MOLECULAR AND CELLULAR BIOLOGY, June 1988, p. 2651–2654
0270-7306/88/062651-04$02.00/0

Vol. 8, No. 6

B-*raf*, a New Member of the *raf* Family, Is Activated by DNA Rearrangement

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Received 19 November 1987/Accepted 14 March 1988

Complementary DNA clones of a putative transforming gene were isolated from NIH 3T3 cells transformed with human Ewing sarcoma DNA. The gene was termed B-*raf* because it is related to but distinct from c-*raf* and A-*raf*. It appears that substitution in the amino-terminal portion of the normal B-*raf* protein confers transforming activity to the gene.

Most human transforming genes detected by the NIH 3T3 cell transfection assay belong to the *ras* gene family (19), although many other transforming genes have recently been isolated. However, in more than half the human tumors so far examined, no activation of any known oncogene could be detected. Thus, more information is required on transforming genes involved in human cancers. Therefore, we have been searching for new oncogenes and have reported the identification of a novel transforming gene from human Ewing sarcoma DNA, which turned out to be a very large gene (7). In this report we describe the isolation and molecular characterization of the transforming gene related to c-*raf* and A-*raf* (27; U. R. Rapp, J. L. Cleveland, and T. I. Bonner, *in* P. Reddy, T. Curran, and A. Skalka, ed., *The Oncogene Handbook*, in press).

Isolation of a probe specific for the transforming gene. High-molecular-weight DNA was prepared (3) from the secondary transformants (7), partially digested with restriction enzyme *Mbo*I, and fractionated by sucrose density gradient centrifugation (22). Fractions containing fragments of 35 to 45 kilobase pairs (kbp) of DNA were collected and inserted into the *Bam*HI site of the cosmid vector pHC79 (11, 15). The ligated DNAs were packaged in vitro (in vitro packaging kit GP10; Vector Cloning Systems Inc.) and introduced into *Escherichia coli* 490A. Clones were screened as described (9) with the human repetitive sequence *Alu* probe BLUR8 (18). By screening 2×10^6 independent colonies, we obtained 300 *Alu*-containing clones. A set of clones that together were thought to contain the entire sequence of the transforming gene were selected. Each cosmid DNA was digested with the restriction enzyme *Hin*dIII to give good separation of the digested fragments. The fragments were then fractionated by agarose gel electrophoresis and transferred to nitrocellulose filters. One filter was probed with cDNA made against transformant mRNA (Fig. 1a), and the other filter was probed with cDNA made against normal NIH 3T3 mRNA (Fig. 1b). Most of the bands of clones containing two or three *Hin*dIII fragments with *Alu* sequences (lanes 7 to 13) were detected by both transformant cDNA and NIH 3T3 cDNA, which suggested that these fragments were of mouse origin. On the contrary, in clones containing more than four *Hin*dIII fragments with *Alu* sequences (lanes 1 to 6), hybridization with cDNA was observed specifically in the transformant panel. The hybridizing fragments could not be detected in the NIH 3T3 panel even after longer exposure of the filter. The *Hin*dIII fragments that were specifically detected with transformant cDNA (fragments A to D in Fig. 1a and c) were prepared for further Northern (RNA) blot experiments. Two of these, the 5.4-kbp fragment A and the 8.2-kbp fragment D (Fig. 1a and c), could detect a faint 5.2-kb mRNA in the transformants (data not shown). We then partitioned fragment A into eight fragments with restriction endonuclease *Hae*III. One of these fragments, the 600-bp fragment, detected two species

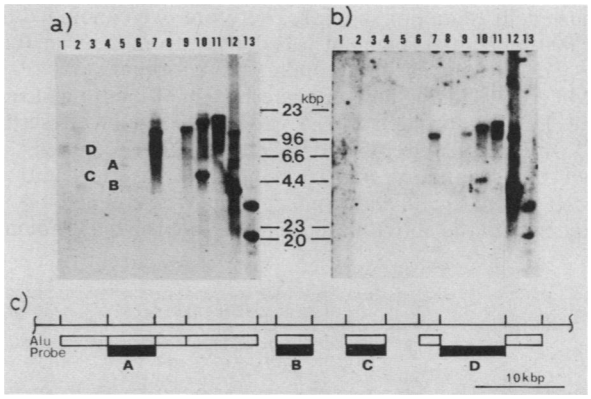

FIG. 1. Determination of DNA fragments specific for the transforming gene. *Alu*-positive cosmid clones were digested with the restriction enzyme *Hin*dIII, and the digests were subjected to Southern blot analysis. As probes, cDNAs made against mRNA extracted from the transformants (a) and normal NIH 3T3 cells (b) were used. Lanes 1 through 13 are for individual cosmid clones: lanes 1 to 6, clones containing more than four *Hin*dIII fragments with *Alu* sequences; lanes 7 to 13, clones containing two or three *Hin*dIII fragments with *Alu* sequences. Part of the transforming gene is illustrated schematically in panel c. The *Hin*dIII recognition sites are shown by vertical bars. Open boxes indicate fragments containing the *Alu* sequence, and solid boxes indicate fragments specifically detected in panel a. A, B, C, and D in panel a correspond to A, B, C, and D in solid boxes in panel c. Hybridization conditions were as described (14). Complementary DNAs were synthesized with random primer by using cloned Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories) in a total volume of 50 μl. The reaction conditions were as recommended by the supplier except that 2 μg of random hexadeoxynucleotide per ml, 1 mM dATP, 1 mM dGTP, 1 mM TTP, and 100 μCi of [α-^{32}P]dCTP (3,000 Ci/mmol) were used. The specific activity of the probe was about 1.5×10^8 cpm/μg of RNA.

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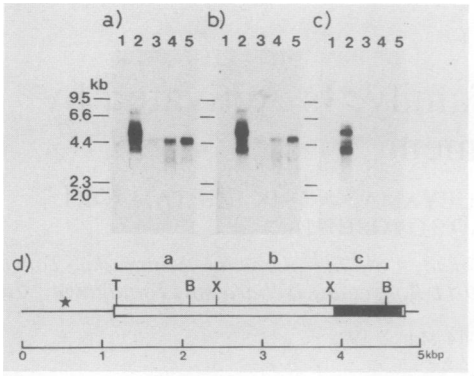

FIG. 2. Expression of B-*raf*-specific mRNA in the transformant. Poly(A)$^+$ RNAs (1 μg) extracted from cells and tissues were subjected to Northern blot analysis (21). As probes, fragments a (a), b (b), and c (c), depicted along with the schematic illustration of B-*raf* cDNA (d), were used. Lane 1, NIH 3T3 mRNA; lane 2, transformant mRNA; lane 3, mRNA extracted from Ewing sarcoma maintained in nude mice; lane 4, human placenta mRNA; lane 5, human embryo fibroblast TIG-1 mRNA. Sizes were determined from mobilities of denatured λ DNA digested with *Hin*dIII. (d) The star shows the location of the original 600-base-pair probe used in screening cDNA clones; the open box indicates a predicted open reading frame; the solid box indicates the kinase domain of B-*raf*. Hybridization and washing of the filter were done in the stringent condition as described (31). Abbreviations: T, *Tth*III1 site; B, *Bgl*II site; X, *Xho*I site.

of mRNA of 5.2 and 4.6 kb that were specifically expressed in transformants and in human cells, respectively, but not in mouse cells (data not shown). Therefore, we concluded that the 600-bp *Hae*III fragment was derived from the transforming gene present in the secondary transformants.

Molecular cloning and characterization of the transforming gene. Double-stranded cDNA was synthesized as described (20) with the polyadenylated [poly(A)$^+$] RNA prepared (1, 6) from the secondary transformants and inserted into the *Eco*RI site of λgt10 vector phage (13). We screened 4×10^6 plaques with the above-described 600-bp probe. Two clones, λEBR1 and λEBR2, with 5-kbp inserts that gave virtually identical restriction maps were isolated (Fig. 2d). Southern blot hybridization of the transformant DNA with the λEBR1 insert gave an *Eco*RI digestion pattern similar to that observed with the *Alu* probe (data not shown), suggesting that the cloned inserts represented the transforming gene. Nucleotide sequence data showed that the original probe had only 74 bp of the hybridizing sequence to the λEBR1 insert. Sequence analysis also revealed that the gene contained a sequence that was highly homologous with but distinct from the corresponding portion of c-*raf* (Fig. 2d, Fig. 3) (5). Since the sequence was also different from that of A-*raf* (2, 23), we named this transforming gene B-*raf*. The initiation codon of the transforming B-*raf* could not be identified in the λEBR1 insert. We next divided the sequence encoding the open reading frame into three portions (a, b, and c; Fig. 2d) and used them as probes to analyze the expression of the transforming gene in the transformants. By use of probes a and b, the 4.6-kb mRNA was detected in human cells (Fig. 2a and b, lanes 3, 4, and 5), which is consistent with the fact that the original probe, the location of which is shown by an asterisk in Fig. 2 (see below), detected the same 4.6-kb mRNA. On the other hand, probe c did not hybridize with mRNA of human origin (Fig. 2c, lanes 3, 4, and 5). Each of these probe detected two major mRNA species of 5.2 and 3.8 kb in the transformants, together with a few minor species. These minor mRNA species may represent precursor forms. It is also possible that mRNA species of different higher structures were generated during gel electrophoresis. These data suggested that the λEBR1 cDNA insert was derived from at least two different human genes which were rearranged in the transformants. Since no apparent rearrangement was observed in a tumor maintained in nude mice (Fig. 2a, b, and c, lane 3), activation of B-*raf* seemed to be induced during in vitro transfection.

Although the B-*raf*-specific mRNA was undetectable in human fibroblasts or placental cells (Fig. 2), we could detect a low level of expression as the 9.5-kb mRNA in the brains of a human fetus (data not shown). In contrast, the 9.5-kb B-*raf* mRNA was well expressed in many cell lines estab-

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   1  AAAACACTTGGTAGACGGGACTCGAGTGATGATTGGGAGATTCCTGATGGGCAGATTACAGTGGGACAAAGAATTGGATCTGGATCATTT
      LysThrLeuGlyArgArgAspSerSerAspAspTrpGluIleProAspGlyGlnIleThrValGlyGlnArgIleGlySerGlySerPhe

  91  GGAACAGTCTACAAGGGAAAGTGGCATGGTGATGTGGCAGTGAAAATGTTGAATGTGACAGCACCTACACCTCAGCAGTTACAAGCCTTC
      GlyThrValTyrLysGlyLysTrpHisGlyAspValAlaValLysMetLeuAsnValThrAlaProThrProGlnGlnLeuGlnAlaPhe

 181  AAAAATGAAGTAGGAGTACTCAGGAAAACACGACATGTGAATATCCTACTCTTCATGGGCTATTCCACAAAGCCACAACTGGCTATTGTT
      LysAsnGluValGlyValLeuArgLysThrArgHisValAsnIleLeuLeuPheMetGlyTyrSerThrLysProGlnLeuAlaIleVal

 271  ACCCAGTGGTGTGAGGGCTCCAGCTTGTATCACCATCTCCATATCATTGAGACCAAATTTGAGATGATCAAACTTATAGATATTGCACGA
      ThrGlnTrpCysGluGlySerSerLeuTyrHisHisLeuHisIleIleGluThrLysPheGluMetIleLysLeuIleAspIleAlaArg

 361  CAGACTGCACAGGGCATGGATTACTTACACGCCAAGTCAATCATCCACAGAGACCTCAAGAGTAATAATATATTTCTTCATGAAGACCTC
      GlnThrAlaGlnGlyMetAspTyrLeuHisAlaLysSerIleIleHisArgAspLeuLysSerAsnAsnIlePheLeuHisGluAspLeu

 451  ACAGTAAAAATAGGTGATTTTGGTCTAGCTACAGTGAAATCTCGATGGAGTGGGTCCCATCAGTTTGAACAGTTGTCTGGATCCATTTTG
      ThrValLysIleGlyAspPheGlyLeuAlaThrValLysSerArgTrpSerGlySerHisGlnPheGluGlnLeuSerGlySerIleLeu

 541  TGGATGGCACCAGAAGTCATCAGAATGCAAGATAAAAATCCATACAGCTTTCAGTCAGATGTATATGCATTTGGAATTGTTCTGTATGAA
      TrpMetAlaProGluValIleArgMetGlnAspLysAsnProTyrSerPheGlnSerAspValTyrAlaPheGlyIleValLeuTyrGlu

 631  TTGATGACTGGACAGTTACCTTATTCAAACATCAACAACAGGGACCAGATAATTTTTATGGTGGGACGAGGATACCTGTCTCCAGATCTC
      LeuMetThrGlyGlnLeuProTyrSerAsnIleAsnAsnArgAspGlnIleIlePheMetValGlyArgGlyTyrLeuSerProAspLeu

 721  AGTAAGGTACGGAGTAACTGTCCAAAAGCCATGAAGAGATTAATGGCAGAGTGCCTCAAAAAGAAAAGAGATGAGAGACCACTCTTTCCC
      SerLysValArgSerAsnCysProLysAlaMetLysArgLeuMetAlaGluCysLeuLysLysLysArgAspGluArgProLeuPhePro

 811  CAAATTCTCGCCTCTATTGAGCTGCTGGCCCGCTCATTGCCAAAAATTCACCGCAGTGCATCAGAACCCTCCTTGAATCGGGCTGGTTTC
      GlnIleLeuAlaSerIleGluLeuLeuAlaArgSerLeuProLysIleHisArgSerAlaSerGluProSerLeuAsnArgAlaGlyPhe

 901  CAAACAGAGGATTTTAGTCTATATGCTTGTGCTTCTCCAAAAACACCCATCCAGGCAGGGGGATATGGTGCGTTTCCTGTCCACTGA
      GlnThrGluAspPheSerLeuTyrAlaCysAlaSerProLysThrProIleGlnAlaGlyGlyTyrGlyAlaProProValHisEnd
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FIG. 3. Nucleotide sequence of putative B-*raf* kinase domain. The nucleotide sequence and the amino acid sequence of the region with homology to the c-*raf*-1 and A-*raf* kinase domains is shown. Numbers indicate number of bases from the beginning of the homologous region. The cDNA inserted into pUC119 was unidirectionally deleted (10), and its nucleotide sequence was determined by the dideoxy-chain termination method with the 7-DEAZA sequencing kit (Takara Shuzo Co., Kyoto, Japan) (24).

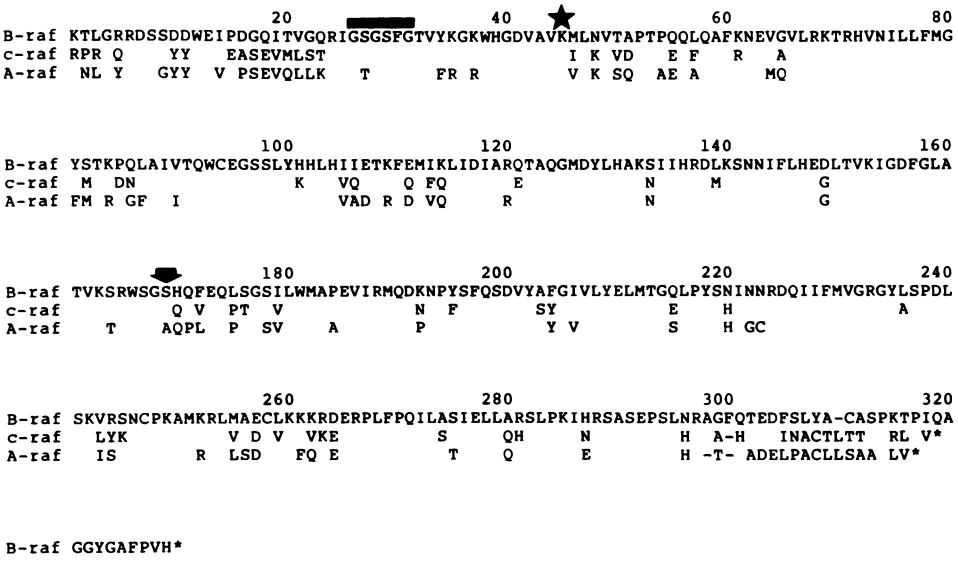

FIG. 4. Comparison of B-*raf* with other *raf* family genes. The amino acid sequence of the B-*raf* kinase domain is compared with those of c-*raf*-1 and A-*raf*. Only amino acid residues of c-*raf*-1 and A-*raf* that are different from those of B-*raf* are shown. The solid box and solid star indicate the ATP-binding site and its reactive lysine, respectively. The arrow indicates a residue corresponding to the phosphotyrosine of tyrosine kinases (corresponding to Tyr-416 of $pp60^{src}$). Dashes indicate gaps introduced to align sequences. Asterisks indicate the carboxyl terminus of each protein. Numbers indicate amino acid residues from the beginning of the homologous region of these genes.

lished from malignant tumors of human origin (data not shown). This suggests that B-*raf* is expressed in a tissue-specific manner and might play a role in malignant transformation.

The inserts of λEBR1 and λEBR2 were thought to be cDNAs against the 5.2-kb mRNA of the transformants. cDNAs against the 3.8-kb mRNA, which is also unique in the transformants, are being analyzed.

Identification of B-*raf*, a new member of the *raf* gene family. Inspection of the amino acid sequence predicted from the λEBR1 insert showed that the sequence from portion c had marked homology with the kinase domain of c-*raf*-1, but no protein was found in the data bank (NBRF-PIR) to contain a sequence homologous to that of portion a or b (Fig. 2d). The homology of the nucleotide sequence of B-*raf* (Fig. 3) and the corresponding sequence of c-*raf* (5) was 75% in the corresponding region. A comparison of the amino acid sequences of the kinase domains of B-*raf*, c-*raf* (5), and A-*raf* (2) is shown in Fig. 4. The overall homology in the amino acid sequence of the kinase domains of B-*raf* and c-*raf* was 76% and that of those of B-*raf* and A-*raf* was 74%. The most highly conserved amino acid residues in the members of the kinase superfamily are also conserved in B-*raf*. Like other serine/threonine kinases such as c-*raf* (5) and *mos* (30), B-*raf* has serine (position 169 in Fig. 4) instead of the tyrosine corresponding to Tyr-416 of $pp60^{src}$, although the putative ATP-binding site is exactly conserved.

Within each family of the kinase superfamily, individual members that have a closely related kinase domain, usually more than 70% homology in amino acid sequence, show extensive similarities in their overall structures (12). Thus, the B-*raf* protein, posessing a kinase domain closely related to that of c-*raf* and A-*raf*, is also expected to carry a second region of high homology to the A-*raf* and c-*raf* proteins (2, 17, 27) in its amino-terminal half.

Activation of the *raf* gene family. There is accumulating evidence that c-*raf*-1 expression is involved in carcinogenesis (27; Rapp et al., in press). Therefore, the mechanisms of activation of c-*raf*-1 should be clarified. The v-*raf* protein of 3611 murine sarcoma virus (26) and the v-*mil* protein of avian sarcoma virus MH2 (29) were generated by fusion of viral *gag* protein with the carboxyl-terminal half of c-*raf*-1 protein. In most other activated c-*raf*'s, the polypeptides in the amino-terminal portion are replaced by foreign polypeptides (8, 16, 28). Also, viral LTR insertion between exon 5 and 6 of c-*raf*-1 also activated the gene (25). In addition, A-*raf* became tumorigenic when its carboxyl-terminal half was fused with viral *gag* and expressed by using a retroviral vector (2). The activated form of B-*raf* protein also seems to lack the authentic amino-terminal sequence, because mRNA of the activated B-*raf* is a chimeric transcript in which the sequence for the B-*raf* kinase domain is fused at its 5′ side to a foreign sequence. From these findings, we speculate that the amino-terminal portions of the gene products of *raf* family proto-oncogenes have a regulatory role in normal function, and loss of the sequence results in cellular transformation (2, 4, 5, 17).

This work was supported by Grants-in-Aid for Special Project Research on Cancer-Bioscience from the Ministry of Education, Science and Culture of Japan.

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