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Structure and Biological Activity of Human Homologs of the *raf/mil* Oncogene

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Two human genes homologous to the *raf/mil* oncogene have been cloned and sequenced. One, c-*raf*-2, is a processed pseudogene; the other, c-*raf*-1, contains nine exons homologous to both *raf* and *mil* and two additional exons homologous to *mil*. A 3′ portion of c-*raf*-1 containing six of the seven amino acid differences relative to murine v-*raf* can substitute for the 3′ portion of v-*raf* in a transformation assay. Sequence homologies between c-*raf*-1 and Moloney leukemia virus at both ends of v-*raf* indicate that the viral gene was acquired by homologous recombination. Although the data are consistent with the traditional model of retroviral transduction, they also raise the possibility that the transduction occurred in a double crossover event between proviral DNA and the murine gene.

Oncogenes are evolutionarily conserved genes which have been identified because they induce cellular transformation either when naturally incorporated into a retrovirus or when their DNA is transfected into tissue culture cells (4). Most of the approximately 20 known oncogenes were originally isolated as viruses containing genes of nonprimate origin. However, their conservation allows the identification of the homologous genes in other vertebrate species, including humans, thereby facilitating the study of their role in human tumorigenesis. Jansen et al. recently reported (9) that the *raf* oncogene isolated from the murine transforming retrovirus 3611-MSV (20) and the *mil* (or *mht*) oncogene isolated from the avian transforming virus MH2 (8, 10, 11) are homologous genes derived from different species. This observation has been confirmed by direct sequence comparison (12, 27). Their deduced amino acid sequences are distantly related to the oncogenes which encode tyrosine-specific kinases as well as others such as *erb-B*, *fms*, and *mos* which apparently do not (14, 27). Although it was initially reported that the viral *raf* and *mil* gene products do not have tyrosine-specific kinase activity (21, 27), both are associated with a protein kinase which phosphorylates serine and threonine (17, 27). In this report, we describe the structure of two human genes which contain *raf*- and *mil*-related sequences and show that one of these genes, c-*raf*-1, is capable of causing transformation. Since it has been shown (6) that this gene is located at human chromosomal band 3p25, its structure should be useful in determining whether the gene is rearranged in human tumors which involve chromosomal rearrangements in this region. Sequences from within the gene should also be useful in characterizing alternately spliced messages.

MATERIALS AND METHODS

Selection of λ clones and restriction mapping. The human genomic library of Lawn et al. (13) obtained from *Hae*III and *Alu*I partial digests of human DNA inserted in Charon 4A was screened by hybridization to the *Xho*I-*Bst*E2 fragment of cloned v-*raf* (20). Plaque lift filters were hybridized and washed at 60°C in 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), conditions which had previously been shown to be capable of detecting the human genes on genomic blots. Restriction fragments, usually *Eco*RI fragments, from the cloned λ DNAs were nick translated and used as substrates for restriction mapping as previously described (3).

Sequencing. The sequence of c-*raf*-2 and exons 2 and 3 of c-*raf*-1 were determined by the chemical cleavage method (15). The sequence of the remainder of c-*raf*-1 was determined by the dideoxynucleotide chain termination method (23) on appropriate restriction fragments inserted into pBR322. Sequencing reactions were performed as described previously (32), with primers appropriate for the *Eco*RI, *Hin*dIII, and *Sal* sites of pBR322, except that chain elongation reactions were done at 37°C.

Immunoprecipitation of *gag-raf* proteins. Since the amino terminal of the Moloney leukemia virus (MoLV) *gag* polyprotein is myristilated, [^{3}H]myristate was used to label NIH/3T3 cells which had been transfected with 3611-MSV/c-*raf*-1 hybrid DNA. This choice of label results in the labeling of relatively few proteins, resulting in very clean immunoprecipitation gels. The cells were radioactively labeled by incubation for 2 h in medium containing 100 μCi of [^{3}H]myristic acid (5.22 pCi/nmol; New England Nuclear Corp., Boston, Mass.) per ml. Immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 5 to 20% gradient gels were performed as previously described (21).

RESULTS

Two human genes homologous to *raf/mil*. The human genomic library was screened by moderate-stringency hybridization with a cloned v-*raf* sequence as probe. Restriction mapping of the seven positive clones revealed that they represented two distinct sets of overlapping sequences (Fig. 1). When these clones were used as probes for hybridization to restriction digests of cloned 3611-MSV DNA, it was clear that both sets of clones contained sequences homologous to the 5′ and 3′ portions of v-*raf*. This observation was confirmed by nucleotide sequencing (see below). Thus the two sets of clones represent two different loci, which we hereafter refer to as c-*raf*-1 and c-*raf*-2. The *Eco*RI, *Sph*I, and *Pst*I fragments of these two sets of clones which hybridized to the

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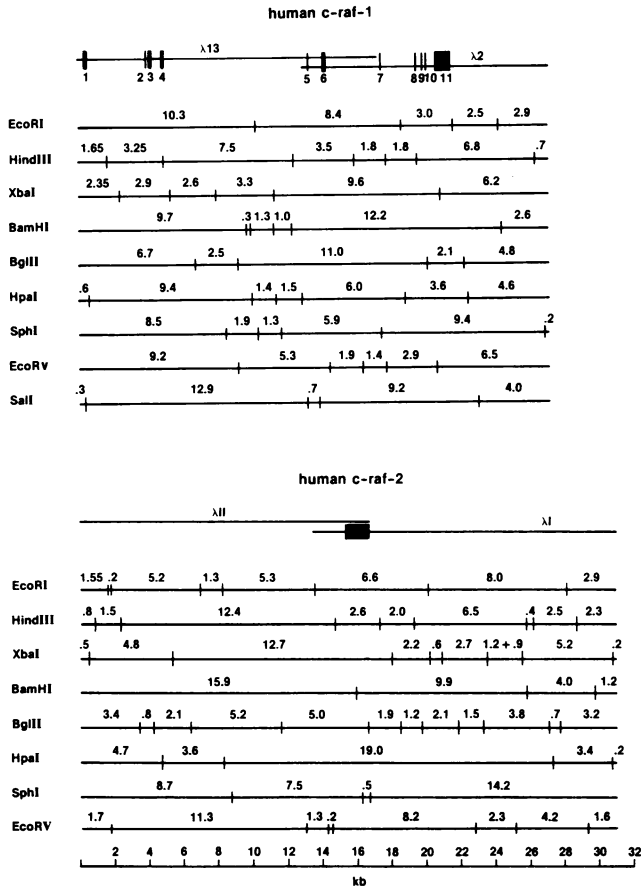

FIG. 1. Restriction maps of the human c-*raf* loci derived from overlapping λ phage clones. The c-*raf*-1 locus is defined by two clones, λ2 and λ13. The positions of the two clones and the exons which they contain are shown above the restriction map. Sizes of restriction fragments are shown in kilobases. The c-*raf*-2 locus is defined by clones λ1 and λ11, which contain a single region of homology to v-*raf* indicated by the black box. Three other clones which were similar to λ1 are not shown.

v-*raf* probe corresponded to the human restriction fragments which were previously detected (20) in Southern blots of human DNA. It therefore appeared that the two loci represent all the v-*raf*-related sequences in the human genome. To determine whether there are any other distantly related sequences in human DNA, we used the v-*raf* probe to hybridize at low stringency (3× SSC, 60°C) to *Eco*RI and *Sph*I digests of human DNA. The results (Fig. 2) show strong *Eco*RI bands at 8.4 and 3.0 kilobases (kb) and *Sph*I bands at 9.4 and 5.9 kb which correspond to the fragments of c-*raf*-1. Somewhat fainter bands are detected at 6.6 and 7.4 kb for *Eco*RI and *Sph*I, respectively, which correspond to the fragments of c-*raf*-2. No other significant bands are evident in this blot, although we have detected the 0.45-kb *Sph* fragment of c-*raf*-2 in other blots. Similar blots (data not shown) with *Hin*dIII, *Xba*I, *Bgl*II, *Eco*RV, *Bgl*I, *Pvu*II, *Pst*I, and *Kpn*I revealed no bands which are not accounted for by the restriction maps of c-*raf*-1 and c-*raf*-2. We therefore conclude that there are no other loci in the human genome which are as closely related to v-*raf* as the c-*raf*-2 locus is. However, screening a human cDNA library at lower stringency has yielded clones which indicate the presence of additional *raf* related gene(s) (G. Mark, unpublished observations).

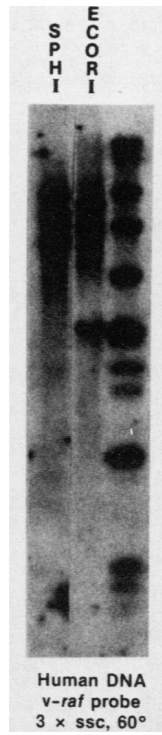

FIG. 2. Blot of v-*raf* related sequences in human cellular DNA. Human DNA (2 μg) was digested with *Sph*I (lane 1) or *Eco*RI (lane 2), electrophoresed in a 0.7% agarose gel, transferred to nitrocellulose, and hybridized to the v-*raf* probe in 3× SSC at 60°C. Lane 3 contains a marker composed of ^{32}P-end labeled restriction fragments from *Hin*dIII digestion of λ DNA and *Taq*I digestion of φX 174 DNA.

c-*raf*-1 is a gene with introns. The approximate positions of the v-*raf*- and v-*mil*-related sequences within the clones were determined by a combination of electron microscopy of heteroduplexes of v-*raf* DNA with subclones of c-*raf*-1 and c-*raf*-2 and hybridization of v-*raf* and v-*mil* probes to restriction digests of the clones. The appropriate regions of the clones were then sequenced and compared with each other and with the v-*raf* and v-*mil* sequences (Fig. 3). The sequence of the c-*raf*-1 clones demonstrates that 11 exons are homologous to the v-*mil* sequence. Since the homology to v-*mil* begins within exon 1, the 5′ end of this exon was defined as the point at which homology to the c-*raf*-2 sequence disappears. This point corresponds to a sequence which appears to be a splice acceptor sequence, i.e., an AG preceded by 10 to 12 nucleotides of pyrimidine-rich sequence (18). All the other exon-intron boundaries are defined by a break in homology to v-*mil* or v-*raf* and show characteristic splice acceptor sequences as just described for exon 1 and splice donor sequences beginning with a GT and usually followed by $\frac{A}{G}$AG. The 5′ boundary of exon 1 has recently been verified by comparison with a human cDNA sequence (T. I. Bonner, H. Oppermann, P. Seeburg, S. B. Kerby, M. A. Gunnell, R. Nalewaik, and U. R. Rapp, submitted for publication). Homology to v-*raf* begins within exon 3. Homology to both v-*raf* and v-*mil* continues through the termination codon in exon 11. As previously noted (27), v-*mil* homology terminates 12 nucleotides beyond the terminator, whereas v-*raf* homology extends ca. 190 nucleotides into the 3′ untranslated sequence. The overall homology of the coding regions of c-*raf*-1 and v-*raf* is 90%, and their

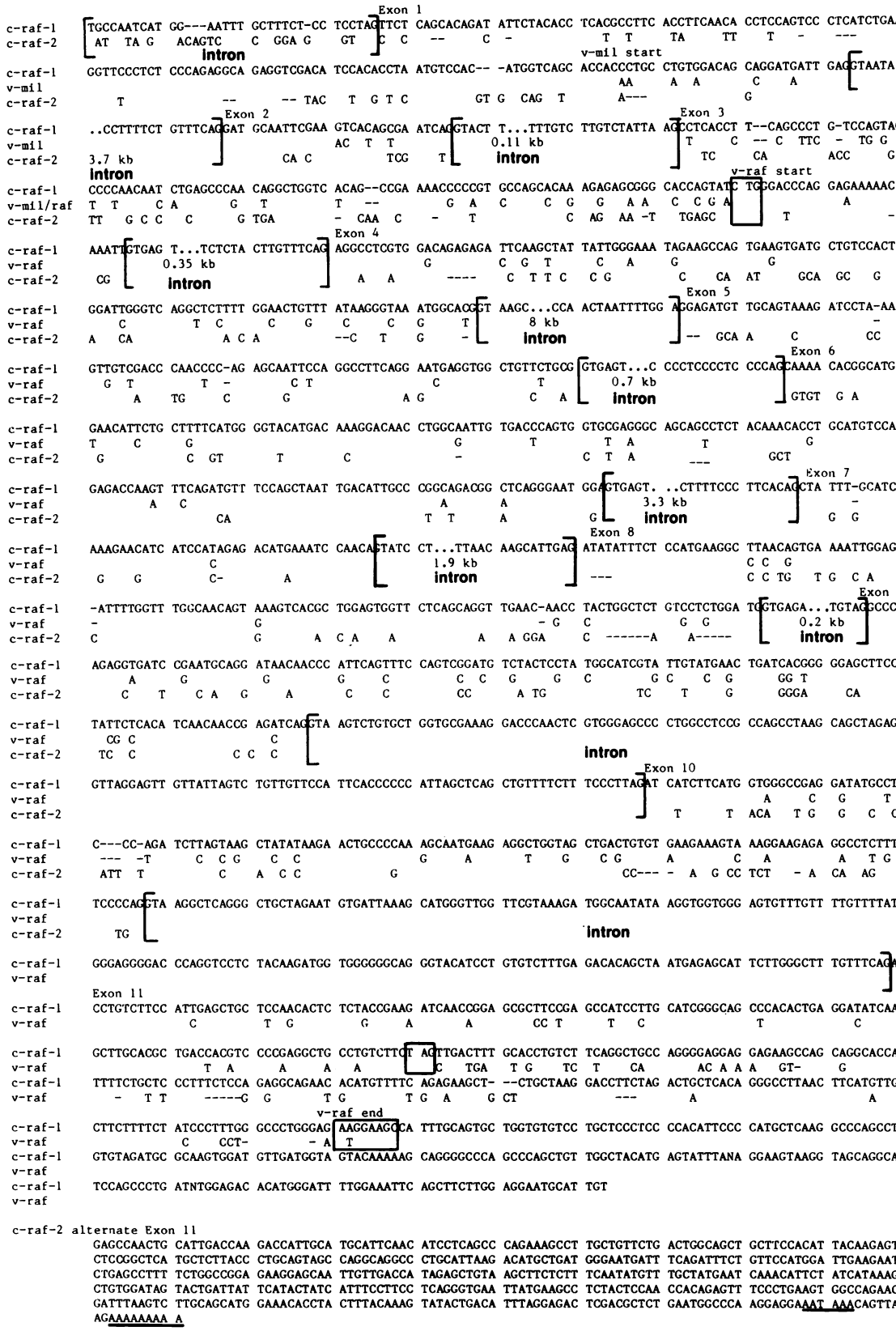

FIG. 3. Sequences of c-*raf*-1 and c-*raf*-2 compared with the viral *mil* or *raf* sequences. The exon sequences of c-*raf* are shown, along with part of the adjacent intron sequence. The other sequences are shown only where they differ from the exons c-*raf*-1. Dashes indicate gaps introduced to align the sequences. The beginning and ending of v-*raf* homology as well as the terminator codon, TAG, are boxed. The sequence for v-*mil* is used for comparison 5′ of the beginning of v-*raf* homology. A putative alternate exon sequence for c-*raf*-2 is shown at the bottom, with the poly(A) signal sequence and the presumptive poly(A) sequence underlined.

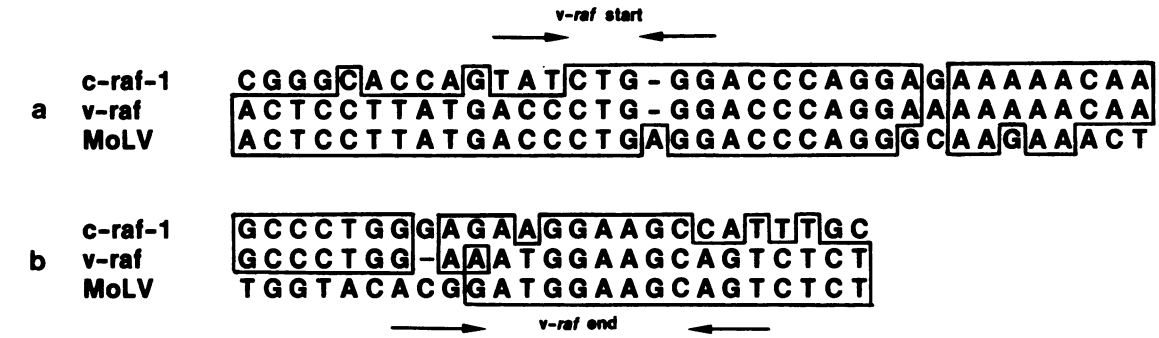

FIG. 4. Comparison of the c-*raf*-1 sequence with those of v-*raf* and MoLV at the junctions between viral sequence and *raf* sequence. (a) 5′ junction; (b) 3′ junction. Homologous nucleotides are boxed.

homology in the 3′ untranslated region is 73%. The 3′ end of the message has been identified by cloning of cDNAs from normal human liver and placenta (Bonner et al., submitted for publication). Sequence comparisons with c-*raf*-1 indicate that exon 11 has about 900 nucleotides of 3′ untranslated sequence ending with a polyadenylate [poly(A)] addition site which is located 90 nucleotides 5′ of the *Eco*RI site at 21.7 kb on the restriction map. Since a 2.97-kb liver cDNA clone extends 810 nucleotides 5′ of the beginning of exon 1, there is a substantial amount of sequence corresponding to the 5′ end of the message which has yet to be identified within the genome. The size of the exons, excluding the 3′ untranslated sequence of exon 11, ranges from 28 to 177 nucleotides and is consistent with the observation that the number of amino acids encoded by the average exon is approximately 45 (5). The introns range in size from 141 nucleotides to 8 kb and are generally larger than their counterparts in chicken (10) and mouse genes (M. Goldsborough, personal communication). Since *raf* is distantly related to the avian *src* gene, we have examined whether the exon boundaries are conserved. Using a previously published amino acid sequence alignment (27), which spans the junctions between *raf*/*mil* exons 4 to 10 and *src* exons 7 to 12, we found that although some of these boundaries occur within the more conserved regions of the amino acid sequences, none of the boundaries coincide.

c-*raf*-2 is a processed pseudogene. The sequence of the c-*raf*-2 locus indicates the absence of any introns. With the exception of numerous small deletions and insertions, the c-*raf*-2 sequence is homologous with exons 1 to 10 of c-*raf*-1. These insertions and deletions introduce numerous shifts in the reading frame of c-*raf*-2 relative to that of c-*raf*-1. In addition, there are approximately 20 termination codons in each of the three possible reading frames, with only three open reading frame stretches of more than 200 nucleotides, the largest being 350 nucleotides. Thus, c-*raf*-2 is a pseudogene. Similar pseudogenes which lack the introns found in their normal counterparts have been termed processed pseudogenes and are thought to have been generated by a reverse transcription of spliced mRNA (24). Until the 5′ end of c-*raf*-1 is defined, it is not possible to determine whether c-*raf*-2 is a complete copy of the 5′ end of the message. However, it is clear that c-*raf*-2 does not maintain homology with c-*raf*-1 all the way to its 3′ end. It is difficult to precisely locate the point at which the homology is lost, since the homology goes from ca. 95% in the middle of exon 10 to unrecognizable at approximately 50 nucleotides into exon 11. However, the point of divergence appears to be near the boundary between exons 10 and 11. This suggests that c-*raf*-2 might have arisen from a message with an alternative splicing pattern of its 3′ end. Such alternative splicing was originally observed in the calcitonin (1) gene and has since been observed in the k-*ras* oncogene (16). We have therefore presented the remaining c-*raf*-2 sequence as a putative alternate exon 11. This sequence terminates with a string of nine A residues which might represent the poly(A) tail of the message from which c-*raf*-2 was derived. The oligoadenylate sequence is preceded, with nine intervening nucleotides, by the sequence AATAAA which generally appears about 20 nucleotides 5′ of the poly(A) addition site in previously described mRNA sequences (19). If c-*raf*-2 is actually derived from alternative splicing, then the related exon(s) should be found in the c-*raf*-1 locus. To test for the presence of such exons, we used the *Sph*I-*Hin*dIII fragment of the alternative c-*raf*-2 exon (spanning nucleotides 28 to 255 of the alternate exon 11) as probe. Low-stringency hybridization to restriction digests of human DNA reveals the presence of one or two bands in each digest which are not accounted for by the c-*raf*-2 locus. However, this probe does not hybridize to the cloned DNA of the c-*raf*-1 locus. Therefore, if there is such an alternative exon 11 at the c-*raf*-1 locus, it is located beyond the 3′ end of the existing clones.

Although the overall homology of c-*raf*-2 to c-*raf*-1 (comparing exons 1 to 10) is only ca. 80%, c-*raf*-2 is considerably more closely related to c-*raf*-1 than to v-*raf*. Considering only exons 4 to 10, where all three sequences can be compared, there are 26 nucleotide positions at which c-*raf*-2 matches v-*raf* but not c-*raf*-1, 54 positions at which it matches c-*raf*-1 but not v-*raf*, and 160 positions at which it differs from both. There are in addition 10 positions at which all three differ from one another. Since a pseudogene does not appear to exist in mice (M. Goldsborough, personal communication), the pseudogene was formed after the evolutionary divergence of the rodent and primate lineages. The existence of a substantial number of positions at which c-*raf*-2 matches v-*raf* but not c-*raf*-1, coupled with the large number of positions at which c-*raf*-2 differs from *v-raf* and c-*raf*-1, suggests that the pseudogene was formed relatively early in primate evolution.

Transduction of v-*raf*. The transforming retroviruses are generally considered to have arisen from a recombination event between a nontransforming retrovirus and a normal cellular gene (4). In the case of the 3611-MSV virus, this recombination event would have involved a murine retrovirus which is closely related to the Moloney leukemia virus (MoLV) and the mouse gene which is homologous to the human c-*raf*-1. In comparing the sequences of c-*raf*-1, v-*raf*, and MoLV (25) (Fig. 4) to determine the precise points at which this recombination would have occurred, we find that there is a stretch of approximately 10 nucleotides at each end

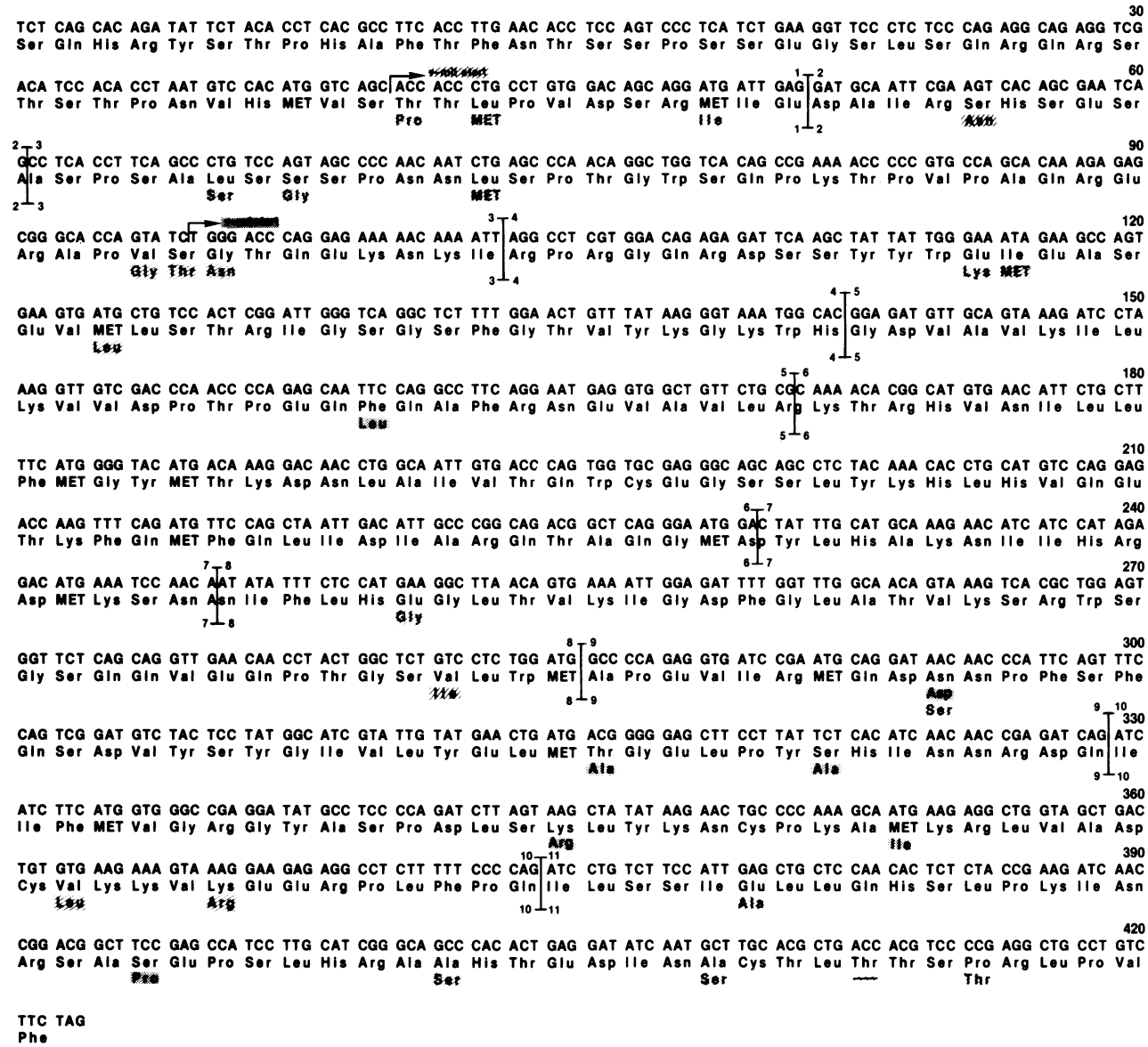

FIG. 5. Deduced amino acid sequence that would be encoded by exons 1 through 11 of c-*raf*-1. Amino acids which are different in v-*mil* or v-*raf* are shown below the c-*raf*-1 amino acid sequence and are shaded with slashes or stippling, respectively. The exon boundaries are indicated by brackets, and the beginnings of the v-*mil* and v-*raf* sequences are indicated by arrows.

of the v-*raf* sequence in which the c-*raf*-1, v-*raf*, and MoLV sequences are nearly identical. Therefore, the beginning of v-*raf* homology occurs in exon 3 of c-*raf*-1 within the sequence CTGGGACCCAGG, which is identical in c-*raf*-1 and v-*raf* and differs from the corresponding MoLV sequence only by an extra A in the MoLV sequence (Fig. 4a). Allowing additional mismatch, this homology region can be extended in the 3′ direction to include 20 nucleotides with 16 matches. On the basis of a comparison of only the v-*raf* and MoLV sequences, Rapp et al. had previously identified (20) the beginning of the *raf*-specific sequence in v-*raf* as occurring immediately after this 12-nucleotide sequence. Furthermore, Rapp et al. hypothesized that the A which is present in MoLV but absent in v-*raf* was deleted after the recombination event to place the v-*raf* sequence in the proper reading frame. However, since this A is missing in the human c-*raf*-1 sequence, it is likely that it is also missing in the murine homolog. It therefore appears that the point of recombination is within the CTG which is common to the three sequences. Similarly, the end of v-*raf* homology occurs within the 3′ untranslated sequence of exon 11 (Fig. 4b) within the sequence AAGGAAGC, which is nearly (7 of 8 nucleotides) identical to the corresponding sequence, ATGGAAGC, of v-*raf* and MoLV. The single nucleotide difference presumably reflects evolutionary divergence between the human and mouse genes.

These sequence homologies imply that the point of recombination cannot be assigned to a specific nucleotide at either end of the v-*raf* sequence. More importantly, the sequence homologies provide clear evidence that the transduction occurred via homologous recombination. Although short stretches of homology between viral and cellular sequences have been observed in other transforming retroviruses, only in the case of Finkel-Biskis-Jinkins virus, which contains the *fos* oncogene, has such homology been observed at both ends of the acquired sequence (31). The sequence data for

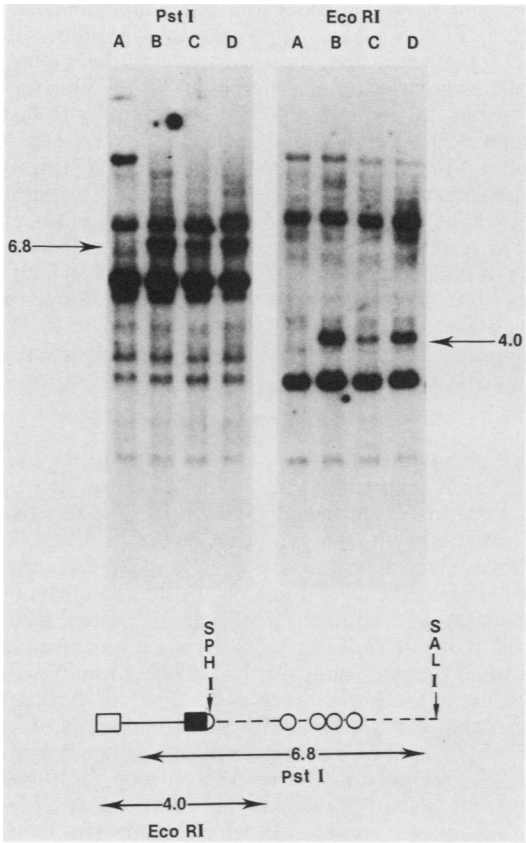

FIG. 6. Presence of transfected DNA in transformd NIH/3T3 colonies. NIH/3T3 cells were transfected with the hybrid v-*raf*/c-*raf*-1 DNA depicted at the bottom. The open box, the solid line, and the closed box represent the long terminal repeat, *gag*, and v-*raf* sequences of the cloned DNA of 3611-MSV virus. The open circles represent exons 7 through 11 of c-*raf*-1, and the dashed line represents the intervening intron and 3′ flanking sequence. The 4.0-kb *Eco*RI and 6.8-kb *Pst*I restriction fragments which are characteristic of hybrid DNA are also indicated. These fragments are detected in DNA isolated from transformed colonies (lanes B through D above) but not in the control NIH/3T3 DNA (lane A) with a v-*raf* probe (the 0.7-kb *Xho*I-*Sst*II fragment of 3611-MSV) hybridized in 3× SSC at 60°C and washed in 1× SSC at 60°C.

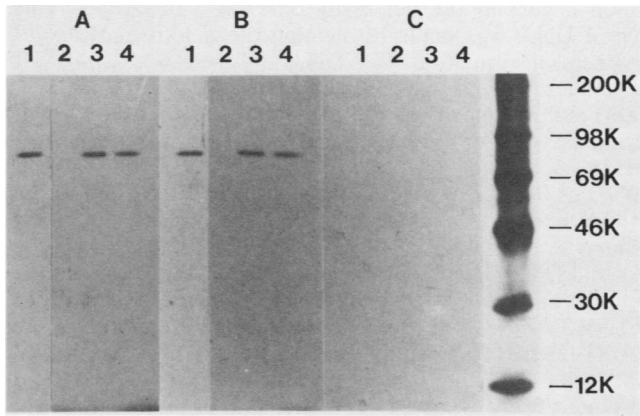

FIG. 7. Immunoprecipitation of *gag-raf* proteins from NIH/3T3 cells transfected with hybrid DNA described in Fig. 6. Cells were labeled with [^{3}H]myristate, lysed, and immunoprecipitated with anti-*gag* and anti-*raf* sera as described previously (A. Schultz, T. Copeland, G. Mark, U. Rapp, and S. Oroszlan, Virology, in press). Panel A, Cells transformed by 3611 MSV; panel B, cells transformed with hybrid DNA; panel C, untransfected NIH/3T3 control cells. Lanes: 1, anti-p30 (*gag*) serum; 2, anti-SP63 serum competed with SP63 peptide; 3, anti-SP63 serum; 4, anti-v-*raf* protein serum; marker lane contains myosin (200 kilodaltons [kd]), phosphorylase B (97 kd), bovine serum albumin (69 kd), carbonic anhydrase (30 kd), and cytochrome *c* (12 kd). Anti-SP63 is a rabbit antiserum to a synthetic peptide corresponding to the 12 carboxy-terminal amino acids of the v-*raf* or c-*raf*-1 protein. Anti-v-*raf* is a rabbit serum against v-*raf* protein (corresponding to amino acids 178–421 of Fig. 4) expressed in *Escherichia coli* (H. Oppermann, unpublished observations).

raf and *fos* are consistent with the current model of retroviral transduction of oncogenes (28), which envisions (i) the integration of a retroviral provirus upstream of the gene, (ii) deletion of the 3′ end of the provirus and the 5′ end of the gene to form the 5′ virus oncogene junction, (iii) transcription and splicing to remove the introns from the gene, (iv) packaging of the transcript into heterozygous virions, and (v) recombination between the chimeric RNA and parental viral RNA during subsequent reverse transcription to establish the 3′ junction. The presence of clear homology between the parental virus and the gene at both junctions for v-*raf* and v-*fos* suggests a simpler model in which there is (i) a double crossover via homologous recombination between the gene and either integrated or unintegrated proviral DNA, followed by (ii) transcription and splicing to remove the introns and (iii) packaging of the RNA in a virion to establish the new viral genome. In this case, the homologous recombination is presumably not based on long stretches of near-perfect homology as is the case for chromosomal crossover during meiosis. Instead, we suggest that it is similar to the partially homologous recombination which occurs when mammalian cells are transfected with two nonhomologous DNAs (2). In this case it has been shown that recombination junctions between integrated copies of the exogenous DNAs occur within 20- to 50-nucleotide stretches of partial (62 to 75%) homology. However, in formation of the junction, some or all of the homology region may be deleted in multiples of 13 to 14 nucleotides, deletions of 0 to 54 nucleotides having been observed. It is not clear from the published report whether the recombination took place before or after the exogenous DNAs were integrated, and it is therefore not clear that it is directly applicable to the process of transduction. It does, however, suggest that there is a mammalian recombination protein which recognizes homologous DNA regions and produces staggered recombination joints. If such a protein were active in oncogene transduction, the relatively poor homology at a sizeable distance from the apparent joint may have been overlooked in previous examinations of oncogene sequences.

Transforming activity of the *raf* gene. Since the *ras* oncogenes appear to acquire their transforming ability through mutation of a single amino acid (7, 22, 29, 30), it is of some interest to know whether the c-*raf*-1 gene is capable of transformation or whether the v-*raf* gene is transforming because of an altered amino acid sequence. The amino acid sequence encoded by exons 1 to 11 of the c-*raf*-1 gene (Fig. 5) shows seven amino acid changes relative to v-*raf* and 22 amino acid changes relative to v-*mil*. Of the changes relative to v-*raf*, one occurs in exon 5 and the remaining six are clustered at the carboxy terminal in exons 9 to 11. To test whether any of the last six changes are essential for the transforming activity of v-*raf*, we have constructed a hybrid DNA making use of the conserved *Sph*I restriction site

which occurs at the beginning of exon 7 in c-*raf*-1. This hybrid DNA was made by ligating the 2.3-kb *Eco*RI-*Sph*I fragment of viral 3611-MSV DNA and the 5.6-kb *Sph*I-*Sal*I fragment of c-*raf*-1 into the *Eco*RI and *Sal*I sites of pBR322. The resulting plasmid (Fig. 6) thus contains the 5′ long terminal repeat of 3611-MSV, the viral *gag* gene sequences, and the portion of v-*raf* which is 5′ of the *Sph* site followed by exons 7 to 11 of c-*raf*-1, the poly(A) addition site at the end of exon 11, and an additional 1.5 kb of 3′ flanking sequence. When NIH/3T3 cells were transfected with the plasmid DNA (linearized by cutting with *Sal*I), transformed colonies appeared. Although the transformation efficiency of 3611-MSV DNA was 4,000 FFU/μg of DNA and that of the *Eco*RI-*Hin*dIII fragment containing the 5′ half of 3611-MSV was 1,000 FFU/μg, the hybrid DNA gave only 4 to 5 FFU/μg. There were no spontaneous foci in an equivalent number of untransfected cells.

To verify that the colonies resulted from expression of the hybrid gene, single-cell clones from individual foci were grown and tested for the presence of the transfected DNA and expression of hybrid protein. When tested by Southern blotting with a v-*raf* probe, all foci showed the presence of *Eco*RI and *Pst*I bands characteristic of the hybrid DNA (Fig. 6). The intensity of these bands indicated that they were present at approximately 1 copy per cell. The absence of bands corresponding to those of 3611-MSV viral DNA eliminated the possibility that the transformation results from a contamination with either 3611-MSV virus or a plasmid containing v-*raf*. Seven cell clones were also tested for the presence of a polyprotein analogous to the 3611-MSV *gag-raf* polyprotein. As shown for a representative clone in Fig. 7, there is a 75,000-dalton protein present in five of the clones which, like the similar protein of 3611-MSV-transformed cells, is immunoprecipitated by *gag* (MoLV p30) and *raf* antisera. We therefore conclude that the cells are transformed as a result of the presence of the transfected DNA. However, the very low transformation efficiency requires an explanation. A clue can be found in the results of Sodroski et al. (26), who found a similar low efficiency (1 to 2 FFU/μg) for a similar hybrid (*Rec-Kpn*) between viral *fes* and its homologous human gene. With a selectable marker (pSV2*gpt*, which confers resistance to mycophenolic acid) linked to the *fes* DNAs, they found that fewer of the resistant cells were transformed with the hybrid DNA than with the viral DNA. Furthermore, when untransformed but resistant clones were examined, they were found to have either entirely deleted or rearranged the *fes* DNA. This observation suggests that the transforming region of the hybrid DNA, which is more extended as a result of the presence of introns, may be more susceptible to breakage or other rearrangement, thereby lowering its transformation efficiency. To test this possibility, the human c-*raf*-1 DNA in the hybrid was replaced with the corresponding portion of a human cDNA clone (Bonner et al., submitted for publication). This new hybrid is identical to the original hybrid except for the removal of the intron sequences and the 1.5 kb of 3′ flanking sequence. On transfection, this DNA transforms NIH/3T3 cells with an efficiency (2,000 FFU/μg) comparable to that of the 3611-MSV controls. Although this experiment does not establish the mechanism by which the transformation efficiency is reduced when the introns and flanking sequence are present, it does allow us to conclude that the last six amino acid differences between murine v-*raf* and human c-*raf*-1 are not essential to the transforming ability.

The remaining amino acid difference in exon 5 is presumably also not essential, since the phenylalanine of c-*raf*-1 also occurs in v-*mil*. This supposition is consistent with the report (18) that the transforming potential of the mouse c-*raf* gene can be activated by insertion of an MoLV long terminal repeat within the genome. Preliminary attempts to test this last amino acid difference by substituting exons 4 to 6 into v-*raf* did not result in transformation. However, this hybrid DNA was constructed by deleting 5 kb from the 8-kb intron between exons 4 and 5. Analysis of the transfected cells by neomycin selection indicates that abnormally small hybrid proteins of molecular weight 65,000 and 58,000, which react with both *gag* and *raf* antisera, are produced. Since the *raf* antiserum is against sequences found in exons 6 to 11, this result suggests that the deletion has induced aberrant splicing of the RNA.

DISCUSSION

We have shown that there are two human genes homologous to the *raf*/*mil* oncogenes. One of these two genes, c-*raf*-2, is a processed pseudogene which appears to have been formed early in the evolution of the primates. It has been shown elsewhere that this gene is located on chromosome 4, whereas the c-*raf*-1 gene is located on chromosome 3 (6). Since c-*raf*-2 contains numerous insertions and deletions relative to c-*raf*-1 as well as numerous termination codons in all reading frames, it is unlikely to be functional. The location of the c-*raf*-1 gene at band p25 of chromosome 3 suggests that it might be involved in some types of human tumors which contain characteristic chromosomal aberrations in this region (6). The restriction maps of this locus should prove useful in evaluating these possibilities, as should the probes provided by the clones. The clones as described here contain all the sequences which are homologous to the viral transforming genes, v-*raf* and v-*mil*, and contain the 3′ end of the gene as defined by the poly(A) position of cDNA clones from placental and liver mRNAs (Bonner et al., submitted for publication). However, the sequence of the c-*raf*-2 gene suggests that it may have been produced from an alternatively spliced message in which exon 11 was replaced by another exon(s). If such an exon exists, it is not located in the present clones and must be at least 5.5 kb 3′ of the end of the present exon 11.

The exon sequences of the present clones account for 2.2 kb of a 2.97-kb human liver cDNA (Bonner et al., submitted for publication) and there must therefore be more of the gene to be identified beyond the 5′ end of the present clones. Analysis of cDNA clones indicates that only 0.6 kb of the additional 0.8 kb is actually coding sequence. Preliminary analysis of clones from the 5′ end of c-*raf*-1 indicates that these additional coding sequences are distributed over another 19 kb. Thus, the entire gene spans more than 40 kb. As a first step in assessing what changes in the gene are necessary to activate its tumorigenic potential, we have shown that the c-*raf*-1 gene can be substituted for the 3′ two-thirds of v-*raf* without impairing its transformation capability in transfection assays. This eliminates all but one of the seven amino acid changes between murine v-*raf* and human c-*raf*-1 as being essential for transformation. Since transformation of mouse NIH/3T3 cells by promoter insertion activation of the cellular mouse gene has been achieved (17a), it is unlikely that isolated amino acid changes are necessary to activate the *raf* gene. It therefore appears that the *raf* genes cause transformation either by an inappropriately high level of expression of the normal gene product or by expression of a truncated gene product which does not function normally.

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