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Characterization of Murine A-*raf*, a New Oncogene Related to the v-*raf* Oncogene

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A 1.6-kilobase cDNA (A-*raf*) has been isolated from a murine spleen cDNA library which encodes part of a protein related to the *raf* oncogene. Its amino acid sequence has 85% homology to *raf* in a central portion of 100 amino acids. In contrast to *raf*, A-*raf* shows a highly restricted tissue distribution of expression, with highest levels observed in epididymis, followed by intestine. When incorporated into a retrovirus, the resulting *gag*-A-*raf* fusion gene causes transformation in vitro and induces tumors in newborn mice. Thus, A-*raf* represents a new proto-oncogene. Transformation by A-*raf* is independent of *ras* gene function, as is the case for *raf* and *mos* but not other oncogenes.

The v-*raf* oncogene was originally identified as the transforming gene of the murine retrovirus 3611-MSV (MSV = murine sarcoma virus) (26, 27). A homologous gene, v-*mil*, was subsequently found in the genome of the avian carcinoma virus MH2 (12, 13, 36), which also carries the oncogene v-*myc*. The avian homolog of v-*raf* in MH2 appears to be biologically active (25), although it may not have been maintained as an effective oncogene in this virus (1, 39). In contrast, 3611-MSV efficiently transforms established epithelial (14) and primary as well as established fibroblastic cells in vitro (22a), immortalizes interleukin 3-dependent primary hematopoietic cells (11, 23), and induces predominantly fibrosarcomas and erythroleukemias in newborn mice (25). Combination of v-*myc* with v-*raf* in a retrovirus results in the addition of lymphomas and carcinomas to the tumor spectrum of v-*raf* and a dramatic shortening of the latency period of both v-*raf* and v-*myc* specific tumors in mice (25). In humans, the *raf* homologous gene c-*raf*-1 (3, 4, 4a) was found to be activated in primary stomach cancer (35) and glioblastoma (9) as determined by DNA transfection into NIH 3T3 cells.

3611-MSV expresses v-*raf* as part of two differentially modified *gag* fusion proteins, a myristilated and phosphorylated 79-kilodalton (kDa) protein, p79, and a glycosylated form, p90. However, unlike v-*src*, which requires myristilation for transforming activity (5, 6, 20, 21, 33), transformation by the normally cytosolic (18, 23) *raf* protein does not require myristilation. Furthermore, *gag*-derived functions known to contribute to transformation by other *gag-onc* fusion proteins (8) are not necessary, since mouse c-*raf* (18) can be made transforming by promoter insertion (32).

The deduced amino acid sequences of v-*raf* (16) and its avian counterpart v-*mil* (36) are distantly related to the tyrosine kinase-encoding oncogenes such as *src*, *erbB*, *fes*, *abl*, and others. However, these viral *raf* proteins have serine-threonine- rather than tyrosine-specific kinase activity (17). Recently the genomic structure of c-*raf*-1 (3, 4, 4a), the active human homolog of v-*raf*, has been determined, and the complete amino acid sequence has been deduced from the cDNA sequence (4a). In addition to the active c-*raf*-1 gene there is a second v-*raf* homologous gene in humans, the c-*raf*-1-derived pseudogene c-*raf*-2 (4).

We now report the isolation of a cDNA representing a new member of the *raf* gene family, A-*raf*, which is related to v-*raf*. The A-*raf* gene is transcribed with a different tissue distribution than c-*raf*, the highest levels being observed in epididymis. Incorporation of A-*raf* cDNA into a retroviral genome generates a transforming virus; thus, A-*raf* is a new proto-oncogene. Functionally, A-*raf* is similar to *raf* and *mos* in that transformation is independent of *ras* gene function.

MATERIALS AND METHODS

Library screening and hybridization. A mouse spleen cDNA library in the vector λgt10 (2) was screened by hybridization to a v-*raf*-specific probe (the *Xho-Sst*II fragment) in 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 60°C followed by washing of filters in 1× SSC at 60°C. A phage containing a 1.6-kilobase (kb) *Eco*RI insert was purified, subcloned into plasmids pUC12 and pUC13, and characterized by restriction mapping and hybridization to digests of v-*raf* and the corresponding human gene c-*raf*-1 (4a).

Sequencing. The cDNA was sequenced by the dideoxynucleotide chain termination method (30) on fragments inserted in pUC18 and pUC19. The fragments were either specific restriction fragments or deletions generated by exonuclease III digestion.

Construction of transforming A-*raf* DNA. An analog of 3611-MSV was constructed using a cloned murine leukemia virus, LEUK (22), which is closely related to Moloney leukemia virus, and A-*raf* cDNA. A plasmid containing the complete, unpermuted LEUK DNA was linearized by cutting with *Xho*I at a site homologous to the *Xho*I site of Moloney leukemia virus. The *Xho*I ends were filled using *Escherichia coli* DNA polymerase in the presence of dATP, dTTP, dCTP, and dGTP. After treatment with bacterial alkaline phosphatase, these molecules were ligated with the 1.4-kb A-*raf* *Nco*I-*Eco*RI fragment, which had previously been blunt ended by filling the ends using *E. coli* DNA polymerase in the presence of dATP, dCTP, and dTTP, but not dGTP, and treating with S1 nuclease to remove the single unpaired nucleotide from the *Nco*I site.

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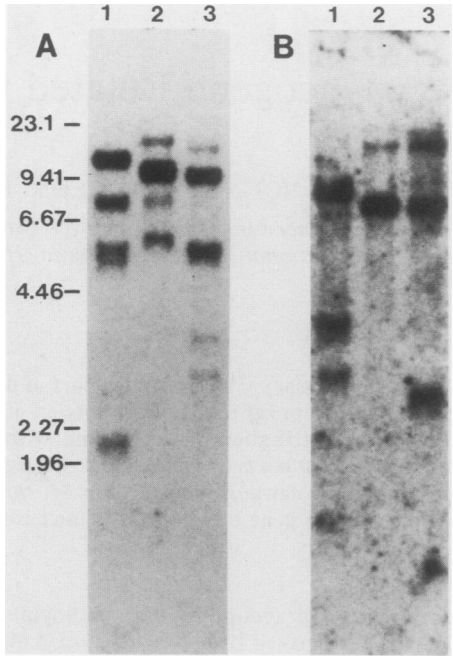

FIG. 1. Southern blot analysis of the human A-*raf* locus. High-molecular-weight DNA from the human embryonic cell line HEL 299 (ATCC) was restricted to completion with *Bgl*II (lanes 1), *Sph*I (lanes 2), and *Xba*I (lanes 3), and the fragments were separated electrophoretically on a 1% agarose gel and blotted onto Gene Screen. Blots were hybridized with ^{32}P-labeled, nick-translated c-*raf*-1 (A) or A-*raf* (B) cDNA probes in 1.5× SSC at 60°C, washed with 1× SSC at 60°C, and exposed to Kodak XAR-5 film for 4 days. Sizes of hybridizing fragments were determined from their migration relative to ^{32}P-labeled *Hin*dIII fragments of bacteriophage lambda.

RNA analysis. Total poly(A)$^+$ RNAs from mouse tissues were the generous gift of Rolf Müller (European Molecular Biology Laboratory). Polysome-associated RNA from the cell line NFS-60 was isolated as described previously (24), and poly(A)$^+$ RNA was selected by oligo(dT)-cellulose chromatography. Equal quantities of poly(A)$^+$ RNA (20 μg) were denatured by glyoxalation (29), transferred to nitrocellulose, and hybridized under stringent conditions to ^{32}P-labeled nick-translated probes as described by Thomas (38).

DNA analysis. High-molecular-weight DNA was prepared from isolated nuclei of the human embryonic lung cell line HEL 299 (ATCC) as described previously. DNAs were digested to completion with *Bgl*II, *Sph*I, and *Xba*I, separated electrophoretically on 1% agarose, and blotted onto Gene Screen (New England Nuclear Corp.). Blots were hybridized with the 2.97-kb c-*raf*-1 cDNA (4a) and 1.6-kb A-*raf* cDNA probes in 1.5× SSC at 60°C, washed extensively with 1× SSC at 60°C, and exposed to XAR-5 film.

Immunoprecipitation. Cells were labeled and lysed as previously described (3, 32). Clarified lysates were incubated in 1-ml portions with 10 μl of antibody and 100 μl of protein A-agarose containing 1 mg of bovine serum albumin per ml. Antibodies included rabbit sera raised against p30^{gag} and against a v-*raf* C-terminal synthetic peptide, SP63. Incubation was overnight at 4°C with gentle shaking. Immunoprecipitates were washed three times with lysis buffer, and pellets were dissolved in 50 μl of sample buffer, heated for 2 min at 100°C, and run on a 5 to 20% sodium dodecyl sulfate-polyacrylamide gel. The gel was fixed in 200 ml of 14% acetic acid–10% methanol for 0.5 h and then transferred to 200 ml of Amplify (Amersham Corp.) for an additional 0.5 h. The gel was then dried and exposed to SB-5 film (Kodak) overnight.

DNA transfection. Transfection of NIH 3T3 cells with cloned DNA was carried out by the calcium phosphate precipitation procedure as previously described (26). Transformed foci were counted 8 days posttransfection.

RESULTS

A-*raf* is related to v-*raf*. The 1.6-kb A-*raf* cDNA was isolated from a mouse spleen cDNA library by using a v-*raf*-specific probe at moderate stringency. However, its restriction map did not correspond to that of v-*raf*. In particular, it lacked the *Sph*I site present in v-*raf* and human c-*raf*-1 even though it hybridized to portions of v-*raf* on both sides of the v-*raf* *Sph*I site. Moreover, A-*raf* appeared to be a different gene as judged from a comparison of Southern blots of restricted human genomic DNA (Fig. 1) hybridized with c-*raf*-1 (Fig. 1A) and A-*raf* (Fig. 1B) probes.

To further characterize the homology of A-*raf* to v-*raf*, the cDNA clone was sequenced (Fig. 2). Comparison of the sequences of A-*raf* and v-*raf* established that the sequences are related (Fig. 2), showing 77% homology between nucleotides 700 and 980 but less outside this range. The A-*raf* sequence has an open reading frame of 1,308 nucleotides beginning at the 5′ end which encodes a protein that is closely related to the v-*raf* and v-*mil* proteins (Fig. 3). However, the A-*raf* termination codon occurs 6 nucleotides earlier than in viral and human c-*raf*-1. A 280-nucleotide 3′ untranslated region ends with a poly(A) stretch of 14 nucleotides. The 3′ untranslated region is thus considerably shorter than the 900-nucleotide 3′ untranslated region of the human c-*raf*-1 message. Since there is no terminator at the 5′ end of this open reading frame, the cDNA must be incomplete. Indeed, the stretch of 436 amino acids encoded by the A-*raf* cDNA is substantially smaller than the 648 amino acids of the complete human *raf* protein, and the 1.6-kb size of the A-*raf* cDNA is substantially less than the 2.4-kb A-*raf* mRNA (see below).

Comparison of the deduced amino acid sequence of murine A-*raf* with those of murine v-*raf* and human c-*raf*-1 indicates that the amino acid homology is as high as 85% for amino acids 187 to 287 but decreases to 70 to 75% at the carboxy terminus and between amino acids 130 and 187 (Fig. 3). Before amino acid 130 there is almost no homology of murine A-*raf* to human c-*raf*-1 and avian v-*mil*, although c-*raf*-1 and avian v-*mil* have substantial homology to each other in this region. Indeed, this region showed no significant homology with any other protein in the National Biomedical Research Foundation protein data bank. The one exception is amino acids 40 to 61, which match the c-*raf*-1 sequence at 19 of 22 amino acids if a gap of 4 amino acids is introduced elsewhere in the A-*raf* sequence. This homology region coincides with the 5′ end of v-*mil*. Thus, the homology between A-*raf* and *raf* appears to be restricted to the kinase domain as defined by homology to other kinase genes as well as the position (amino acids 146 to 151) of the apparent nucleotide binding site. Based on the sequence comparison, the observation that A-*raf* hybridizes to different restriction fragments in human DNA than does v-*raf* (Fig. 1), and the fact that these fragments map to different chromosomes, 7 and X (K. Huebner, A. ar-Rushdi, C. A. Griffin, M. Isobe, C. Kozak, B. S. Emmanuel, L. Nagarajan, J. L. Cleveland, M. A. Gunnell, M.D. Goldsborough, C. M. Croce, and U. R. Rapp, Proc. Natl. Acad. Sci. USA, in press) rather than 3

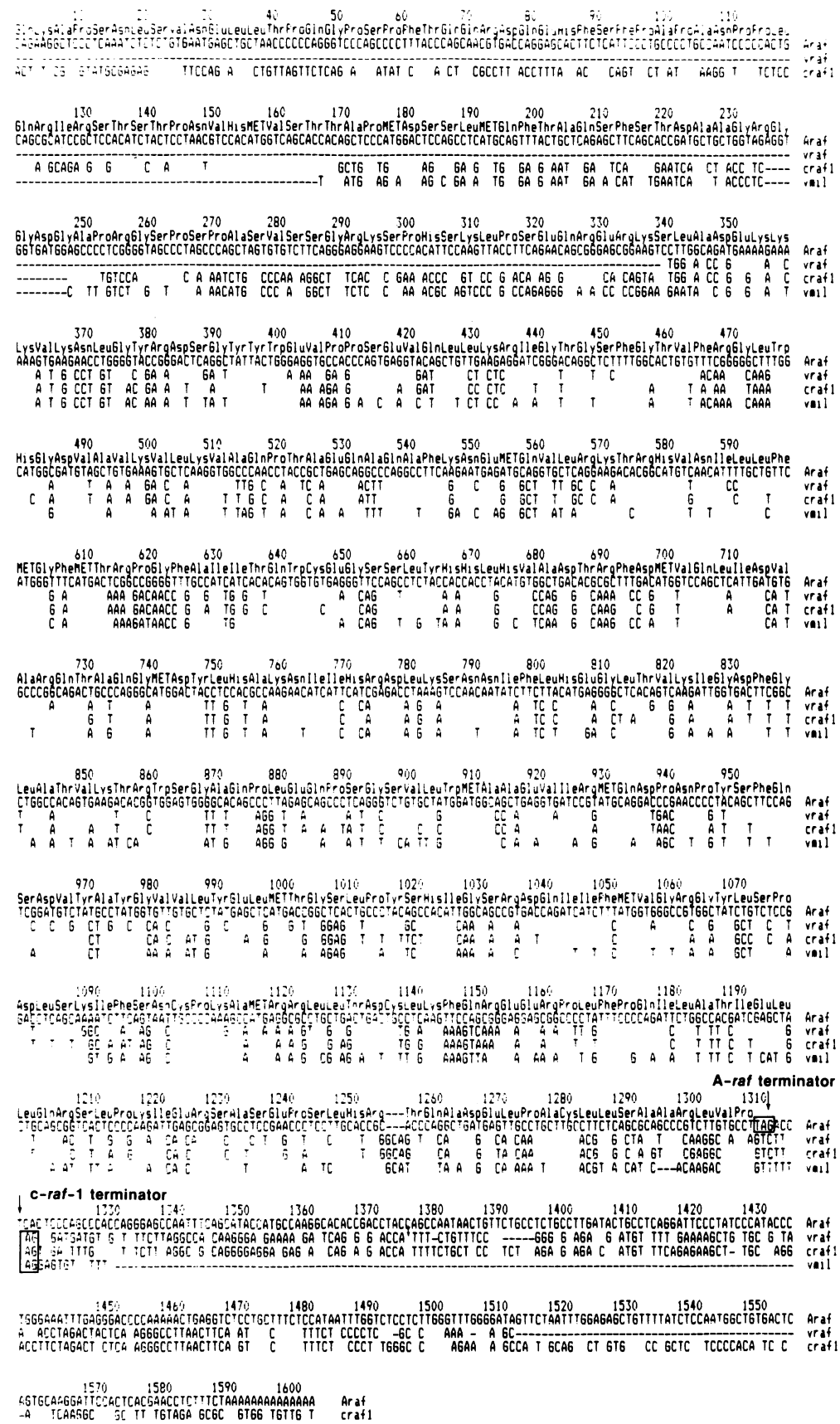

FIG. 2. Nucleotide sequence of A-*raf* cDNA in comparison with sequences of v-*raf*, c-*raf*-1, and v-*mil*. Dashed lines indicate gaps introduced to align sequences and the lack of corresponding sequence in v-*raf* and v-*mil*.

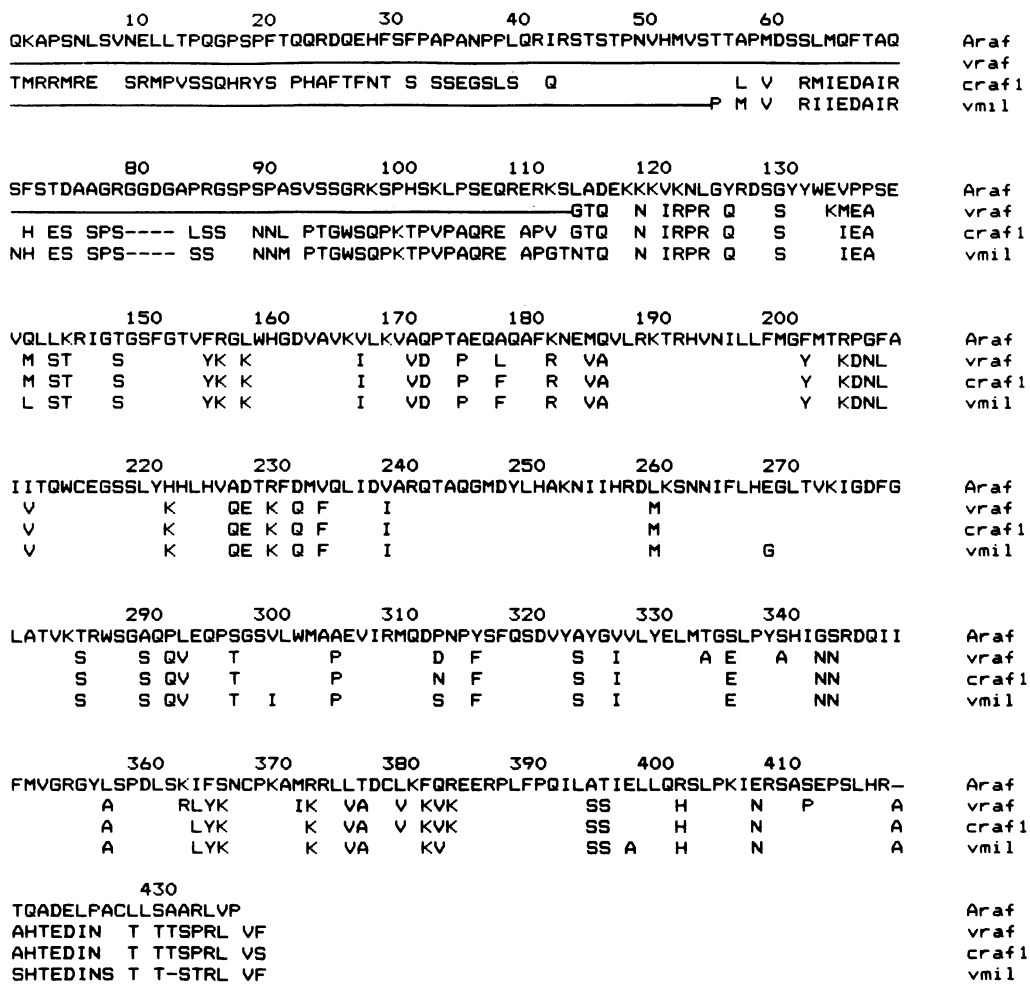

FIG. 3. Amino acid sequence of A-*raf* in comparison with other *raf* family members. Dashed lines indicate gaps introduced to align sequences, and solid lines demarcate the end of corresponding sequence in v-*raf* and v-*mil*.

and 4 for *raf* (4), we conclude that A-*raf* is a new member of the *raf* gene family. Since v-*mil* is more closely related to *raf* than is A-*raf*, it appears that the A-*raf* and *raf* genes were derived from a gene duplication event which substantially predates the evolutionary divergene of birds and mammals.

Tissue-specific expression of A-*raf*. Expression of A-*raf* was investigated in a series of mouse tissues (Fig. 4) and in whole mouse embryos at two stages of development. c-*raf* was expressed as a 3.1-kb RNA at high levels in 14-day embryo, heart, lung, testes, epididymis, brain, and intestine; lower levels were detectable in spleen, liver, placenta, and 18-day embryo (Fig. 4A). In contrast to c-*raf*, expression of A-*raf*-related RNA was remarkably tissue specific. A 2.4-kb transcript was detected at high levels in epididymis and at lower levels in liver and intestine (Fig. 4B).

A-*raf* is capable of transformation. To test whether A-*raf* has transforming potential, an analog of 3611-MSV, the virus carrying v-*raf*, was constructed by cloning the A-*raf* cDNA in-frame into the *gag*-p30 region of the LEUK strain of Moloney leukemia virus (22). This new recombinant retrovirus should express a *gag*-A-*raf* fusion protein of 75,302 calculated molecular weight, slightly smaller than the *gag*-v-*raf* fusion protein of 3611-MSV, which has a calculated molecular weight of 79,652. When tested for transforming ability on NIH 3T3 cells, the A-*raf* recombinant (designated 9IV A-*raf* MSV) transformed fibroblasts with an efficiency comparable to 3611-MSV (see Table 1). In contrast, a construct containing the A-*raf* insert in the opposite orientation (8IV) failed to induce any foci. The foci were made up of crisscrossing cells that were less refractile than 3611-MSV-transformed NIH 3T3 cells and were more dense than foci induced by J1 virus (25), a variant of 3611-MSV carrying a *raf*/*mil* hybrid gene. Infected cells grew readily in soft agar at a rate intermediate between J1 and 3611-MSV. Moreover, high-titer stocks of 9IV A-*raf* MSV have been prepared and found to be tumorigenic in NFS/N mice injected intraperitoneally as newborns. Two different helper viruses were used for rescue of 9IV A-*raf* MSV from nonproducer-transformed, soft agar-derived NIH 3T3 cells. Both pseudotype stocks, one with the LEUK strain of Moloney leukemia virus and the other with Cas Br-M murine leukemia virus (R. Yetter, unpublished data), induced rapid fibrosarcoma and erythroid hyperplasia after a latency of 5 to 10 weeks. This tumor type was not induced by either of the helper viruses alone. Nonproducer cell clones transformed by the 9IV A-*raf* recombinant virus were isolated from soft agar and tested for the expression of A-*raf* by Northern blotting and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Northern blots using A-*raf* and murine leukemia virus long terminal repeat probes demonstrated a genome-size RNA of

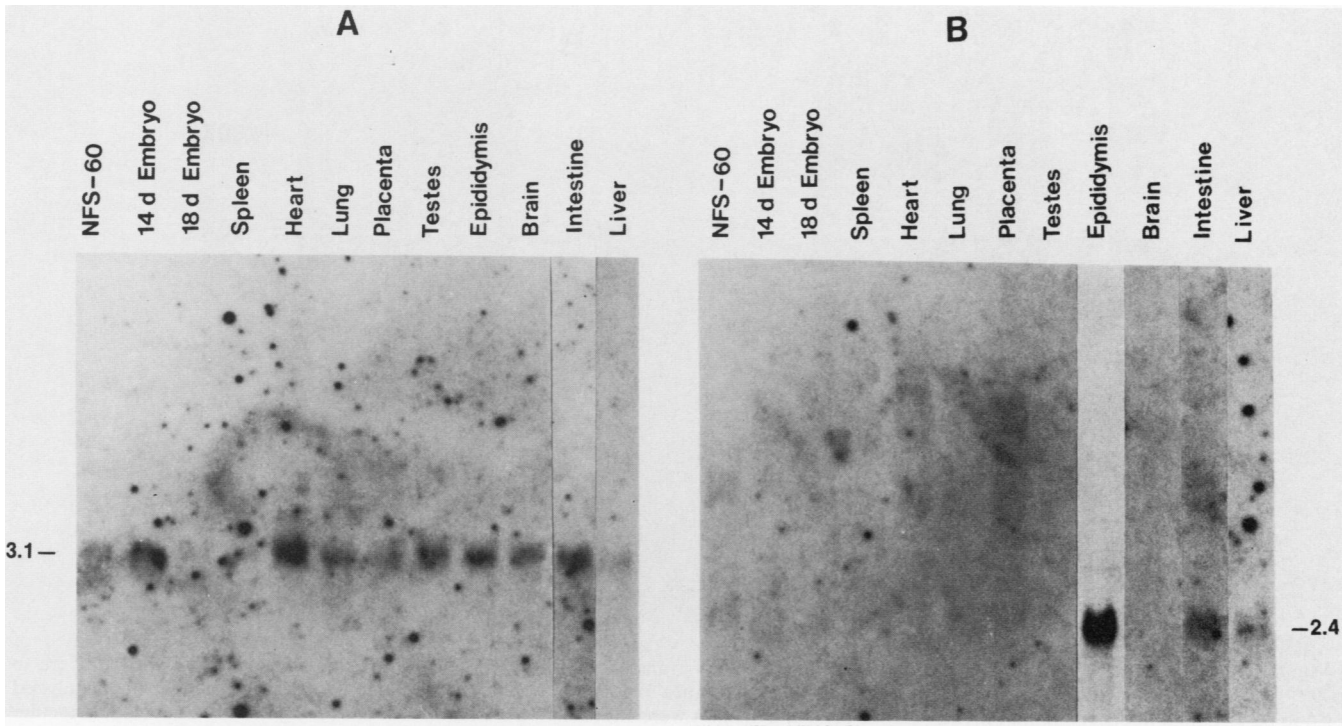

FIG. 4. Northern hybridization analysis of c-*raf*-1 (A) and A-*raf* (B) transcripts expressed in mouse tissues. Total poly(A)$^+$ RNA (20 μg) was glyoxalated, blotted, and hybridized with ^{32}P-labeled, nick-translated c-*raf*-1 (A) and A-*raf* (B) cDNA probes. The sizes of the RNAs were determined from their migration relative to ^{32}P-labeled, denatured *Hin*dIII fragments of bacteriophage lambda. The blots were exposed for 1 week.

9.6 kb, the predicted size of this virus (data not shown). As was observed with v-*raf* and c-*raf*-1, and unlike the situation with v-*myc* versus c-*myc* (24), expression of the cellular A-*raf* gene was not suppressed in cells expressing A-*raf* from the viral genome. When we looked for expression of the predicted *gag*-A-*raf* fusion protein, using p30-specific antiserum, we observed the expected polypeptide as 75 kDa (Fig. 5B, lane 1); the 85-kDa polypeptide in the same lane is presumably a glycosylated version of *gag*-A-*raf*, analogous to the glycosylated form of *gag*-v-*raf* of 90 kDa (Fig. 5A). Immunoprecipitations were also performed with antibody SP63, with specificity for the 12 C-terminal amino acids of c-*raf*-1 (32). As expected from the sequence (Fig. 5), this antibody recognized the *gag*-v-*raf* polypeptides present in J2 virus (25)-transformed cells (Fig. 5A, lane 3) and the endogenous cellular c-*raf*-1 protein of 72 kDa, but failed to detect *gag*-A-*raf* proteins.

Transformation by A-*raf* is independent of *ras* gene function. Cellular *ras* proteins are required for growth of NIH 3T3 fibroblast cells in serum-containing medium (19). However, the cells continue to replicate in the absence of active *ras* proteins if they express v-*raf* or v-*mos*. In contrast, cells transformed by v-*sis*, v-*fms*, v-*src*, and v-*fes* do not replicate in the absence of active *ras* proteins (35a). We therefore determined whether expression of A-*raf* from the viral genome might also overcome the dependence of NIH 3T3 cells on cellular *ras* proteins by injecting anti-*ras* antibody 259 (19) into NIH 3T3 cells before and after transformation by either v-*raf* (3611-MSV) or mouse A-*raf* (virus construct 9IV), in the presence of helper virus (LEUK strain of Moloney leukemia virus or 4070A). Determination of tritiated thymidine incorporation by cells after antibody injection (Table 2) showed that transformation of cells either by v-*raf* or by virus containing A-*raf* obviated the cellular dependence on *ras* proteins for growth in standard medium. We conclude that the N-terminally truncated portion of A-*raf* present in virus construct 9IV can substitute for v-*raf* in this assay.

TABLE 1. Transforming efficiency of v-*raf* and A-*raf*

Transfected DNA[a]	Transformation efficiency (FFU[b]/μg of DNA)
LEUK (helper)	0
PM1 + helper	12,000
3611-MSV + helper	4,000
9IV + helper	6,000
8IV + helper	0

[a] PM1, A plasmid containing an active v-*mos* gene. 9IV, A hybrid of the LEUK strain of Moloney leukemia virus (*Xho*I) with the *Nco*I-*Eco*RI fragment of A-*raf*. 8IV, A reverse orientation of the insert (A-*raf*) in hybrid 9IV.
[b] FFU, Focus-forming units.

DISCUSSION

By characterizing a 1.6-kb cDNA derived from mouse spleen, we have identified a new proto-oncogene, which we have called A-*raf* in recognition of its relatedness to the *raf* oncogene. The protein encoded by the cDNA has amino acid homology as high as 85% with the kinase region of c-*raf*-1 but very little similarity elsewhere. In this sense it is similar to the *neu* gene, which has recently been shown to be related to the *erbB* oncogene and the EGF receptor. The *neu* oncogene (31) has been shown by hybridization to be related only to the kinase region of *erbB*, and the apparently identical DNA fragment MAC117 (31) has been shown by sequence analysis to have 85% amino acid homology to a

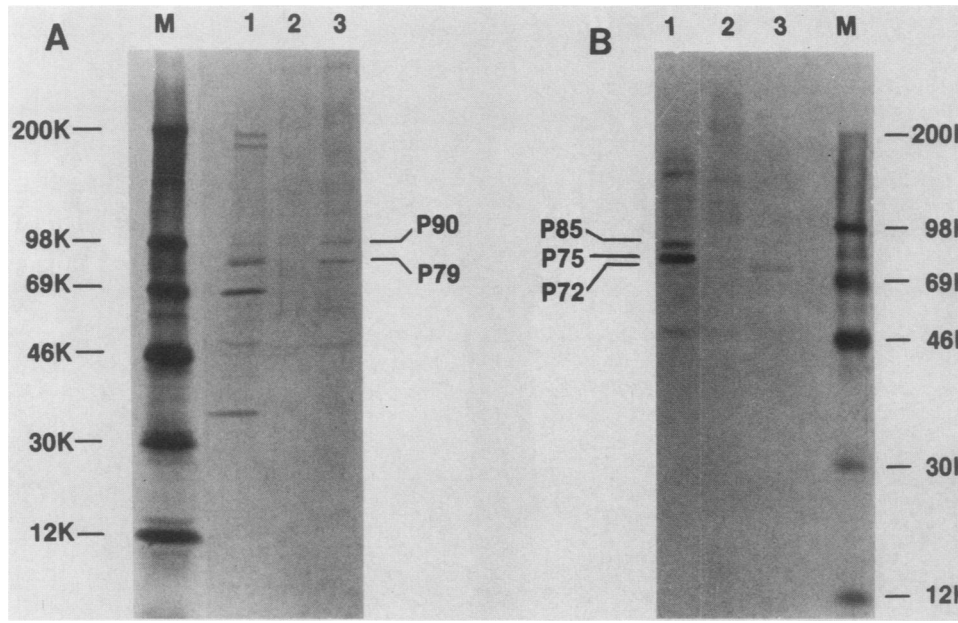

FIG. 5. *raf* proteins expressed in fibroblasts transformed by v-*raf* (J2) and A-*raf* (9IV) recombinant retroviruses. Lysates of [^{35}S]methionine-labeled J2 virus-transformed NIH 3T3 (A) and 9IV-transformed FRE (B) cells were immunoprecipitated with antisera to p30^{gag} (A and B, lanes 1) and SP63 (33) with (lanes 2) and without (lanes 3) competing SP63 peptide. Immunoprecipitates were then analyzed on 5 to 20% sodium dodecyl sulfate-polyacrylamide gels, and the gels were exposed to SB-5 film (Kodak) overnight. M, ^{14}C-labeled molecular weight standards (New England Nuclear Corp.).

portion of the kinase region. Other genes, N-*myc* and r-*fos*, which are less closely related to the previously known oncogenes *myc* and *fos*, have been described (7, 15, 10, 34, 37). These results suggest that a number of oncogenes belong to small families with members having related yet distinct functions. In the case of A-*raf* this supposition is supported by the observed differences in tissue distribution of RNA expression relative to *raf*. Although the A-*raf* and *raf* proteins are closely related, we have shown that they can be distinguished by using antibodies directed against a synthetic peptide corresponding to the C-terminal 12 amino acids of *raf*. Presumably antibodies against the C-terminal peptide of A-*raf* will allow the detection of cellular A-*raf* without cross-reaction with c-*raf*.

Incorporation of A-*raf* cDNA into the genome of murine leukemia virus, such that it was expressed as a *gag* fusion protein, revealed a strong potential transforming activity of the A-*raf* proto-oncogene. Similar constructions with the human homolog of mouse A-*raf* extend this observation to include the human A-*raf* gene (M. Huleihel and U. R. Rapp, unpublished data). We conclude that point mutations within the tested coding segment of the gene are not required for oncogenic activation. In analogy to *raf*, we suspect that linkage to viral *gag* sequences and the concomitant N-terminal myristilation of the fusion protein are probably not critical for oncogenic activity, and we consider amino-terminal truncation as the most likely contributing factor to the transforming activity of A-*raf*. Comparison between c-*raf*-1 and A-*raf* shows an abrupt loss of homology 5′ of the kinase domain of the A-*raf* protein, suggesting a functional separation of the molecule into catalytic and regulatory domains. NH_2-terminal truncation might thus release the catalytic portion of the protein from its normal regulation and trigger constitutive enzymatic activity. However, more constructions with the complete A-*raf* cDNA as well as additional deleted forms will have to be tested to evaluate this possibility.

TABLE 2. Labeling efficiences of NIH 3T3 cells transformed by various oncogenes[a]

Oncogene	Cell line	Labeling efficiency[b]	
		Avg % (±SE)	No.[c]
None	NIH 3T3	10 (1.3)	7
v-*raf*	NIH F4-3611-LEUK	93 (4.0)	10
v-*raf*	NIH F4-3611-4070A	91 (2.3)	6
A-*raf*	NIH-9IV-LEUK	92 (3.1)	4

[a] Antibody 259 was microinjected into the cytoplasm of the cell types listed. A 3-h pulse of [^{3} H]thymidine between 18 and 24 h after injection was followed by methanol fixation and fluorescent-antibody staining to identify injected cells (14). Injections with nonneutralizing antibody 238 have also been performed in these cell lines. In all cases of antibody 238 injections, average labeling efficiencies varied from 95 to 105% (35a).

[b] Labeling efficiencies were obtained by first determining the percentage of injected cells which had incorporated thymidine. This percentage was then divided by the percentage of uninjected cells on the same plate which were labeled. SE, Standard error value.

[c] Number of determinations, each of which averaged between 150 and 200 injected cells.

The expression of c-*raf*-1 in many normal tissues suggests some common regulatory function for this gene. In contrast, A-*raf* appears to be more exclusively employed due to differential transcription of the gene in a select set of tissues. Nevertheless, both genes can substitute for each other when expressed in growth-restricted fibroblast cells. This finding comes from experiments, reported here for A-*raf* and elsewhere for other transformants (35a), in which a functional classification of oncogenes was attempted according to their dependence on intact *ras* gene function for replication of fibroblast cells in culture. They involve injection of *ras* antibody into the cytoplasm of normal and oncogene-transformed cells. Whereas normal cells respond with growth arrest to antibody injection when grown in serum-

containing medium (19), cells transformed by v-*raf*, A-*raf*, and v-*mos* (35a) continue to replicate. In contrast, oncogenes v-*src*, v-*fms*, v-*src*, and v-*fes* do not overcome this growth arrest (35a). v-*raf* and v-*mos* have other similarities besides their ability to complement inactive *ras* proteins; they are both cytoplasmic proteins and have associated serine-threonine-specific protein kinase activity. These shared properties suggest a common position of *raf*, *mos*, and A-*raf* in the transduction of mitogenic signals. Whether their observed independence on the functioning of *ras* genes is due to a more downstream position of *ras* and *mos* in signal transduction pathways involving *ras* or indicates the existence of independent pathway(s) remains to be determined. Use of anti-*raf* antibodies injected into *ras*-transformed cells should help clarify this issue.

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