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Definition of the Human *raf* Amino-Terminal Regulatory Region by Deletion Mutagenesis

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Activation of transforming potential of the cellular *raf* gene has uniformly been associated with the deletion of amino-terminal coding sequences. In order to determine whether 5′ truncation alone could activate cellular *raf*, we constructed 21 human c-*raf*-1 cDNAs with variable BAL 31-generated deletions distal to a Moloney murine sarcoma virus long terminal repeat and a consensus translation initiation sequence. The deletions ranged from 136 to 1,399 nucleotides of coding sequence and shortened the 648-amino-acid *raf* protein by 44 to 465 amino acids. The full-length c-*raf*-1 cDNA was nontransforming upon transfection of NIH 3T3 cells, as were four mutants with deletions of 142 or fewer amino acids. Seven of nine mutants with deletions of 154 to 273 amino acids induced transformation with efficiencies ranging from 0.25 to 70 foci per μg of DNA. Mutants with deletions of 303 to 324 amino acids displayed high transforming activities (comparable with that of v-*raf*), with a peak activity of 2,400 foci per μg of DNA when 305 amino acids were deleted. Deletions of >383 amino acids, extending into the *raf* kinase domain, lacked transforming activity. Northern (RNA) blotting and immunoprecipitation assays indicated that transfected NIH cells expressed *raf* RNAs and proteins of the expected sizes. Thus, 5′ truncation alone can activate *raf* transforming potential, with a sharp peak of activation around amino acid 300. Analysis of three *raf* genes previously detected by transfection of tumor DNAs indicated that these genes were activated by recombination in *raf* intron 7 and encoded fusion proteins containing amino-terminal non-*raf* sequences. The extent of deletion of *raf* sequences in these recombinant genes corresponded to BAL 31 mutants which did not display high transforming activity, suggesting that the fused non-*raf* coding sequences may also contribute to biological activity.

The *raf* oncogene was first identified in the acutely transforming retrovirus 3611-MSV, isolated from a methylcholanthrene-transformed mouse cell line (34). Both v-*raf* and its independently isolated avian retroviral homolog, v-*mil*, are members of the protein kinase gene family and encode cytoplasmic *gag* fusion proteins with associated serine-threonine kinase activity (25, 28, 40). Recently, a small family of *raf*-related genes has been described in humans (1, 4, 5, 16, 26). One of these genes, c-*raf*-1, is the human homolog of v-*raf* (4). It spans at least 44 kilobases (kb) of genomic DNA, contains 17 exons, and encodes a 648-amino-acid protein (5). The *raf* protein contains a carboxy-terminal domain, encoded by exons 10 through 16, which is 33% homologous to the *src* tyrosine kinase domain (25).

Transfection of NIH 3T3 cells with genomic DNAs of normal and neoplastic human and rodent cells has led to the identification of at least 12 activated cellular *raf* genes (7, 13, 16, 18, 21, 30, 35, 37, 41) in addition to the viral oncogenes v-*mil* and v-*raf*. In each of the activated *raf* genes which has been characterized, there was a deletion at the 5′ end of the normal cellular gene. In two cases, the 5′ deletion extends to intron 5 (29, 31); in one case (v-*mil*), the deletion extends to exon 7 (4, 40); in seven cases, the deletion extends to intron 7 (14, 19, 37, 41); in one case (v-*raf*), the deletion extends to exon 9 (4); and in one case (B-*raf*), the deletion extends to the equivalent of intron 9 (16). These results have suggested the hypothesis that the *raf* amino-terminal sequences encode a regulatory domain, the deletion of which results in constitutive activity of the carboxy-terminal kinase domain.

Molecular characterization of the truncated *raf* genes has not, however, excluded the possibility that alterations other than truncation contribute to *raf* activation. Replacement of the normal *raf* regulatory region by a novel promoter and translation initiation site would affect *raf* expression at the levels of transcription and translation. In addition, the fusion of novel upstream coding sequences with the remainder of *raf* could alter protein stability, localization, or biochemical activity.

We sought to directly address whether 5′ deletion alone is sufficient to activate *raf* transforming potential and, further, to begin to define the topology of the putative 5′ regulatory domain. To this end, we generated a series of human *raf* genes with 5′ deletions distal to a long terminal repeat (LTR) promoter and a translation initiation sequence. Since identical transcription and translation signals were provided to each deletion mutant, the effect of varying amino-terminal truncation on biological activity of the mutants could be directly compared.

MATERIALS AND METHODS

Plasmids, oligonucleotides, and molecular cloning. The plasmid pm1sp consists of a Moloney murine sarcoma virus (MSV) LTR with flanking mink genomic sequences cloned into the *Eco*RI site of pBR322 (3). p627 is a full-length normal human c-*raf*-1 cDNA cloned into the *Eco*RI site of pUC13 (5). pF4 is a cloned 3611-MSV provirus (33). Standard methods were employed for oligonucleotide synthesis, purification, and molecular cloning procedures (24, 39).

Vector construction. The construction of the vector used for deletion mutagenesis, pΔ*raf*, is illustrated in Fig. 1. Plasmid pm1sp was digested with *Kpn*I and *Xma*I, which cut 29 nucleotides downstream of the transcription start site of the LTR promoter (11) (Fig. 1C). Next, two complementary synthetic oligonucleotides, CCGGAATTCACCATGGG

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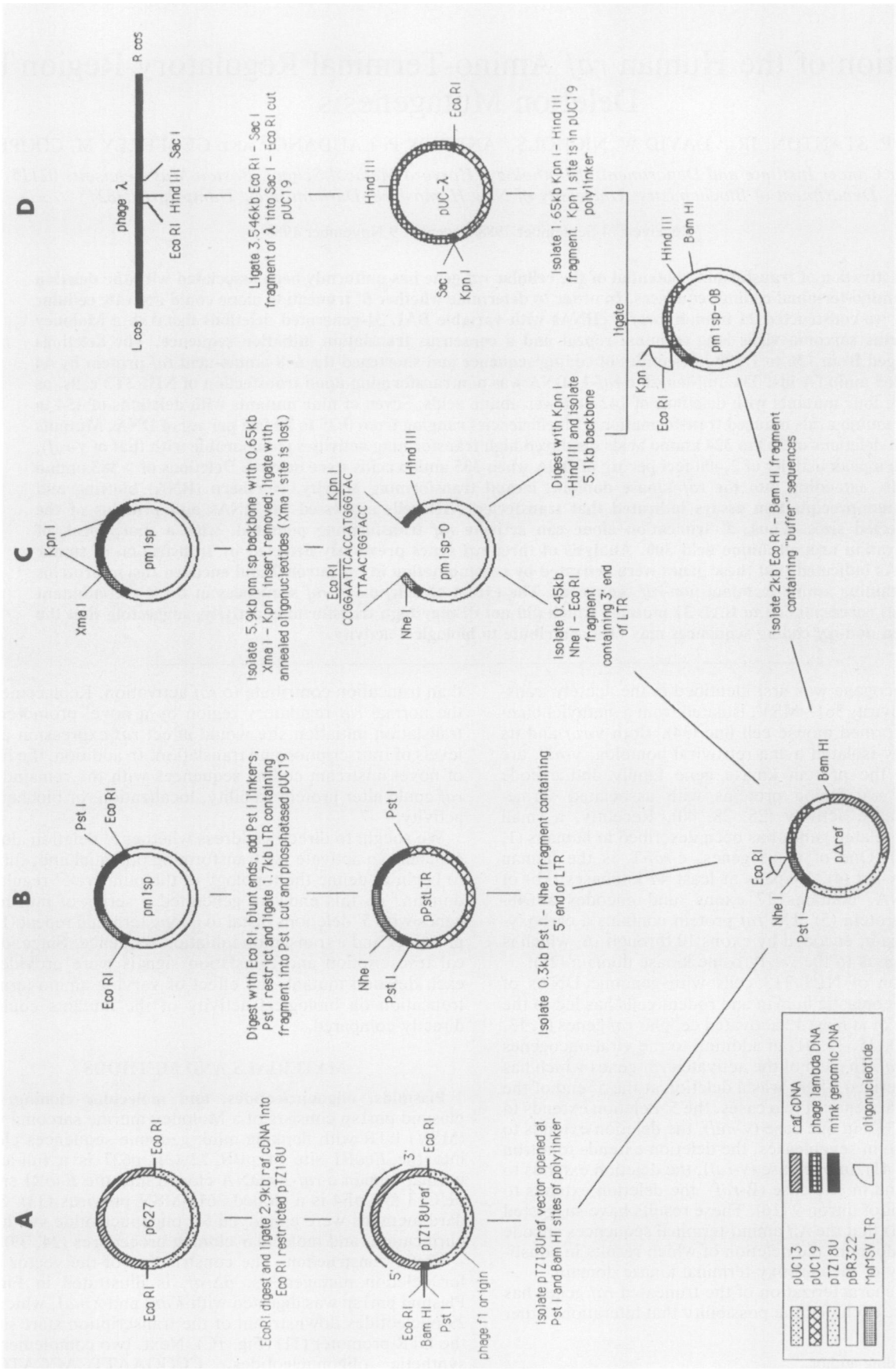

FIG. 1. Construction of p*Δraf*. The vector was assembled from three different plasmids and λ DNA as shown and described in Materials and Methods. Only restriction endonuclease sites relevant to cloning manipulations are shown. The constituent parts of each plasmid are drawn roughly in correct proportion to each other, but the relative sizes of the plasmids are not drawn to scale. Details of the plasmids and indicated manipulations are provided in Materials and Methods. MoMSV, Moloney MSV; L, left; R, right.

TAC and CCATGGTGAATT, were annealed, producing a duplex encoding the Kozak consensus translation sequence (CACCATGG) (22) with internal *Eco*RI and *Nco*I sites and overhanging *Xma*I (5′) and *Kpn*I (3′) ends. This annealed oligomer (designated by a pie splice in subsequent constructions) was ligated with *Xma*I-*Kpn*I-cut pm1sp to form pm1sp-O, in which the LTR promoter was immediately followed by the ATG-containing oligonucleotide (Fig. 1C). Next, to form a buffer to protect vector sequences from BAL 31 deletion, a 3.546-kb *Eco*RI-*Sac*I fragment of λ DNA (positions 21266 through 24772) was subcloned into *Eco*RI-*Sac*I-cut pUC19 (Fig. 1D). A 1.65-kb *Hin*dIII (λ position 23130)-*Kpn*I (pUC19 polylinker site) fragment was then ligated into *Kpn*I-*Hin*dIII-digested pm1sp-O to form pm1sp-OB (Fig. 1, product of C and D). Next, the *Eco*RI LTR insert of pm1sp was converted to *Pst*I ends and subcloned into pUC19 to form pPstLTR (Fig. 1B). Finally, the 2.9-kb *Eco*RI *raf* cDNA insert of p627 was ligated into the *Eco*RI site of pTZ18U (U.S. Biochemical Corp.) to form pTZ18U*raf*. A clone was selected with the cDNA oriented 5′ to 3′ away from the *Hin*dIII end of the polylinker (Fig. 1A). pΔ*raf* was then constructed by way of a four-fragment ligation with the following: (i) a *Pst*I-*Bam*HI-cut pTZ18U*raf* backbone (Fig. 1A), (ii) a *Pst*I-*Nhe*I fragment of pPstLTR containing the 5′ 0.3 kb of the LTR (*Nhe*I recognizes a unique site in the LTR [11]) (Fig. 1B), (iii) an *Nhe*I-*Eco*RI fragment of pm1sp-O containing the 3′ 0.45 kb of the LTR and extending to the *Eco*RI site of the ATG-containing oligonucleotide (Fig. 1C), and (iv) a 2-kb *Eco*RI-*Bam*HI fragment of pm1sp-OB containing the 3′ end of the oligonucleotide, 1.65 kb of λ DNA, and 346 nucleotides of pBR322 extending from *Hin*dIII to *Bam*HI (Fig. 1, product of C and D). The final vector was mapped extensively to confirm that the desired sequences were properly arrayed.

The vector pLTR*raf*, which contains the full-length *raf* cDNA of p627 distal to the Moloney MSV LTR of pm1sp, was constructed by inserting the *Pst*I-*Sma*I LTR fragment of pPstLTR into *Pst*I-*Sma*I-cut p627.

DNA sequencing of deletion mutants. The F′ *Escherichia coli* XL1-Blue (Stratagene Cloning Systems) was transformed to ampicillin resistance by pΔ*raf* DNA subjected to the BAL 31 deletion procedure. Single-stranded DNA was prepared by superinfection with the helper bacteriophage M13 K07 by the protocol supplied with the Genescribe pTZ vectors (U.S. Biochemical Corp.). Dideoxy chain termination sequencing reactions were performed by a protocol supplied by U.S. Biochemical Corp., using the primer GCGCCAGTCTTCCGATAG, which is complementary to the MSV LTR between 23 and 40 nucleotides upstream of the synthetic ATG.

Cell culture. The BR2-215, CA1-154, and RC1-138 *raf*-transformed cell lines have been described previously (37). Transfection assays were performed with NIH 3T3 cells as previously described (10). Transformed NIH cells were grown in 5% calf serum. Cells transfected with pSV2neo (36) were grown in medium supplemented with G418 (GIBCO Laboratories) at a concentration of 400 μg/ml.

RNA isolation and analysis. Total cell RNA was isolated by the guanidinium isothiocyanate method (15, 24) or by a two-step phenol method (8). In some cases, cytoplasmic RNA was prepared by a rapid cell lysis method (12) using RNAsin (Promega Biotec) as the RNase inhibitor. RNAs (15 μg) were electrophoresed in 1% agarose-formaldehyde gels, blotted onto GeneScreen Plus membranes (Dupont, NEN Research Products), and probed with ^{32}P-labeled *raf* cDNA by using hybridization conditions recommended by the supplier. The DNA probe was radiolabeled by the random-primed synthesis method with a Boehringer Mannheim kit.

Peptide synthesis and antibody production. The peptide (Lys)-Thr-Leu-Thr-Thr-Ser-[^{14}C]Pro-Arg-Leu-Pro-Val-Phe, which contains the carboxyl-terminal 11 residues of the c-*raf*-1 protein, was synthesized by the solid-phase method described by Merrifield (27, 38). The composition of the peptide was verified by amino acid analysis. The peptide was coupled to bovine serum albumin with glutaraldehyde (20, 42). New Zealand White rabbits were immunized with 1 mg of the bovine serum albumin-peptide conjugate in 1 ml of 0.15 M NaCl–0.01 M $NaPO_4$ (pH 7), emulsified with 1 ml of complete Freund adjuvant. Immunizations were repeated four times at 2-week intervals with incomplete Freund adjuvant. Antisera used for immunoprecipitations were collected 60 and 67 days after the primary immunization.

Immunoprecipitations. Nearly confluent monolayer cultures were washed and incubated for 30 min in methionine- or phosphate-free Dulbecco modified Eagle medium containing 10% dialyzed calf serum and labeled for 4 h in the same medium containing [^{35}S]methionine (0.25 to 0.5 mCi/ml) or $^{32}P_i$ (1 mCi/ml). Cells were washed and extracted under denaturing conditions as described previously (23). Cell extracts were normalized by dilution with lysis buffer containing 1% Nonidet P-40 to give equal concentrations of trichloroacetic acid-precipitable counts and precleared with protein A-agarose coupled to nonimmune immunoglobulin G (23). After being centrifuged briefly, 0.2 ml of the precleared supernatant was incubated for 2 h on ice with 0.01 ml of preimmune serum, immune serum, or immune serum in which peptide-specific antibodies were blocked with 0.01 ml of 0.01 M synthetic peptide. Immune complexes were analyzed by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels (9% polyacrylamide) as described previously (23). [^{35}S]methionine-labeled proteins were detected by fluorography with sodium salicylate (6) for 4 to 10 days.

RESULTS

Generation of deletion mutants. In order to facilitate the rapid production and analysis of 5′ *raf* deletion mutants, we constructed a specialized vector (pΔ*raf*) which provided a strong LTR promoter and uniform transcription and translation control sequences to each mutant gene. Also, the entire mutagenesis procedure, sequence analysis of deleted DNAs, and expression of deleted sequences in NIH 3T3 cells could be accomplished with the same vector. pΔ*raf* was assembled by inserting four DNA segments into the polylinker of pTZ18U, a pUC-derived vector which contains the phage f1 intragenic region, allowing production of single-stranded DNA for sequencing. From *Pst*I to *Eco*RI sites of the polylinker, the vector (Fig. 2) contained the following: (i) a Moloney MSV LTR, (ii) complementary synthetic oligonucleotides encoding the Kozak consensus translation sequence (22), (iii) a 1.988-kb segment of buffer DNA (λ and pBR322 sequences) to protect the LTR and ATG sequences from BAL 31 deletion, and (iv) a full-length normal human c-*raf*-1 cDNA (5).

The deletion strategy consisted of linearizing the vector at the unique *Bam*HI site located immediately upstream of the *raf* cDNA (Fig. 2) and digesting with nuclease BAL 31 (0.03 U/μg of DNA) for 8 to 35 min, resulting in variable deletion into both the *raf* cDNA and the λ-pBR322 buffer DNA. The length of the buffer DNA was close to the length of *raf* coding sequences, thus allowing deletion of virtually the entire *raf* cDNA. Deleted DNA was digested with *Kpn*I,

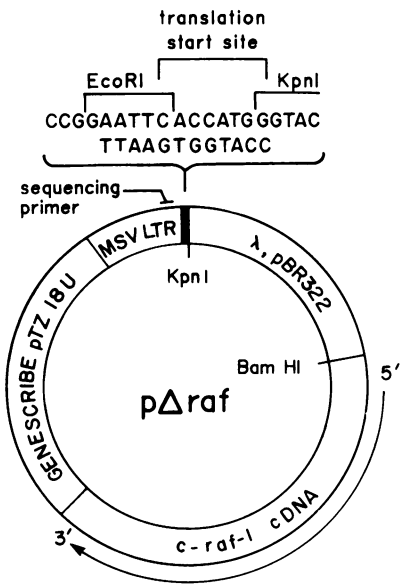

FIG. 2. pΔ*raf* vector for deletion mutagenesis. The unique restriction endonuclease sites and functional DNA segments employed in the generation of BAL 31 deletion mutants are indicated. Sequencing across the resulting ATG-*raf* cDNA fusion was primed from just upstream in the LTR as shown.

excising any buffer DNA which remained after BAL 31 digestion (Fig. 2). The ends of the deleted vector molecule were blunted by exonucleolytic removal of the 3′ *Kpn*I overhang, exposing the sequence ATGG (Fig. 2). Blunt-ended vector DNA was separated from the buffer DNA fragment, and vector DNA was circularized by blunt-end ligation, fusing the synthetic ATGG sequence with *raf* cDNA with a deletion at the 5′ end. The ligation product was used to transform competent F′ *E. coli* XL1-Blue to ampicillin resistance.

By comparing the sequences of mutants with that of the c-*raf*-1 cDNA (5), it was possible to isolate a series of precisely defined deletion mutants which were in-frame with respect to the synthetic ATG initiation codon. Single-stranded DNA was purified and sequenced by the chain termination method, using a primer complementary to the MSV LTR just upstream of the synthetic ATG to allow sequencing across the fused ATGG-*raf* junction. Sequencing reactions were performed on a total of 106 deletion mutants isolated after different periods of BAL 31 digestion. In six cases, no sequence could be obtained from apparently pure single-stranded DNA, probably a result of deletion of the primer-complementary region of the LTR. In 18 of the 106 mutants, part or all of the synthetic ATG codon was deleted, possibly as a result of BAL 31 digestion or damage occurring after *Kpn*I digestion. Of the remaining 82 mutants, comparison with the *raf*-1 cDNA sequence indicated that 26 were in-frame with respect to the synthetic ATG. However, of the 26 in-frame mutants, four were independent isolates of the identical mutant and two other mutants were isolated twice. Thus, 21 unique in-frame mutants were identified (Table 1). The deletions spanned the 5′ two-thirds of the *raf* coding sequence, ranging from 136 to 1,399 nucleotides deleted from a total coding sequence of 1,944 nucleotides. The mutant genes encode *raf* proteins shortened at their amino termini by 44 to 465 amino acids, with a methionine added to the beginning of each protein. Of the 21 mutant genes, 15 also encoded a non-*raf* amino acid in position 2 because the terminal G of the synthetic ATGG became nucleotide 1 of codon 2 (Table 1).

TABLE 1. *raf* deletion mutants

Mutant[a]	No. of nucleotides deleted	No. of amino acids deleted[b]	Size (kDa) of predicted *raf* protein	Transforming activity[c]
8B	136	44	68	<0.25
8K	208	68	65.5	<0.25
8A	274	90	63	<0.25
12E	430	142	57	<0.25
16E	466	154	55.5	4
20H	502	166	54.5	<0.25
16A	544	180	52.5	3
20R	583	193	51	70
22G	642	213	49	9
22M	667	221	48	40
24H	685	227	47.5	0.25
20D	766	254	44.5	<0.25
16G	823	273	42	1
24G	913	303	39	750
22W	919	305	38.5	2,400
20A	943	313	37.5	550
22D	976	324	36.5	140
26I	1,153	383	30	<0.25
35E	1,162	386	29	<0.25
26J	1,165	387	29	<0.25
35D	1,399	465	20.5	<0.25

[a] Mutants were named according to the time of BAL 31 digestion (8, 12, 16, 20, 22, 24, 26, or 35 min) and the order of isolation (A, B, C, etc.).

[b] Amino acids deleted were calculated by subtracting the size of the mutant *raf* protein (after fusion to the synthetic ATGG) from that of the normal *raf* protein (648 amino acids). In one case (mutant 22G), the terminal G of ATGG was deleted; thus ATG is fused to the deleted *raf* gene. Replacing the first nucleotide of the second codon of the mutant genes with G of ATGG resulted in a change from the normal *raf* amino acid at that position in 15 cases. These changes can be summarized as follows: Gln→Glu for 8A, 16E, 20A, 22W, 24H, and 35E; Arg→Gly for 16G, 20D, 22M, and 35D; Leu→Val for 20R; Thr→Ala for 16A; Lys→Glu for 12E; Trp→Gly for 24G; Cys→Gly for 20H.

[c] Plasmid DNA of each deletion mutant was transfected onto two or more plates of NIH 3T3 cells in at least three doses (10, 100, and 1,000 ng) in two or more separate experiments. Transforming efficiencies are indicated as the number of foci per microgram of transfected DNA. Those deletion mutants with transforming activities marked <0.25 failed to produce a transformed focus.

Transforming activity of *raf* deletion mutants. The biological activities of full-length c-*raf*-1, v-*raf*, and each of the 21 *raf* deletion mutants were determined by transfection of NIH 3T3 cells. Samples (10, 100, and 1,000 ng) of each deletion mutant were transfected as intact plasmid DNA. The results are summarized in Table 1 and in Fig. 3, which depicts transforming activity as a function of the extent of 5′ *raf* deletion. A full-length *raf* cDNA expressed from the Moloney MSV LTR (pLTR*raf*) lacked detectable transforming activity (<0.25 foci per μg of DNA). Mutants with deletions of 180 or fewer amino acids had undetectable or low transforming activity (<5 foci per μg). A group of three mutants with deletions of 193 to 221 amino acids induced transformation with efficiencies of 9 to 70 foci per μg of DNA, whereas deletions extending from 227 to 273 amino acids were associated with little or no transforming activity. Still further deletions, however, revealed a group of mutants (303 to 324 amino acids deleted) with potent transforming activity, peaking at 2,400 foci per μg of DNA when 305 amino acids were deleted (mutant 22W). By comparison, the cloned v-*raf* provirus (3611-MSV plasmid pF4) had a transforming activity of ~12,000 foci per μg of DNA. Further deletion into the kinase domain (383, 386, 387, and 465 amino acids removed) abolished transforming activity.

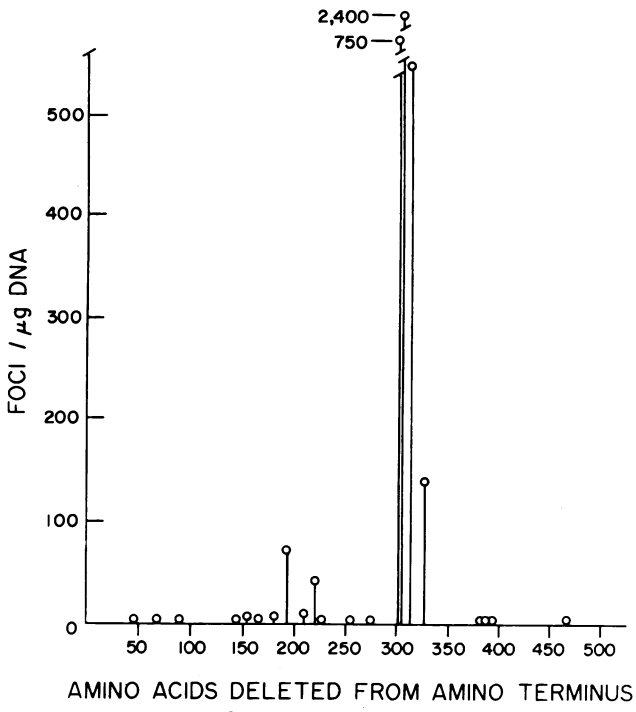

FIG. 3. Transforming activity of 21 *raf* deletion mutants as a function of the number of amino acids deleted. This figure was prepared from the data presented in Table 1.

Expression of transforming and nontransforming mutant *raf* genes. The observed differences in transforming activity could be a result of differences in deletion mutant mRNA or protein stability rather than of intrinsic biochemical differences between mutant proteins. In order to determine whether transfected NIH cells expressed *raf* transcripts and polypeptides of the expected sizes, we analyzed RNA and protein from cells containing pLTR*raf* and each of the 21 deletion constructs. For those mutants which induced transformation, individual transformed foci were expanded for analysis. Cell lines containing pLTR*raf* and the nontransforming deletion mutants (8B, 8K, 8A, 12E, 20H, 20D, 26I, 35E, 26J, and 35D) were established by cotransfecting NIH 3T3 cells with 0.5 to 10 μg of *raf* mutant DNA plus 10 ng of pSV2neo DNA. G418-resistant colonies, usually numbering 50 to 500, were pooled and grown to mass culture. The mutant 24H, which yielded a single transformed focus (24H3-1; transforming activity, 0.25 foci per μg of plasmid DNA), was also cotransfected with pSV2neo to generate the nontransformed cell line 24H-neo.

Results of Northern analysis of RNA from deletion mutation-containing cell lines probed with ^{32}P-labeled c-*raf*-1 cDNA are presented in Fig. 4. All cell lines expressed *raf* transcripts of the expected sizes. The levels of *raf* message in the different cell lines varied more than 10-fold, with generally higher expression seen in morphologically transformed cell lines compared with G418-selected cell lines harboring nontransforming constructs. *raf* mRNA was not

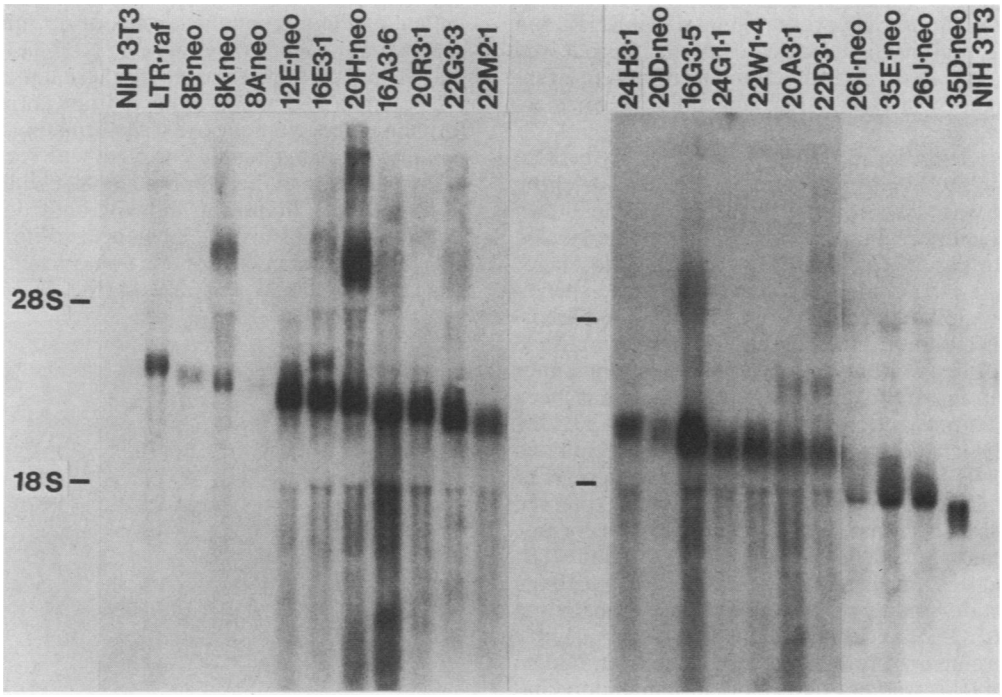

FIG. 4. NIH cells transfected with *raf* deletion mutants contain *raf* message of expected size. RNA isolated from 11 transformed and 10 nontransformed cell lines harboring *raf* deletion constructs and from NIH 3T3 cells was analyzed by blot hybridization with radiolabeled *raf* cDNA as described in Materials and Methods. Cell lines derived from transformed foci were named according to the name of the transfected *raf* deletion mutant, the exponent (base 10) of the nanograms of DNA transfected (followed by a hyphen), and the number of the focus (in order of foci cloned). Thus, 22F3-1 is the first transformed cell line (number 1) derived from transfection experiments with 10^3 ng (number 3) of mutant 22F plasmid DNA. Deletion constructs that failed to produce transformed foci were cotransfected with pSV2neo, and G418-resistant colonies were selected and pooled to form cell lines, such as 12E-neo. Filters were exposed to film for 30 h with intensifying screens, except for the final five lanes of the panel on the right (26I-neo, 35E-neo, 26J-neo, 35E-neo, and NIH 3T3) which were exposed for 95 h. The positions of 28S (~5.1-kb) and 18S (~2.0 kb) rRNAs are indicated.

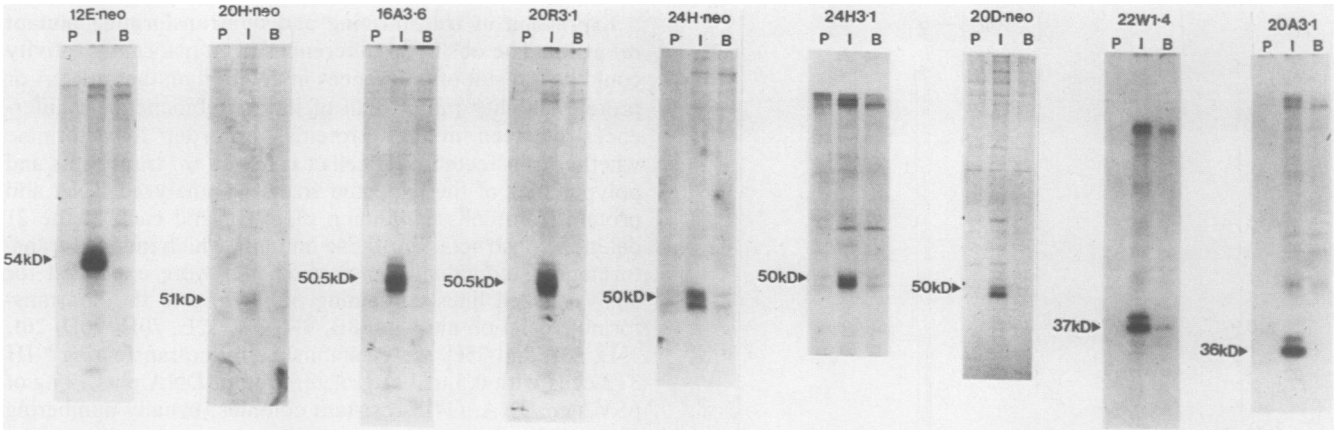

FIG. 5. Antipeptide antiserum raised against the 11 carboxy-terminal c-*raf*-1 amino acids specifically precipitates truncated *raf* proteins. Representative *raf* transfected cell lines were metabolically labeled with [^{35}S]methionine, and lysates were precipitated with preimmune serum (P), anti-*raf* immune antiserum (I), or immune serum with blocking *raf* peptide (B) as described in Materials and Methods. 12E-neo, 20H-neo, 24H-neo, and 20D-neo are nontransformed cell lines established by cotransfection of deletion constructs with pSV2neo. The five other cell lines were established from transformed foci. The sizes of the marked protein species were determined from the mobility of α2-macroglobulin (180 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97.4 kDa), fructose-6-phosphate kinase (84 kDa), bovine serum albumin (66.2 kDa), pyruvate kinase (58 kDa), fumarase (48.5 kDa), ovalbumin (42.7 kDa), lactic dehydrogenase (36.5 kDa), carbonic anhydrase (31 kDa), and triosephosphate isomerase (26.5 kDa). kd, Kilodaltons.

detected in NIH 3T3 cells at exposure times which allowed clear visualization of all 21 deletion mutant RNAs, indicating that even cell lines such as 35D-neo, with comparatively low levels of *raf* mRNA, were still expressing higher levels of *raf* message than were NIH 3T3 cells. RNAs of some cell lines, particularly 16G3-5 and 20H-neo, contained, in addition to mutant transcripts of expected size, larger *raf* homologous bands. Such bands were only present in total cell RNAs and may therefore represent unprocessed or partially processed RNA species. Alternatively, they might represent transcripts of *raf* genes which were rearranged during transfection.

A rabbit antiserum raised against a synthetic peptide corresponding to the 11 carboxy-terminal amino acids of the c-*raf*-1 protein was used to investigate expression of *raf* protein by immunoprecipitation of [^{35}S]methionine-labeled cell extracts. Representative results are presented in Fig. 5. Proteins of approximately the predicted sizes were specifically immunoprecipitated from 14 of the 21 cell lines harboring *raf* deletion mutants, including all 11 lines established from *raf*-transformed foci and 3 of the 10 G418-resistant lines into which nontransforming *raf* deletion mutants had been introduced by cotransfection (12E-neo, 20H-neo, and 20D-neo). *raf* protein was also specifically immunoprecipitated from cell line 24H-neo, the nontransformed counterpart of 24H3-1 (Fig. 5). Thus, truncated *raf* proteins were expressed in all deletion mutant-transfected cell lines, except for one group of three mutants at the extreme amino terminus (8B, 8K, and 8A) and a second group of four mutants in which the putative nucleotide-binding region of the kinase domain was deleted (26I, 26J, 35D, and 35E).

Although normal *raf* protein has been demonstrated in NIH 3T3 cells (9), it was not detectable in immunoprecipitates of [^{35}S]methionine-labeled cell extracts. We therefore sought to increase the sensitivity of immunoprecipitation by labeling with $^{32}P_i$. Under these conditions, normal *raf* protein was detectable in NIH 3T3 cells and in deletion mutant-transfected cell lines (Fig. 6). Comparison of *raf* protein immunoprecipitated from cell line 20H-neo in Fig. 5 ([^{35}S]methionine labeled) and 6 (^{32}P labeled) illustrates the differing sensitivity of the two methods; 20H-neo *raf* protein was near the limit of detection in [^{35}S]methionine-labeled extracts but was >20-fold stronger than was NIH 3T3 *raf* protein in ^{32}P-labeled extracts. The increased sensitivity afforded by ^{32}P labeling did not permit detection of mutant *raf* protein in the 8K, 26I, 35D, and 35E cell lines, despite the detection of endogenous NIH *raf* protein in the same immunoprecipitates (Fig. 6; also data not shown). Since these mutant cell lines contained levels of *raf* mRNA which were substantially higher than those of NIH 3T3 cells, it is likely that the *raf* proteins encoded by these mutants are unstable.

The amount of *raf* protein did not correlate with transforming potency among the transformation-positive deletion mutants but did generally correlate with levels of *raf* mRNA in the cell lines studied. For example, cell lines 16A3-6 (Fig. 5) and 16E3-1 (data not shown), both derived from constructs with transforming efficiencies of less than 5 foci per μg of DNA, contained as much or more mutant *raf* protein as did cell lines 20A3-1 and 22W1-4 (Fig. 5), both derived from

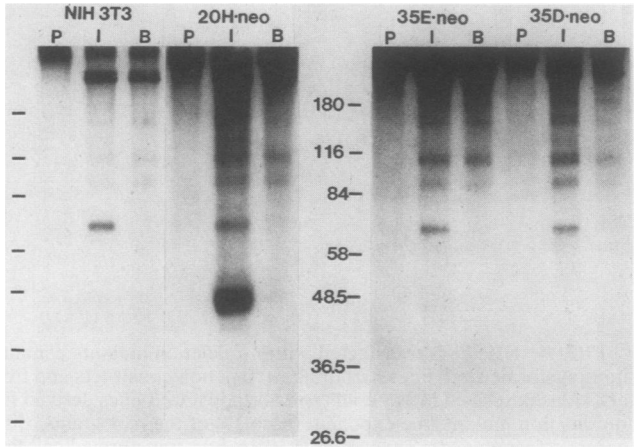

FIG. 6. Immunoprecipitation of *raf* proteins from $^{32}P_i$-labeled cell extracts. Cells were labeled with $^{32}P_i$ and analyzed by immunoprecipitation as described in the legend to Fig. 5. Sizes of molecular weight markers are indicated to the left of the gels.

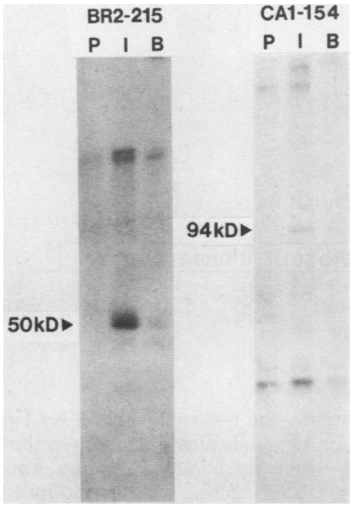

FIG. 7. BR2-215 and CA1-154 *raf* transforming sequences encode hybrid transforming proteins. Extracts of BR2-215 and CA1-154 cell lines were labeled with [^{35}S]methionine and immunoprecipitated as described in the legend to Fig. 5.

mutants with transforming efficiencies of >500 foci per μg of DNA. Also of note, the nontransformed cell line 12E-neo contained the highest *raf* protein level of all cell lines examined, indicating that truncated *raf* protein overexpression was not sufficient for transformation.

Three activated *raf* transforming genes detected by transfection of human DNA are rearranged in intron 7 and encode fusion proteins. Having established a relationship between 5′ deletion and *raf* activation, we sought to better understand the activating events in the BR2-215, CA1-154, and RC1-138 transforming sequences, which we had previously identified as *raf* genes activated during transfection of human tumor DNAs (37). Restriction mapping had shown that all three genes were rearranged within a 2.1-kb segment of genomic DNA spanning the 3′ half of exon 7 and the 5′ half of intron 7 (37). Specifically, the CA1-154 transforming gene recombination site lies within a 0.7-kb segment of intron 7, so this rearrangement resulted in deletion of the amino-terminal 7 exons encoding 278 amino acids. However, the BR2-215 and RC1-138 recombination sites could be mapped only to a ~0.3- to 0.4-kb segment spanning exon 7 and intron 7. To localize these rearrangements more precisely, genomic DNA restriction fragments containing the junction between *raf* (the *Hpa*I site in intron 7) (37) and non-*raf* sequences were cloned into Genescribe pTZ vectors for sequencing. Complete sequencing of both cloned fragments (317 nucleotides for BR2-215 and 440 nucleotides for RC1-138) revealed no exon 7 sequences (data not shown). Thus, both rearrangements occurred in intron 7, similar to the CA1-154 rearrangement.

To determine whether the 5′ fused non-*raf* sequences (which are conserved in independently derived BR2, CA1, and RC1 tertiary transformants) encoded protein, we immunoprecipitated extracts of transformed NIH cells with anti-*raf* antiserum. Proteins of 50 and 94 kilodaltons (kDa) were specifically immunoprecipitated from the BR2-215 and CA1-154 transformant cell lines (Fig. 7). (*raf* protein was not detectable in [^{35}S]methionine-labeled immunoprecipitates of RC1-138 transformed cells.) *raf* exons 8 through 17 encode 370 amino acids; however, there is no ATG in exon 8 or 9. The first potential initiation codon after exon 7 lies in exon 10, 299 amino acids from the C terminus. Thus, if fused upstream sequences did not include a protein-coding sequence, one would expect transforming proteins of ~34 kDa. The significantly greater mass of the immunoprecipitated *raf* proteins indicates that the BR2 and CA1 tumor-derived *raf* genes encoded fusion proteins formed by recombination of non-*raf* amino-terminal coding sequence with the start of *raf* exon 8 (*raf* amino acid 279).

DISCUSSION

The common feature of all activated *raf* genes which have been fully characterized is the loss by retroviral transduction, rearrangement, or insertional mutagenesis of between 423 and 965 nucleotides of 5′ coding sequence. However, the rearrangements which produced these activated *raf* genes also resulted in the fusion of non-*raf* upstream sequences of unknown biological role to the remaining *raf* sequence. Thus, analysis of the foregoing *raf* activations has not determined whether 5′ truncation alone is sufficient to activate the transforming potential of c-*raf*-1.

To address this question, we generated 21 unique 5′ *raf* deletion mutants, each under the control of the Moloney MSV LTR promoter and with identical translation initiation sequences. Analysis of these mutants demonstrated that 5′ deletion was sufficient to activate *raf* transforming potential. The most potent mutants had transforming activities comparable with that of v-*raf*. These results provide a direct indication that the amino-terminal region of the *raf* protein is a regulatory domain, exerting a negative control on those activities of the carboxy-terminal kinase domain which can result in cell transformation. In this respect, *raf* function appears to parallel protein kinase C, where constitutive kinase activity results from the proteolytic cleavage of an amino terminal regulatory domain which shares limited sequence homology with c-*raf*-1 (32).

It is noteworthy that the major peak of *raf* transforming activity is extremely sharp, between deletions of 300 to 320 amino acids, just before the beginning of the *raf* kinase domain (amino acid 342) (25). Further, cells transfected with 14 consecutive mutants in which between 142 and 324 amino acids had been deleted contained comparable levels of mutant *raf* proteins of roughly the expected sizes. Thus, the >10,000-fold difference in transforming activity (from <0.25 to 2,400 foci per μg of plasmid DNA) between these mutants likely reflects intrinsic differences in the biochemical activity of mutant *raf* proteins. This might suggest that the entire amino-terminal regulatory domain must be removed before kinase activity becomes fully constitutive, resulting in full transforming activity.

The smaller peak of *raf* activation (around 200 deleted amino acids) does not correspond to any evident sequence feature of the amino-terminal region. Deletion through the cysteine-rich putative metal/nucleic acid-binding motif (amino acids 151 through 168) (1, 2) was not associated with any significant effect on transforming activity. The partial activation of *raf* transforming potential by more extensive deletions (193 to 221 amino acids) may indicate a secondary role of these amino-terminal sequences in modulating biological activity.

The sites of rearrangements characterized in activated viral and cellular *raf* genes are compared with the peaks of *raf* activation defined by the deletion mutants in Fig. 8. The SCT102 (31, 35) and S1 (29, 30) transforming genes were activated by rearrangement and LTR insertion, respectively, in *raf* intron 5. *raf* exon 6 starts with codon 195, but the first

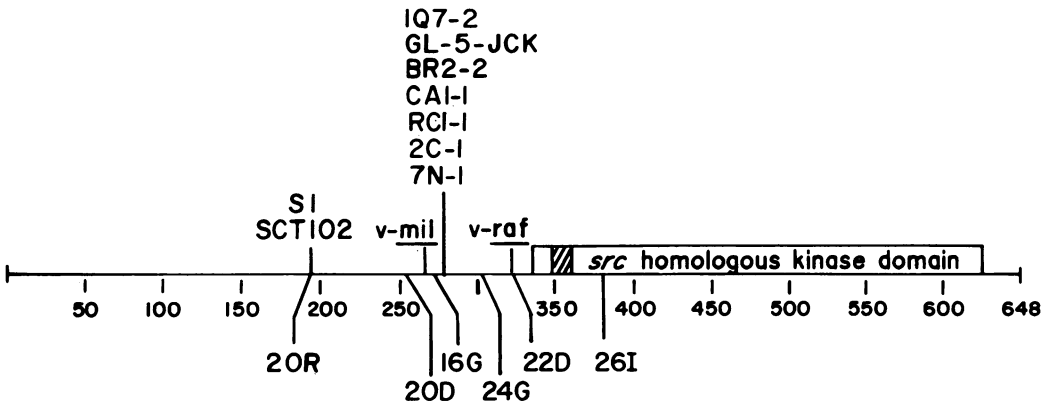

FIG. 8. Diagram of activated *raf* genes described to date. The horizontal line represents the 648-amino-acid c-*raf*-1 protein. The *src* homologous kinase domain (33%) is shown, with the putative nucleotide-binding region (25) indicated (■). Above the line are the 11 characterized *raf* transforming genes reported in the literature, shown at the position where *raf* homology begins. These transforming sequences and their references follow: S1 (29, 30); SCT102 (31); IQ7-2 (17–19); GL-5-JCK (13, 14); BR2-2, CA1-1, and RC1-1 (37); 2C-1 and 7N-1 (41); v-*mil* (40); and v-*raf* (4, 33). Below the horizontal line the positions of several representative deletion mutants are shown to allow comparison of their transforming activities (Table 1) with the extent of deletion in the activated *raf* genes.

in-frame ATG in exon 6 is 213 codons from the start of the c-*raf*-1 cDNA. The S1 transforming gene, with no coding sequences likely contributed by the inserted LTR, probably encodes a protein starting at amino acid 213, consistent with the detection of a 44- to 50-kDa *raf* protein by Molders et al. (29). The SCT102 fusion gene encodes a fusion protein with the *raf* portion beginning at amino acid 195 (31). *raf* deletion mutants corresponding to both of these areas (especially 20R, with 193 codons deleted, and 22G, with 213 codons deleted) exhibited moderate transforming activity, unlike some mutants with greater or lesser deletions. Similarly, the v-*raf* gene has been truncated at codon 323, which lies within the major peak of transforming activity of the deletion mutants.

In contrast, deletion mutants mapping closest to the junction of exons 7 and 8, the most frequent site of activating *raf* rearrangements detected by transfection, had minimal or no transforming activity. For example, mutant 20D, with 254 codons deleted, and mutant 16G, with 273 codons deleted, lay close to the exon 7-8 junction (amino acid 278) but had transforming efficiencies of <0.25 and 1 focus per μg of DNA. Single-copy *raf* genes of comparable transforming potency would not be detectable by transfection of genomic DNA ($<10^{-5}$ foci per μg of DNA). In fact, however, at least five of the seven *raf* transforming genes rearranged in intron 7 encoded fusion proteins. In particular, sequencing of cDNA clones of the 1Q7-2, GL-5-JCK, and 7N-1 activated *raf* genes directly demonstrated fusion of upstream coding sequences to *raf* exon 8 (14, 19, 41). Likewise, the sizes of *raf* proteins immunoprecipitated from cells transformed by the BR2 and CA1 transforming genes requires some contribution from fused non-*raf* protein. In view of the mutant data in the region of the exon 7-exon 8 junction (amino acid 278), it therefore appears likely that fused non-*raf* coding sequences contribute to the biological activity of recombinant *raf* genes activated by rearrangements in intron 7. The amino-terminal non-*raf* sequences may affect protein processing or stability or they may nonspecifically modulate the conformation and activity of the kinase domain.

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