes of lacking in the LTR inserted c-raf.

The difference in transforming activity between the C1 deletion mutant and other activated c-raf genes, suggests that the sequence around the initiation codon plays an important role in activation of c-raf. It is not yet known whether or not only the mutants can explain the difference of transforming activity. The role of sequence downstream of initiating methionine also remains to be clarified.

The proto-trk oncogene has been found to be activated in a similar manner; namely, it was activated by recombination of 3 different kinds of cellular sequences that had no common sequences with the truncated, C-terminal portion of proto-trk (Kozma et al., 1988). The proto-ret oncogene, which is thought to be a tyrosine specific protein kinase, was also found to be activated by replacing its N-terminal portion by two different sequences (Takahashi et al., 1988; Ishizaka et al., 1988). Therefore, this type of activation is not limited to the raf oncogene. Recently, human activated B-raf gene was cloned, and it was shown that the N-terminal portion was substituted by another sequence (Ikawa t al., 1988).

In summary, a region that is rich in serine and threonine residues and is situated just upstream o f the kinase domain of the c-raf product was shown to be important for activation and possibly also for the kinase activity of the product.

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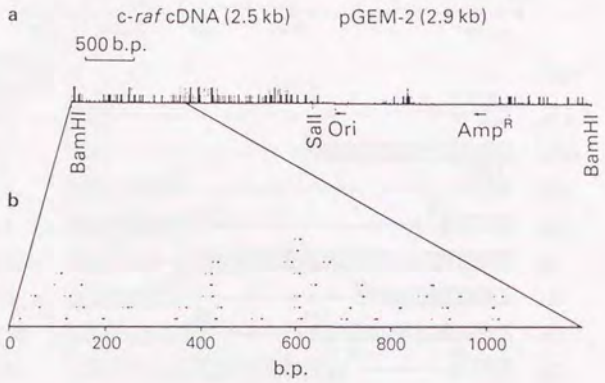


Figure 1 Maps of XhoI linkers in mutants. Panel (a) Positions of inserted linkers determined by restriction mapping. About 300 clones were examined. The smallest bars indicate one clone. Multiple clones mapped at the same position were indicated by the height of bars. The BamHI and SalI sites indicated are from the multiple cloning sites of pGEM2. The replication origin and ampicillin resistant region of this plasmid are shown by boxes. Panel (b) Exact location of linkers in 42 clones on the 5'-half of c-raf cDNA determined by nucleotide sequencing (See Materials and Methods). The small deletions in the clones obtained during the mutation procedure are indicated by horizontal lines.

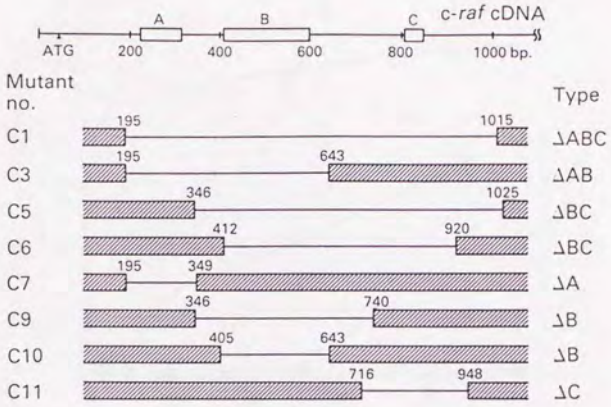


Figure 2 Construction of deletion mutants lacking region A, B or C. The 5'-terminal half of c-raf cDNA is shown. Region conserved in c-raf and A-raf are indicated as A, B and C. Eight mutants lacking one, two or three of these regions were created by combining clones from the random linker insertion mutant library. The deletions are shown by thin lines. These deletion mutants, which had an intact 3'-half, were ligated into expression vectors and subjected to transfection assay.

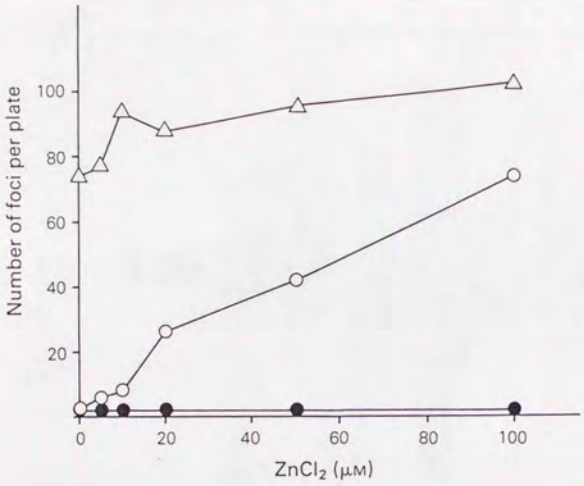


Figure 3 Transforming activity of mutant raf cDNA ligated to the methallothionein promoter. The effects of the zinc concentration on the transforming activities of pEVR3 (●-●), pEVA5 (Δ-Δ) and pEVC11 (○-○) are shown. Plasmids were transfected into NIH 3T3 cells by the calcium-phosphate co-precipitation method, and cultured in medium with the indicated concentrations of zinc. Number of transformed foci per plate (1.7μg plasmid) were scored on day 16

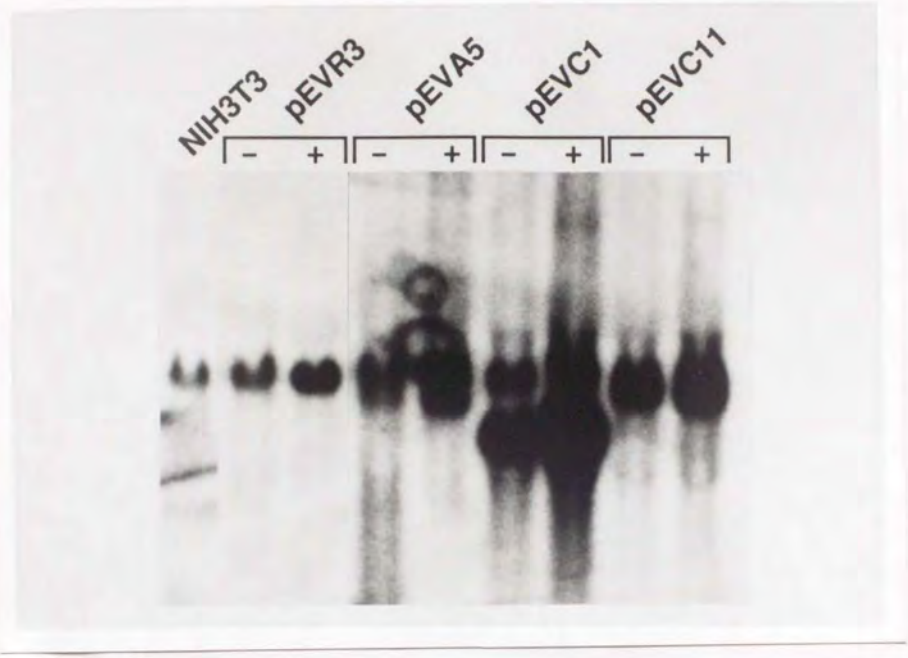


Figure 4 Expression of mutated c-raf genes in various transfectants. Five μg each of pEVR3, pEVA5, pEVC1 and pEVC11 were co-transfected with 150ng of pRSVneo DNA into NIH 3T3 cells as described in Materials and Methods. Cells are cultured with (+) of without (-) 100μM $ZnCl_2$ in the presence of 100μM G418 for 4 weeks. Total RNAs from total survived colonies are obtained and subjected to electrophoresis in 0.9% denaturing gel and analyzed by blot hybridization using [^{32}P]-labelled v-*raf* XB as a probe.

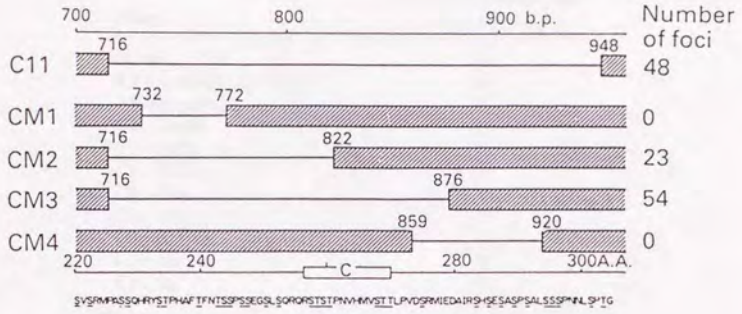
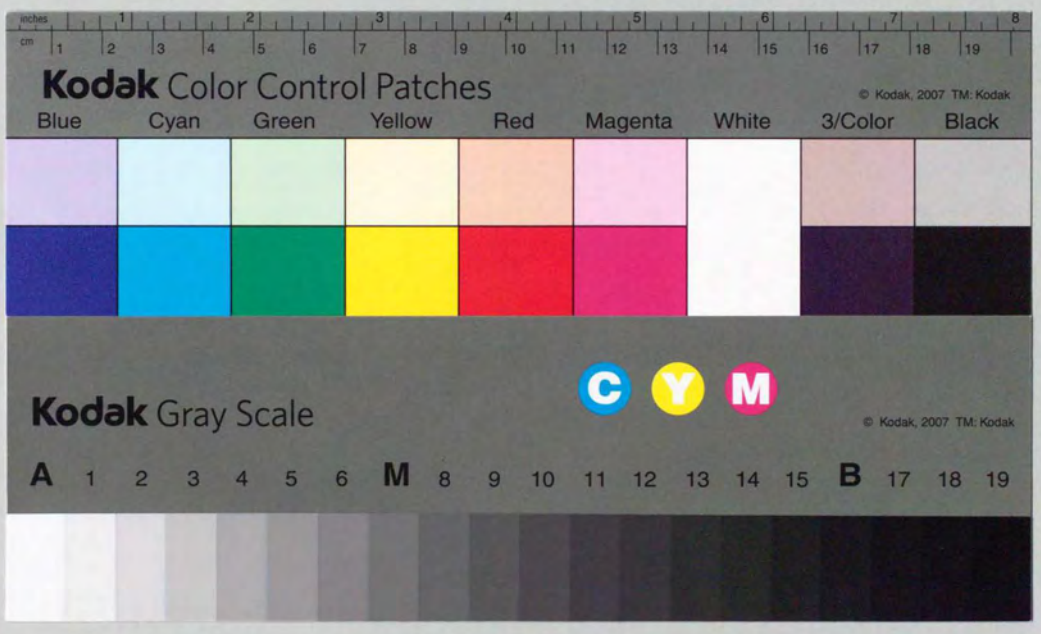


Figure 5 Transforming activities of mutants with small deletions around region C. Four mutants with deletions of parts of the region deleted in C11 mutants were created (CM1, CM2, CM3 and CM4). The extent of deletion in each clone, and the amino acid sequences involved are shown. Region C is indicated by a box. Transforming activity is shown in the right column. Samples of 1.7μg DNA with 100μM zinc were used as described in the legend to Figure 3.

Table 1 Transforming activities of deletion mutants

Insert	*No. of transformed foci (5 μg DNA)*
None	0
R3 (normal c-*raf* cDNA)	0
A5 (activated c-*raf* cDNA)	78
C1 ΔABC	5
C3 ΔAB	0
C5 ΔBC	0
C6 ΔBC	0
C7 ΔA	0
C9 ΔB	0
C10 ΔB	0
C11 ΔC	84

The deletion mutants were constructed, inserted into retrovirus vector pLJ and transfected into NIH3T3 cells as described in Materials and methods. Numbers of transformed foci were counted on day 17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cm
Kodak Color Control Patches
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Blue
Cyan
Green
Yellow
Red
Magenta
White
3/Color
Black
Kodak Gray Scale
C
Y
M
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A 1 2 3 4 5 6 M 8 9 10 11 12 13 14 15 B 17 18 19