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Activation of human c-*raf*-1 by replacing the N-terminal region with different sequences

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ABSTRACT

Two transformants of NIH 3T3 cells, obtained by the transfection of human colon cancer and normal colon DNAs, contained activated c-raf-1. In both the activated c-raf-1, the 5' half of the c-raf-1 sequence was replaced by sequences other than c-raf-1 as a result of recombinations which occurred at the intron between exons 7 and 8. It was suggested, however, that these recombinations, which conferred the transforming activity on the c-raf-1, occurred during the transfection. In one case analyzed, characteristic sequences were found near the breakpoint and these may be involved in the recombination. It was found, upon analysing the structure of the cDNA derived from one of the activated c-raf-1, that fused mRNA had been transcribed from the recombined gene comprising the non-raf gene and c-raf-1. The mRNA possibly encodes a fused protein. One cDNA clone was derived from alternatively spliced mRNA, although its physiological role is unclear. On comparing the structure of the two human activated c-raf-1 and the rat activated c-raf which we have reported previously (5), it was revealed that, in these three cases, the sequences joined to the truncated c-raf(-1)[1] were different. It was suggested from data which we and others have previously reported that various sequences could be capable of activating c-raf(-1) by replacing its 5' half.

INTRODUCTION

Activated c-raf-1 genes have recently been found in several transformants of NIH 3T3 cells obtained by transfecting human cancer DNAs (1,2). We also found rat activated c-raf in a transformant derived from rat hepatocellular carcinoma, and have characterized it by molecular cloning (3,4,5).

Human c-raf-1 comprises at least 17 exons from which it can be deduced that it encodes a protein of 648 amino acid residues (6) possibly having serine-threonine kinase activity, as reported for v-raf protein (7). Among the many transforming genes detected by NIH 3T3 transfection assay, activated c-raf(-1) is the only one which could encode serine-threonine kinase and this product is located in the cytoplasm (8). This suggests that activated c-raf(-1) may have a role distinct from other oncogenes in cellular transformation.

During our studies on the transforming activities of the DNAs from human colon cancer and from the normal colon mucosa of colon cancer patients, we found that c-raf-1 was activated in two primary transformants, 2C-1 and 7N-1. We characterized these two activated c-raf-1, especially that in the 7N-1 obtained by the transfection of normal mucosa DNA, and carried out examinations to find out what DNA changes had led to the activation of the c-raf-1, and whether or not c-raf-1 activation had occurred in tissue DNA. We discuss a feature common to activated c-raf(-1) and claim that c-raf(-1) activation during the process of DNA transfection is not rare.

MATERIALS AND METHODS

DNA transfection

High molecular weight DNAs were prepared from solid tumors and cultured cells as described (9), and the DNAs were transfected into NIH 3T3 cells by the calcium phosphate precipitation method (10). Transformed foci were obtained after 14-21 days. For transfecting cosmid clones, salmon sperm DNA was used as a carrier, and the cosmid clone incorporation was checked by G418 resistance at a concentration of 400 μg/ml.

General methods

Southern and northern blot analyses were performed as previously described (4). The XhoI/BstEII fragment of cloned v-raf, v-raf XB (11), which spans the region corresponding to exons 10-17 of human c-raf-1, was used as a v-raf specific probe. BLUR-8 (12) was used as a human Alu sequence probe. The plasmid, cosmid and phage DNA preparations, and the restriction enzyme mapping were carried out by standard procedures (13).

Molecular cloning

A genomic library was constructed in the cosmid vector, pCV108, which has an SV2-neo-derived selectable marker (14). High molecular weight DNA obtained from the primary transformant 7N-1 was partially digested with MboI, and 35-45 kb fragments were ligated to the BamHI site of pCV108. The human genomic library in the Charon 4A obtained from a partial digest of EcoRI (15) was provided by Dr. T. Maniatis. A cDNA library was constructed in λgt10 from purified poly(A)$^+$RNA obtained from the 7N-1 cells (16). Several DNA fragments were subcloned in pGEM2 (Promega Biotec, Madison, WI).

Nucleotide sequencing

Nucleotide sequence analyses of cDNA and genomic DNA were carried out by the chain termination method (17) after cloning the subfragment into M13mp vectors. In some cases 7-deaza-dGTP, provided by Dr. S. Nishimura of this

Institute, was used in place of dGTP (18). A progressive deletion by exonucleases III and VII was adopted for cDNA sequencing (19).

DNA and protein homology searches

DNA and protein homology searches were performed using a previously developed algorithm (20) against Genbank and NBRF data banks.

RESULTS

Detection of activated c-raf-1 in two primary transformants

The DNAs from surgically removed colon cancers and non-cancerous portions of the colon mucosa of about 30 patients were tested for transforming activity on NIH 3T3 cells. Human c-raf-1 sequences were contained in two primary transformants, 2C-1 and 7N-1. Southern blot analysis, using v-raf XB as a probe, detected 6.9 kb, 3.6 kb and 2.0 kb HindIII bands derived from human c-raf-1 in the DNAs of both transformants (data not shown). 2C-1 was induced by DNA extracted from a mesocolon metastasis of sigmoid colon cancer (2C), and 7N-1 was induced by that from a non-cancerous portion of the colon mucosa (7N) of a different colon cancer patient. Twenty-four and 92 secondary transformants were induced, respectively, by 300 μg of the 2C-1 DNA and 60 μg of the 7N-1 DNA.

Structure of activated human c-raf-1

To determine the structure of one of the activated c-raf-1, a genomic library was constructed from the DNA of 7N-1 cells in the cosmid vector, pCV108 (13). Among 4 x 10^5 clones, 11 clones showing a positive signal with both v-raf XB and BLUR-8 were purified. Two of the cosmid clones, pCN4 and pCN13, showed transforming activity in NIH 3T3 cells, giving 121 and 176 foci/μg DNA, respectively.

A composite restriction map made from overlapping cosmid clones (Fig. 1A, B) was compared with that of normal c-raf-1 reported by Bonner et al. (6). We also cloned a part of the normal c-raf-1 represented by the Charon 4A clone, λE2, obtained from a human genomic library (15) (Fig. 1D). The map of the 5' region of this transforming gene differed from that of c-raf-1, suggesting the occurrence of a recombination within the 4.2 kb HindIII fragment. To locate the recombination point precisely, the 3.2 kb HindIII fragment of λE2 and the 4.2 kb HindIII fragment of pCN13 were subcloned to yield pE2 and p13, respectively, the restriction maps of which were compared (Fig. 1C). The recombination point was located approximately 800 bp upstream of the 3' end of the p13 insert, which corresponded to the intron between exons 7 and 8 of normal c-raf-1, as will be shown later. The region 5' to the recombination point had

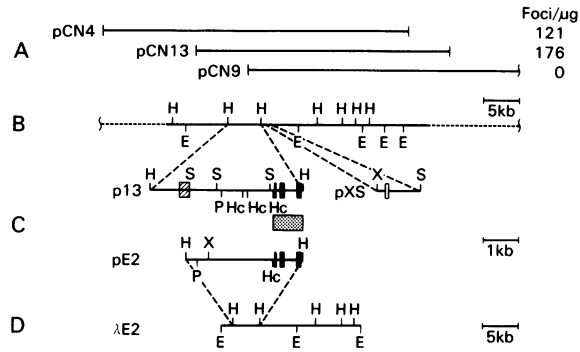


Figure 1.

Structure of activated c-raf-1 and comparison between maps of activated and normal c-raf-1 around recombination point. A: Inserts of cosmid clones isolated from 7N-1 DNA. The transforming activity of each clone on NIH 3T3 cells is shown on the right. B: Structure of activated c-raf-1 deduced from overlapping cosmid clones. EcoRI (E) and HindIII (H) sites were mapped for regions shown by a solid line. C: Enlargement of regions subcloned for further analysis. S, SacI; P, PstI; Hc, HincII; X, XbaI. Solid boxes indicate location of exons 8-10 deduced from reference 6. Locations of 5' end of exon 8 and 3' end of exon 10 were confirmed by nucleotide sequence analysis. Open box indicates location of alternative exon (see Fig. 4). Hatched box indicates part of coding region of non-raf gene corresponding to first 280 bp of activated c-raf-1 cDNA (Fig. 4B). Stippled region indicates HC-7, 0.8 kb HincII-HindIII fragment of p13, which was used as a probe. D: EcoRI and HindIII map of Charon 4A clone λE2 insert, representing portion of normal c-raf-1.

been derived from human DNA, judging from the Southern blot analysis (see below and Fig. 5).

Absence of recombination in original tissues

When a 0.8 kb HincII-HindIII fragment of p13 (HC-7) was used as a probe, a PstI digest of human placenta DNA yielded the 3.0 kb band shown in Fig. 2. The 7N-1 DNA showed a 2.4 kb band derived from the fused gene. This recombinant fragment, however, was detected in the DNA of neither the original normal mucosa (7N) nor the tumor taken from the same patient (7C), even after long exposure.

In the case of another transformant, 2C-1, the size of the corresponding PstI band changed to 7.4 kb. A recombination point must also have been located between exons 7 and 8, since a 0.8 kb HincII-HindIII fragment containing exons 8 to 10 was detected in 2C-1 DNA, as in human placenta DNA and 7N-1 DNA (data not shown). Again, however, the recombinant 7.4 kb PstI band was not detected in the DNA of the original cancer (2C). Thus, in both cases, recombination would appear to have occurred during the transfection process.

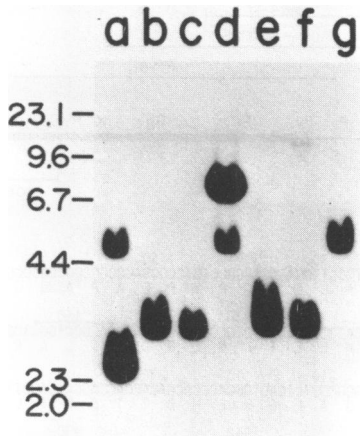


Figure 2
Presence of recombination in primary transformant DNA but not original tissue DNA. DNAs were digested with PstI and probed with HC7. Lanes a, 7N-1 DNA; b, 7N DNA; c, 7C DNA; d, 2C-1 DNA; e, 2C DNA; f, human placenta DNA; g, NIH 3T3 DNA.

Nucleotide sequences around the recombination point

The nucleotide sequences around the recombination point of activated c-raf-1 and the corresponding region of normal c-raf-1 were compared (Fig. 3). The recombination point was located approximately 32 bp upstream of exon 8. An inverted repeat, CTCCT/AGGAG, was found in the intron of normal c-raf-1 surrounding the recombination point. In addition, the 36 bp sequence, complementary to the sequence found in the normal c-raf-1 intron, was positioned 5' to the recombination point of activated c-raf-1 but with an opposite orientation.

Transcripts of activated c-raf-1

RNA blot analysis probed with v-raf XB showed that 7N-1, as well as transformants induced by cosmid clones, pCN4 and pCN13, expressed two kinds of transcript, 3.1 and 2.8 kb in length (data not shown). The 3.1 kb transcript

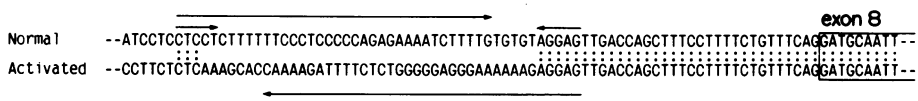
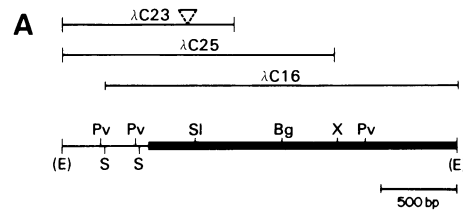
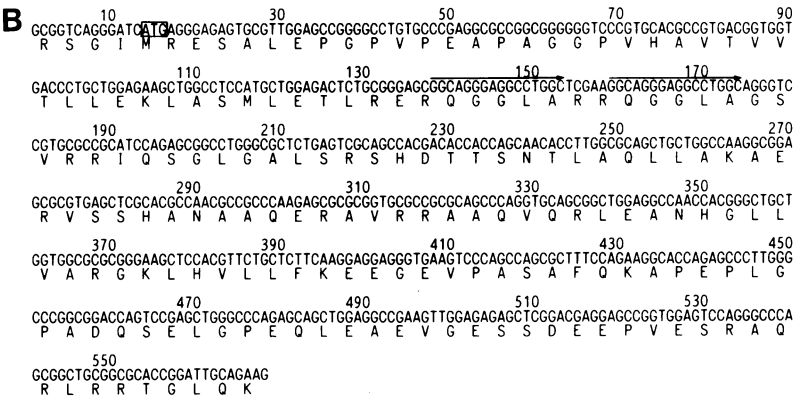


Figure 3
Nucleotide sequences of normal and activated c-raf-1 around recombination point. Short arrows indicate an inverted repeat. Long arrows show characteristic inverted sequences.





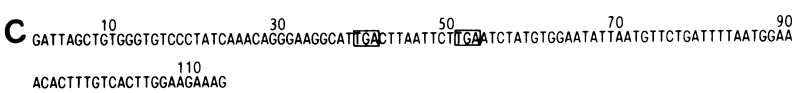


Figure 4

Structure of activated c-raf-1 cDNA obtained from 7N-1. A: cDNA clones representing activated c-raf-1 cDNA. The 113 bp sequence found in clone λC23, but not in λC25 and λC6, is shown separated from the colinear line. In the composite restriction map, the region derived from human c-raf-1 is indicated by a bold line beginning at nucleotide 569. Restriction endonuclease sites: Pv, PvuII; S, SacI; Sl, SalI; Bg, BglII; X, XbaI; (E), EcoRI (derived from linker). B: Nucleotide sequence of the 5' terminal 568 bp of activated c-raf-1 cDNA and putative amino acid sequence in-frame with that of c-raf-1. First ATG is boxed, and 16 bp direct repeat indicated by arrows. C: Nucleotide sequence of 113 bp extra exon found in clone λC23. Two stop codons in-frame with c-raf-1 exon 10 reading frame are boxed.

was also detected in the NIH 3T3 cells and it was, therefore, concluded that 2.8 kb transcript was derived from activated human c-raf-1. The levels of both transcripts were almost the same in 7N-1.

To elucidate the primary structure of the activated c-raf-1 transcript, a cDNA library was constructed from the poly(A)$^+$RNA of 7N-1 in the λgt10 vector. Among 2 x 10^5 clones, approximately 160 were positive with a v-raf XB probe,

and 30 of these were purified. Three inserts containing overlapping cDNA clones, λC6, λC23 and λC25, spanned a total length of 2.6 kb, representing almost the full length of the cDNA, of the activated c-raf-1. The clones were further analysed after subcloning into pGEM2 (Fig. 4A).

Nucleotide and predicted amino acid sequences of activated c-raf-1 cDNA

Nucleotide sequence analysis revealed the cDNA to consist of two regions (Fig. 4A). The region from nucleotide 1 to 568 was a non-raf sequence. The region from nucleotide 569 to the start of the poly(A) tail at nucleotide 2588 was derived from exons 8 to 17 of normal c-raf-1 and was identical to the corresponding normal c-raf-1 cDNA (6) except for four base pairs inserted into the 3' non-coding region: G at nucleotides 1689, 2326, 2385 and T at nucleotide 2434 of the activated c-raf-1 cDNA.

The nucleotide sequence of the non-raf portion of the cDNA and the predicted amino acid sequence in frame with that of the c-raf-1 are illustrated in Fig. 4B. As revealed by nucleotide sequence analysis of the appropriate region, the first 280 bp of the cDNA was contained in the 5' half of a p13 insert (see Fig.1C). The codon utilization of the predicted amino acid sequence agreed with that described by Lathe (21) for human protein-coding sequences. It is likely, therefore, that the coding region of the non-raf gene 5' to the breakpoint, together with the truncated c-raf-1, generated a fused mRNA which should give rise to a fused protein. Since we could not find an in-frame stop codon upstream of the most 5' ATG, the possibility remains of there being a coding sequence further upstream.

To obtain information about recombined upstream sequences, a homology search was conducted at both DNA and protein levels, but no sequence with a significant homology could be found in either GENBANK or NBRF. There are 16 bp direct repeats starting from nucleotides 138 and 159, and the predicted amino acid sequence, Arg-Gln-Gly-Gly-Leu-Ala is also repeated in this region. The hydrophobicity profiles (22) of both normal and activated c-raf-1 products were compared, but no dramatic change was introduced by replacing the N-terminal region.

Presence of an extra exon

In one of the cDNA clones, λC23, a 113 bp sequence insertion was found between nucleotides 842 and 843, corresponding to the junction of exons 10 and 11. The nucleotide sequence of the 113 bp insert is shown in Fig. 4C. It contained two stop codons in-frame with the upstream c-raf-1 coding sequence.

The Southern blot analysis result indicated that, in both normal (λE2) and activated c-raf-1 (pCN13), the 113 bp sequence was in the 1.2 kb XbaI-SacI

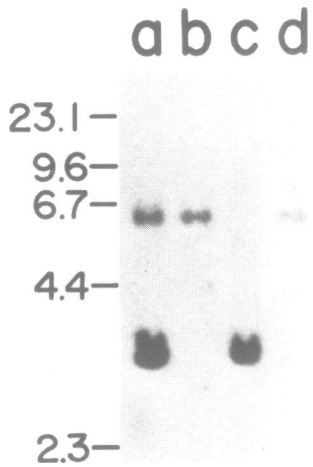


Figure 5
Differences in sequence recombined with c-raf-1 in two primary transformants. DNAs were digested with HincII, and a mixture of 0.28 kb EcoRI-SacI fragment and 0.22 kb SacI fragment of λC23 insert, corresponding to first 530 nucleotides of cDNA (see Fig. 4) was used as a probe. Lanes a, 7N-1 DNA; b, 2C-1 DNA; c, human placenta DNA; d, NIH 3T3 DNA.

fragment in the intron between exons 10 and 11. Nucleotide sequence analyses of appropriate regions of the pXS subclone obtained from pCN13 (Fig.1) revealed the 113 bp sequence to be located approximately 0.5 kb downstream of exon 10. This sequence was flanked by sequence motifs which to a great extent fulfilled the consensus for splicing (23). The 5' junction was TTTGTCCTGCCC-TAG/G and the 3' junction, AAG/GTACTA. These results suggested that it was alternative splicing which produced such an aberrant mRNA. From northern blot analysis, using a 170 bp NcoI-RsaI fragment of the pXS insert containing the extra exon as a probe (data not shown), no band could be detected, suggesting alternative splicing to be rare.

Activation of c-raf(-1) by recombination with three different genes

We used the 0.5 kb 5' terminal fragment of the λC23 insert, which contains most of the non-raf portion of cDNA, as a probe for blot hybridization. As can be seen in Fig. 5, 6.6 kb and 3.6 kb HincII bands were detected in the DNA of 7N-1 cells. The former was clearly shown to have been derived from the mouse genome and the latter from the human genome by Southern blot analysis of DNAs from NIH 3T3 cells and human placenta giving specific bands at 6.6 kb and 3.6 kb, respectively. This result suggests that the sequence which recombined to c-raf-1 in 7N-1 exists as a single copy in the human genome.

The same probe detected no band except an endogeneous one in the DNA of 2C-1 cells. Since we have not cloned the activated c-raf-1 of 2C-1, we do not know whether or not the upstream sequence which recombined to c-raf-1 in 2C-1 is of mouse or human origin, only that it is different from the sequence found in 7N-1.

Table 1 Structure of activated c-raf(-1)

DNA	Recombination	Upstream sequence	Reference
Human			
Stomach cancer	between exons 5 and 6	Unknown (hydrophobic)	27
Glioblastoma line	7 and 8*	Lipocortin II	Yamamoto, T. (Personal communication)
Colon mucosa	7 and 8*	Unknown	This study
Colon cancer	7 and 8*	N.D.	This study
Rat			
Hepatocellular carcinoma	7 and 8*	Unknown	5

N.D.: Not determined, *: Recombination induced during transfection

We have also previously cloned the DNA of the rat activated c-raf gene (4,5). The region 5' to the breakpoint of rat activated c-raf cDNA did not hybridize with the DNA of either 2C-1 or 7N-1. A comparison of the upstream cDNA sequence of the activated c-raf-1 of 7N-1 with that of rat activated c-raf showed no significant homology. These results suggest each of the three activated c-raf(-1) obtained in our laboratory to have a 5' region derived from different sequences.

DISCUSSION

We have described in the present paper the genomic structure and nucleotide sequence of the cDNA of the activated c-raf-1 found in the primary transformant, 7N-1. Some characterization studies were performed on another activated c-raf-1 found in 2C-1. No recombination leading to c-raf-1 activation was detected in the DNA used for the transfection assays in either case. The facts that both 7N-1 and 2C-1 were sole transformants from primary transfections, but that their DNAs are able to induce secondary transformants efficiently, suggest the activated c-raf-1 to have been produced during the course of transfection, although the possibility remains of the rearrangement being present in a small fraction of the original DNA.

As far as we are aware, five different activated c-raf(-1) have been reported, including the three cases in our study (Table 1). In four of them,

a recombination occurred at the intron between exons 7 and 8 which could have been introduced during transfection[2]. Some of the activated oncogenes, detected by NIH 3T3 focus or nude mice assays, were thought to have been created by a rearrangement occurring during transfection; for instance, ret (24), mas (25) and mcf-3 (26). Activation of c-raf(-1) seems to occur at a higher frequency than that of the oncogenes mentioned above, and this fact prompted us to assume the presence of a hot spot for recombination in c-raf(-1). In a previous study (5), we reported the presence of an inverted repeat, CAGGAT/ATCCTG, in the intron of rat normal c-raf around the break point. In this study we found an inverted repeat, CTCCT/AGGAG, in human normal c-raf-1 around the breakpoint. In addition, the 36 bp sequences were found in an inverted form between normal c-raf-1 and the activated c-raf-1 at the region 5' to the breakpoint. Such sequences will produce aberrant secondary structures and may have some role in recombination.

Nucleotide sequence analysis of the c-raf-1 cDNA of the 7N-1 transformant revealed that fused mRNA had been transcribed from the recombined gene. The predicted hybrid protein was composed of a non-raf N-terminal polypeptide and a C-terminal c-raf-1 product encoded by exons 8-17. The region derived from c-raf-1 retained the kinase domain including the putative ATP binding site required for this oncogene's catalytic activity. This region, exons 8-17, was contained in all the activated c-raf(-1) so far analysed (Table 1).

If the N-terminal peptides fused to truncated c-raf(-1) products confer transforming activity by a similar mechanism in each activated c-raf-1, the sequences must have some common characteristics. For as far as our analysis went, however, no common feature was found among the predicted products. For instance, the N-terminal peptide, encoded by the activated c-raf-1 induced by human stomach cancer DNA, was found to be hydrophobic (27), but those encoded by the activated c-raf-1 of 7N-1 and the activated rat c-raf (5) were not hydrophobic. In the case of the activated c-raf-1 obtained from glioblastoma DNA, the 16 amino acid N-terminal of lipocortin II was fused to the truncated c-raf-1 product (Dr. T. Yamamoto, personal communication).

One mechanism of activation would conceivably be the loss of the putative regulatory domain of c-raf-1. It is reported that the insertion of a Molony leukemia virus long terminal repeat into the intron between exons 5 and 6, activates mouse c-raf (8). One characteristic of the N-terminal half of the c-raf-1 product is its having a cysteine-rich region homologous to that present in protein kinase C (28). Protein kinase C is known to be activated into a ligand independent form by the loss by proteolysis of the N-terminal

region containing these cysteine-rich regions. The N-terminal region of c-raf-1 was strongly suggested as a regulatory domain for serine-threonine kinase activity. Truncating the N-terminal coding region and linking the 3' half of the c-raf(-1) gene to the region which is under the control of an efficient promoter, seem to be necessary for c-raf(-1) activation.

We found there to be an alternative splicing in which there was a 113 bp insertion at the junction of exons 10 and 11. In alternatively spliced mRNA, stop codons present in this region may prevent the production of active kinase. The frequency of alternative splicing seems to be low, however, in the cells cultured under the conditions we used, and its physiological role remains to be elucidated.

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1. c-raf(-1) represents both human c-raf-1 and rat c-raf.
2. After this paper was submitted for publicaiton, Stanton et al. reported activation of human raf by similar mechanisms: Mol. Cell. Biol. (1987) 7, 1171-1179.

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