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Mutational Activation of c-*raf*-1 and Definition of the Minimal Transforming Sequence

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A series of wild-type and mutant *raf* genes was transfected into NIH 3T3 cells and analyzed for transforming activity. Full-length wild-type c-*raf* did not show transforming activity. Two types of mutations resulted in oncogenic activity similar to that of v-*raf*: truncation of the amino-terminal half of the protein and fusion of the full-length molecule to *gag* sequences. A lower level of activation was observed for a mutant with a tetrapeptide insertion mapping to conserved region 2 (CR2), a serine- and threonine-rich domain located 100 residues amino-terminal of the kinase domain. To determine essential structural features of the transforming region of *raf*, we analyzed point and deletion mutants of v-*raf*. Substitutions of Lys-56 modulated the transforming activity, whereas mutation of Lys-53, a putative ATP binding residue, abolished it. Deletion analysis established that the minimal transforming sequence coincided precisely with CR3, the conserved Raf kinase domain. Thus, oncogenic activation of the Raf kinase can be achieved by removal of CR1 and CR2 or by steric distortion and requires retention of an active kinase domain. These findings are consistent with a protein structure model for the nonstimulated enzyme in which the active site is buried within the protein.

The *raf* family of proto-oncogenes consists of three active members: A-*raf*-1, B-*raf*, and c-*raf*-1 (2, 4, 5, 20, 22, 23). The three proteins show the greatest stretch of homology in the carboxy-terminal half, which, in the case of c-*raf*-1, has been shown to have serine- and threonine-specific protein kinase activity (33, 48). Physiologically, Raf protein kinases function as information shuttles that communicate between the cell surface and nucleus. From Ki-*ras* revertant and anti-*ras* antibody microinjection studies, it is known that c-*raf*-1 acts independently of and perhaps downstream from Ras in signal transduction (2, 22, 36, 41, 42, 52). In addition, we recently showed that Raf is hyperphosphorylated and enzymatically activated in cells which are transformed by *src*, *fms*, or *ras*, and in cells which have been treated with platelet-derived growth factor or 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (35). In response to platelet-derived growth factor and TPA, Raf protein is also translocated from the cytoplasm to the perinuclear space, as is evident from immune fluorescence and cell fractionation studies (44). Ultimately, *raf* is thought to exert its effect by modulation of transcription factor activity via phosphorylation (42). Evidence for this comes from experiments in which cells transfected with activated *raf* genes showed increased transcriptional activity from an AP1-dependent promoter in transient transfection assays, suggesting that Raf is involved in the regulation of transcription factors of the *jun*-AP1 gene family (56). Raf function is essential for normal rates of cell proliferation, as demonstrated by the lethal effect of mutations in the *Drosophila melanogaster* D-*raf*-1 locus (37).

c-*raf*-1, the most-studied member of the family, encodes a protein containing 648 amino acids (molecular mass, 74 kilodaltons [kDa]). c-*raf* was first identified in its oncogenic form, v-*raf*, the oncogene of the acutely transforming virus 3611-murine sarcoma virus (3611-MSV) (43, 46). v-*raf* is expressed as a myristylated Gag-Raf fusion protein consisting of the amino-terminal 384 amino acids of Gag and the carboxy-terminal 323 residues of mouse c-Raf (43, 46, 47). Amino-terminal truncation and fusion to other genes are also found in activated versions of c-Raf which are frequently obtained by transfection of genomic human and rodent DNAs into NIH 3T3 cells (14, 25, 50, 53). In addition to these cases, in which it was not clear whether protein fusion and myristylation, truncation, or altered gene regulation was the major cause of oncogenic activation of any of these transforming versions, several studies showed that truncation alone is sufficient to oncogenically activate the *raf* gene (34, 54, 56).

Inspection of the primary structure of c-Raf suggests that truncation eliminates a potential ligand-binding site in the amino-terminal half of the protein. Evidence for this derives from the presence of a so-called cysteine finger structure, an amino acid sequence motif with the consensus C-X_2-C-X_{7-13}-C-X_2-C (2, 3, 25, 42). Related cysteine repeats are found in the ligand-binding domains of protein kinase C and other ligand-regulated protein kinases such as growth factor receptors and, in some cases, have been shown to bind divalent cations and/or confer DNA-binding activity (3, 11, 28, 38). The cysteine-rich region is part of the first of two highly conserved regions, termed CR1 (c-Raf amino acids 61 to 192) and CR2 (251 to 266), found in the amino-terminal halves of A-Raf and c-Raf proteins (18, 42). CR2 consists of a stretch of 20 amino acids rich in serine and threonine residues and is found 80 residues upstream of CR3, the highly conserved kinase domain of c-Raf, which extends from position 333 to position 625. These structural considerations are consistent with a mechanism of oncogenic

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activation of Raf proteins which generates a constitutively active kinase by removal or inactivation of an N-terminal negative control domain (2, 4, 5). In this paper, we describe a series of mutants designed to test which mutational events activate or inactivate the transforming activity of Raf protein kinase.

MATERIALS AND METHODS

Cells and viruses. NIH 3T3 clone 7 (Cl 7) cells, a subline of NIH 3T3 cells selected for flat structure, were grown in the Dulbecco modification of Eagle medium supplemented with 10% calf serum, 100 IU of penicillin, and 50 μg of streptomycin per ml. The parental strain for recombinant retroviral constructs was Leuk2, a strain of murine leukemia virus (MuLV) closely related to Moloney MuLV and 3611-MSV (43). Leuk2 was also used as the helper virus for rescue of defective retroviral constructs.

DNA transfection. Plasmid DNAs were linearized by digestion with *Sca*I (New England BioLabs) before transfection of NIH 3T3 cells, which was carried out by the calcium phosphate precipitation procedure described by Graham and van der Eb (16) modified as previously described (43), employing NFS/N mouse high-molecular-weight liver DNA as carrier. If the test construct did not carry a neomycin resistance marker, pSV2neo was included in the transfection mix at a molar ratio of 1:5 (pSV2neo:test plasmid). Transformed foci were counted 4 to 10 days posttransfection.

Site-directed mutagenesis. Single-stranded DNA from a Bluescript phagemid containing nucleotides 92 to 2203 of human c-*raf* cDNA described by Bonner et al. (5) was used as a template for site-directed mutagenesis. Oligonucleotides introducing the desired base changes or looping out the appropriate region of the gene were hybridized to the single-stranded DNA and used as primers for synthesis of the complementary DNA strand, as described previously (15, 59). Mutations were verified by DNA sequencing. Linker insertion mutagenesis was carried out as described previously (55).

Immunoprecipitation of Raf proteins and Western blot (immunoblot) analysis. Unlabeled cell extracts from T75 flasks were immunoprecipitated as previously described with rabbit anti-SP63 antibody is the presence or absence of competing synthetic peptide SP63, which corresponds to the carboxy-terminal 12 amino acids of c-Raf (47) or with anti-p15^{gag} antiserum. The immunoprecipitates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gels were electroblotted onto diazobenzyloxymethyl (DMB) paper, and the blots were reacted with anti-SP63 antibody followed by ^{125}I-labeled protein A. Alternatively, solubilized total cell extracts were separated by SDS-PAGE and transferred to nitrocellulose paper by electroblotting. Blots were reacted with anti-Raf or anti-p15^{gag} antibodies followed by a secondary antibody coupled to alkaline phosphatase. Bands were stained with BCIP (5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt) and Nitro Blue Tetrazolium chloride. To metabolically label cells, the cultures were incubated in the presence of [^{35}S]methionine (50 μCi/ml) in otherwise methionine-free media. Immunoprecipitates from these lysates were separated by SDS-PAGE, and the dried gels were exposed to X-ray film.

RNA analyses. Total RNA was isolated from cultures of uninfected and NIH 3T3 Cl 7 cells transfected with various retroviral vector DNAs as previously described (46). RNA was electrophoresed, blotted, and hybridized, and the blots were washed by standard procedures (7, 8). The probes used were derived from human (5) or mouse (G. Heidecker, unpublished data) c-*raf* cDNA clones.

RESULTS

Construction of retroviral vector Elneo. In order to test the transforming activity of normal and mutated versions of c-*raf*-1, c-*raf*-1 cDNA was incorporated into a retroviral vector termed Elneo. The backbone of the vector is derived from a molecular clone of the MuLV strain Leuk2, a close relative of Moloney MuLV, which gave rise to 3611-MSV (43). The *neo* resistance gene of Tn*5* with a preceding Rous sarcoma virus splice acceptor site displaces most of the envelope gene and is expressed as a subgenomic message (Fig. 1) (21). A multicloning site was inserted between the *Pst*I site at position 563, which precedes the *gag* translational start site and the *Apa*I site at position 5381 (of the Moloney MuLV sequence [51]). Unique restriction sites for *Eco*RI, *Hin*dIII, *Xho*I, and *Apa*I are contained in the polylinker which was derived from the multicloning site of the Bluescript vector (12). In addition, the *Bam*HI site located between the multicloning site and the splice acceptor can be used for insertions.

Deletion activation of c-*raf*-1. The human c-*raf*-1 cDNA was inserted into the Elneo expression vector as an *Eco*RI-to-*Apa*I fragment; this cDNA fragment contains 38 nucleotides of the nontranslated leader sequence and extends 182 nucleotides beyond the translational stop codon. This construct was called EC. The c-*raf*-1 gene is translated from its own AUG start codon, which shows good agreement with the consensus translation initiation sequence (30). Deletions into the coding region of c-*raf*-1 cDNA were introduced by site-directed mutagenesis starting at codon 3 and ending at codons 334 (EC12) and 344 (EC13) (Fig. 1). Both deletions should generate polypeptides that contain the complete kinase domain if the consensus sequence for ATP binding, which starts at c-*raf* codon position 356, is taken as the boundary. However, while the deletion of EC12 retains the complete CR3 domain, EC13 lacks the first 10 amino acids. EC, EC12, and EC13 plasmid DNAs were transfected into NIH 3T3 Cl 7 cells, and transfected cells were selected for resistance to neomycin analog G418. None of the EC and EC13 transfected colonies were transformed. In contrast, all of the EC12 colonies were transformed, as judged by morphology (Fig. 1) and growth in soft agar (data not shown). In addition, virus was rescued from the neomycin-resistant colonies and assayed for focus formation. All rescued virus preparations had comparable titers of neomycin-resistant colony-inducing virus (3×10^3 to 9×10^3 Neo^r units per ml), but only EC12 was able to morphologically transform NIH 3T3 cells.

In order to ascertain proper expression from the EC, EC12, and EC13 construct viruses, we analyzed the level of viral and endogenous c-*raf*-1 RNA by Northern (RNA) blot analysis (Fig. 2A). The steady-state level of EC genome-sized RNA was greater than 10-fold higher than that of the endogenous c-*raf*-1 RNA, even when visualized with mouse c-*raf* cDNA probe. Cells transfected with the EC12 and EC13 constructs expressed considerably less viral RNA; however, the levels of both constructs were comparable to those observed in 3611-MSV-infected cells. When viewing Fig. 2A, one must take into account the fact that a direct comparison is difficult, since different probes have to be used to optimally visualize either 3611-MSV or EC constructs. With human c-*raf* probe, the intensities of the high-molecu-

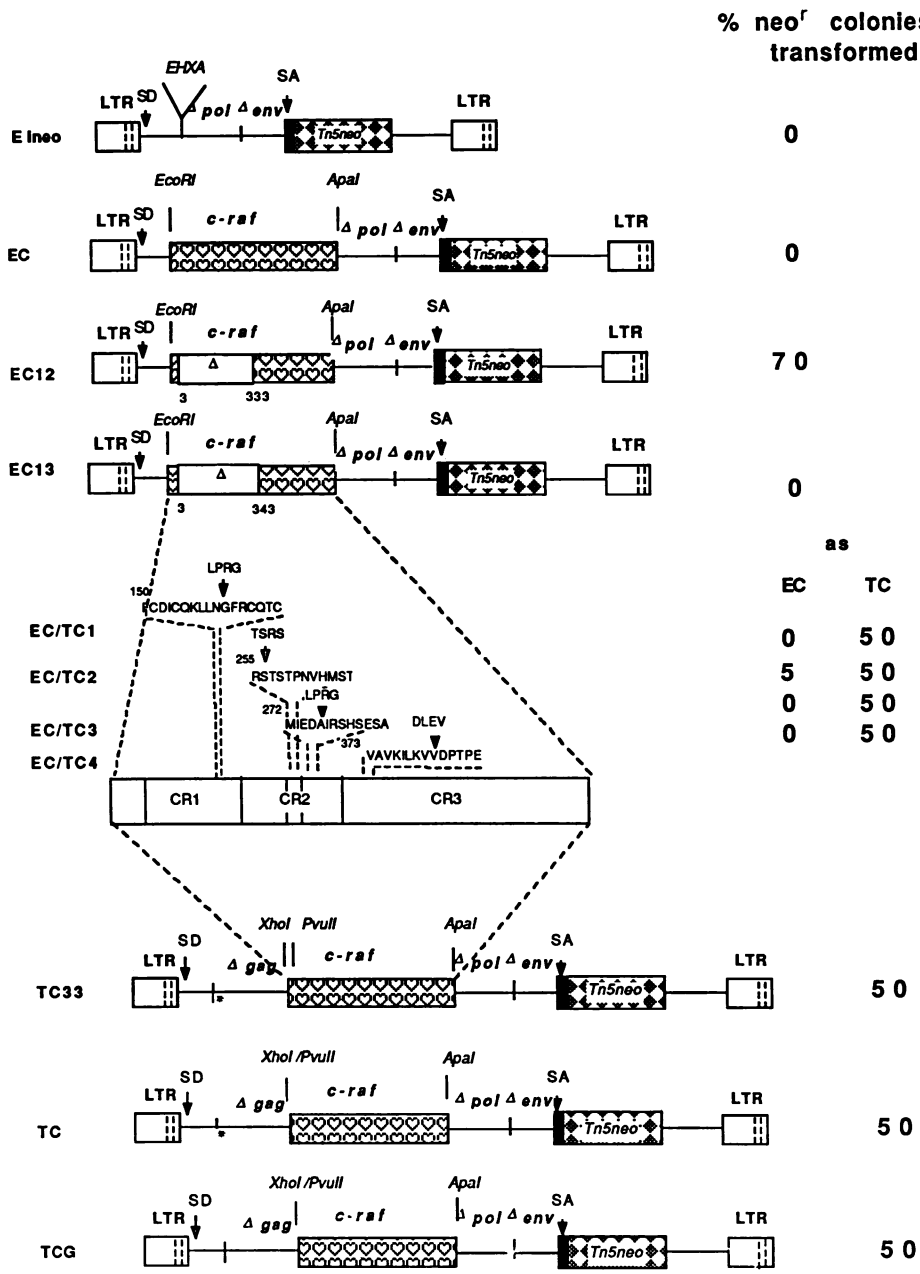

FIG. 1. Genomic organization of retroviral vector Elneo and EC and TC derivatives. The structure of the retroviral vector Elneo is given at the top. The locations of splice donor (SD) and acceptor (SA) sites and useful cloning sites (*E*, *Eco*RI; *H*, *Hin*dIII; *X*, *Xho*I; *A*, *Apa*I) are indicated. The c-*raf*-1 cDNA was inserted as an *Eco*RI-to-*Apa*I fragment to generate ECneo. The codon positions for the deletion junctions in EC12 and EC13 are shown. Also given are the amino acid sequences corresponding to the regions of linker insertion in EC1, EC2, and EC4, and TC1, TC2, and TC4. The asterisk denotes the presence of a myristylated glycine residue in position 2. CR1, CR2, and CR3 stand for Raf conserved regions 1, 2, and 3. LTR, Long terminal repeat.

lar-weight *raf* band are reversed for cells that express EC12 and EC13 and cells that express 3611-MSV (data not shown).

Levels of Raf protein in EC, EC12, and EC13 cells were determined from the anti-Raf immunoprecipitates of [^{35}S] methionine-labeled cells (Fig. 2B). In the case of EC12 and EC13 cells, Raf proteins of expected sizes (35 and 33 kDa, respectively) were clearly detected. The levels of the p35^{raf} (EC12) and p33^{raf} (EC13) proteins were 5- to 10-fold higher than those of the endogenous p74^{raf}. The level of p74^{raf} in EC-transfected cells was similarly elevated (Fig. 2B), as was evident when the p74^{raf} from the EC constructs was compared with that of the NIH control run in parallel or with the level of p79$^{gag\text{-}raf}$ expressed by the same number of cells infected with 3611-MSV.

Amino-terminal fusion activates c-Raf. To test whether steric distortion of the amino-terminal half induces Raf protein transforming activity, we fused near-full-length and full-length c-*raf* cDNA-coding region to the first 313 codons of the Leuk2 *gag* sequences. The first construct was obtained by ligating the c-*raf* sequence at the *Pvu*II site to the *Xho*I site of *gag*, which had been filled in. This construct, called TC, is missing the first 25 c-*raf* codons. The full-length construct (TC33) was obtained by converting the sequence around the translational initiation site of c-*raf*-1 into an *Xho*I

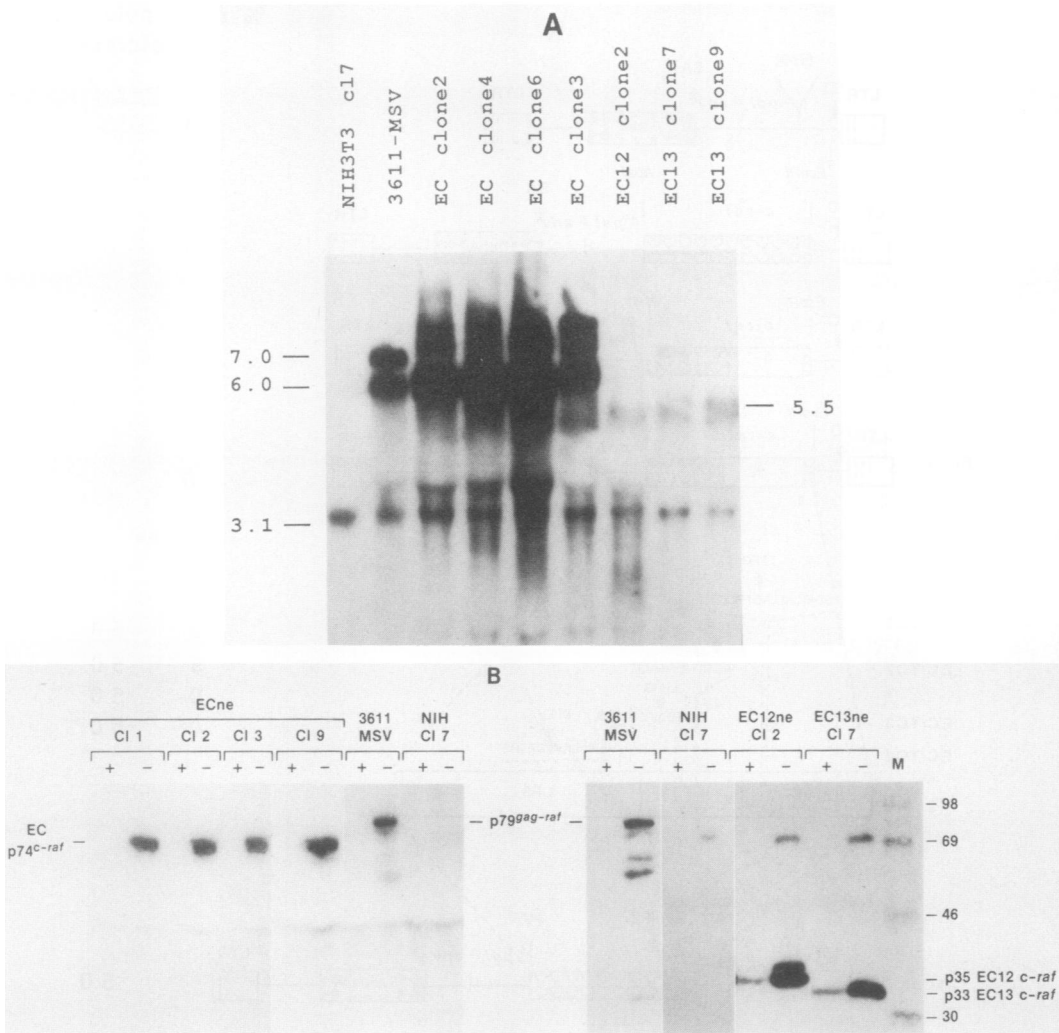

FIG. 2. c-*raf* expression in transfected NIH 3T3 Cl 7 cells. (A) Northern blot analysis of steady-state RNA. The following G418-resistant NIH 3T3 Cl 7-derived cells were analyzed: pSV2neo only (NIH 3T3 Cl 7), 3611-MSV plus pSV2neo, ECneo clones 2, 4, 6, and 3, EC12neo clone 2, and EC13neo clones 7 and 9. The probe was a near-full-length cDNA clone of murine c-*raf*. (B) Immunoprecipitates of ^{35}S-labeled c-Raf protein in cell lines expressing different EC constructs. The left panel shows results obtained with extracts from four independent ECneo transfectant clones compared with results for cells transfected with 3611-MSV and pSV2neo or with pSV2neo alone (NIH 3T3 Cl 7). The right panel compares cells transfected with 3611-MSV and pSV2neo, pSV2neo, EC12neo, and EC13neo. All immunoprecipitations were done in the presence (+) or absence (−) of synthetic peptide SP63 with anti-SP63 antiserum.

site by site-directed mutagenesis. The *Xho*I-*Apa*I fragment containing the whole c-*raf*-1 coding frame was then used to substitute for the *Xho*I-*Apa*I fragment of 3611-MSV (Fig. 1). Both constructs resulted in transformation of NIH 3T3 cells upon transfection, with specific transforming activities comparable to those found with v-*raf* constructs (Fig. 1). Transformed foci were morphologically identical to those induced by 3611-MSV. We investigated the role of myristylation of the fusion protein for transforming activity by converting the myristylation site (Gly at position 2) by site-directed mutagenesis to valine codon (19, 40). The construct expressing this fusion gene, called TCG, retained the transforming activity observed for TC33. The proteins in all three cases were of the expected size of 98 kDa. The protein levels in TC, TCG, and TC33 cells were about equal to that of the *gag*-v-*raf* fusion in 3611 cells and about fivefold higher than the endogenous Raf-1 protein level, as judged by Western blot analysis (Fig. 3). Protein extracted from cells grown in the presence of tritiated myristic acid showed labeled TC33-encoded protein, while the TCG protein was not labeled in this assay (data not shown).

Activation of c-*raf* by linker insertion mutagenesis. Three of the four *Taq*I sites within the c-*raf*-1 cDNA are located in regions encoding sequence motifs characterizing CR1, CR2, and CR3. As a preliminary test for the involvement of these structures in the overall function of the Raf protein in general and to examine a possible role of CR1 (cysteine finger) and CR2 (serine- and threonine-rich region) in negative regulation, we generated linker insertion mutants into these four sites. The linker used was a dodecamer carrying an *Xho*I site. The exact points of insertion and the resultant amino acid residues are given in Fig. 1. Only the linker insertion in CR2 should affect the overall protein structure, as predicted by computer-assisted Chau-Fasman analysis (10). c-*raf* genes carrying these linker insertion mutations were incorporated into the EC and TC backgrounds (Fig. 1) and

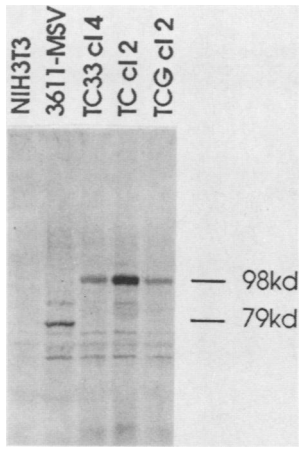

FIG. 3. Western blot analysis of proteins in cells transfected with TC constructs. Total lysates of cells transfected with pSV2neo, pSV2neo and 3611-MSV, TC33, TC, or TCG were separated by SDS-PAGE, transferred to nitrocellulose, and probed with anti-SP63. Bands were visualized with alkaline phosphatase. kd, Kilodaltons.

transfected into NIH 3T3 cells. When expressed as Gag-c-Raf fusion protein from the TC constructs, none of the mutations affected transforming activity, indicating that the mutations did not distort the proteins to a point at which they became unstable. Single-cell neomycin-resistant clones were isolated and expanded, and *raf* expression of the EC linker insertion mutants was examined by Northern (RNA) blot hybridization and Western blot analysis (Fig. 4). Several clones expressed higher levels of viral RNA (Fig. 4A) and protein (Fig. 4B) than that expressed from 3611-MSV. However, of the EC-derived constructs, only EC2, which has the linker insertion into the serine- and threonine-rich CR2, was active as a transforming gene, although the specific transforming activity was 1/50 of that of 3611-MSV. EC4 constructs have the linker insertion close to the ATP-binding site, and even though cells transfected with these constructs showed the highest level of *raf* expression of any EC construct both at the RNA and the protein level, they were not transformed (Fig. 1). Similar results were obtained with EC1 and EC3, which carry linker insertions in the cysteine finger domain (CR1) and in the hinge region between CR2 and CR3, respectively. All four linker insertion mutants had kinase activity in autophosphorylation assays following immunoprecipitation. The activities of the proteins by this crude estimation were comparable to that of the wild-type Raf-1 protein.

Minimal *raf* sequence necessary for transforming activity. The deletion activation experiments generating EC12 and EC13 construct viruses defined the 5′ border of the minimal transforming sequence of c-*raf* within 10 codons. The transduced *raf* sequence in 3611-MSV contains just 10 more codons upstream of the start of *raf* than present in EC12. Consistent with the EC12 data, a v-*raf* deletion mutant (EHN-14) retained transforming activity (Fig. 5). pEHN-14 was generated by restriction enzyme *Apa*I-BAL 31 digestion of pEH, a pBR322 clone containing the 5′ *Eco*RI-to-*Hin*dIII fragment of 3611-MSV (Fig. 5). EHN-14 lacked 66 codons of *gag* and the first 14 codons of v-*raf* sequences. Immunoprecipitation of [^{35}S]methionine-labeled lysates with anti-Raf antiserum revealed high levels of protein of the predicted size (70 kDa; Fig. 6A). Another deletion mutant, EHN-17, lacking just three additional *raf* codons had lost the transforming activity (Fig. 5). EHN-17 was generated by cutting pEH with *Xho*I, removing the sticky ends with single-strand-specific nuclease S1 to retain in-frame expression, and religating the molecule. Although the mutant no longer has transforming activity, high levels of a protein of predicted size (69 kDa) were made by EHN-17 (Fig. 6). Thus the boundaries found for the *gag-raf* minimal transforming sequence are similar to those found for EC12 and EC13 and are pinpointed between v-*raf* codons 15 and 17.

3′ sequences not required for transforming activity were tested with v-*raf* mutants that had stop codons introduced 30, 20, and 16 codons upstream of the normal translational termination codon of v-*raf*. The mutations were generated by site-directed mutagenesis and verified by DNA sequence analysis. The −30 mutant (EHC-30) had lost all transforming activity, whereas the −20 mutant (EHC-20) showed some transforming activity, although these cultures showed less criss-crossing than those transformed by either EHC-16 (the −16 mutant) or the parental construct EH (Fig. 5). All proteins were expressed at levels comparable to those found in pEH-containing cells, as determined by Western blot analysis (Fig. 6C). Although no size difference between the four Raf proteins was apparent, the absence of the carboxy-terminal sequences in EHC-16-, EHC-20-, and EHC-30-encoded proteins could be demonstrated by the fact that they no longer reacted with antisera raised against SP63, a synthetic peptide with the sequence of the 12 carboxy-terminal residues of v-Raf, but did react with antisera raised against Moloney MuLV p15^{gag} (Fig. 6C).

Lys-53 is necessary for v-Raf transformation. The consensus sequence for ATP binding, Gly-X-Gly-X$_2$-Gly-X$_{10-20}$-Lys, is located 15 codons downstream of the 5′ boundary of the minimal transforming sequence (57). The lysine residue at position 53 in the Raf sequence is the primary ATP-binding residue, on the basis of homology to pp60^{src} and other protein kinases (17, 26, 58). As expected, changing the lysine to a tryptophan codon by site-directed mutagenesis resulted in loss of the transforming activity of the construct EH301 (Fig. 5). We also investigated the role of Lys residue 56, which is also conserved in many kinases. EH282 and EH293 carry glutamine and glutamate, respectively. The mutation in EH282 increased the transforming ability of v-*raf*, whereas EH293 showed diminished transforming ability (Fig. 5). Western blot analysis demonstrated high levels of EH282 and EH301 proteins and suggested that the lower transforming activity might be due to a lower steady-state level of the Gag-Raf fusion protein of EH293 (79 kDa; Fig. 7). The differences in transforming activity were also apparent in the morphology of the foci induced by the various constructs. Foci induced by mutant EH282 were denser and larger than those induced by EH. In addition, these cells were more rounded and as a consequence showed less adherence to the plate. In contrast, foci induced by EH293 were smaller and consisted of more adherent cells (45).

DISCUSSION

We conclude that full-length c-*raf*-1 expressed at high levels by a retroviral vector is not transforming, while truncation of c-*raf*-1 is sufficient to cause oncogenic activation as long as an intact CR3 domain is retained. This finding is consistent with our reports showing that the sequence distal to the *Sph*I site of v-*raf* in the *gag-raf* fusion of 3611-MSV can be substituted with c-*raf* sequences without loss of transforming activity (4) and also with a report of

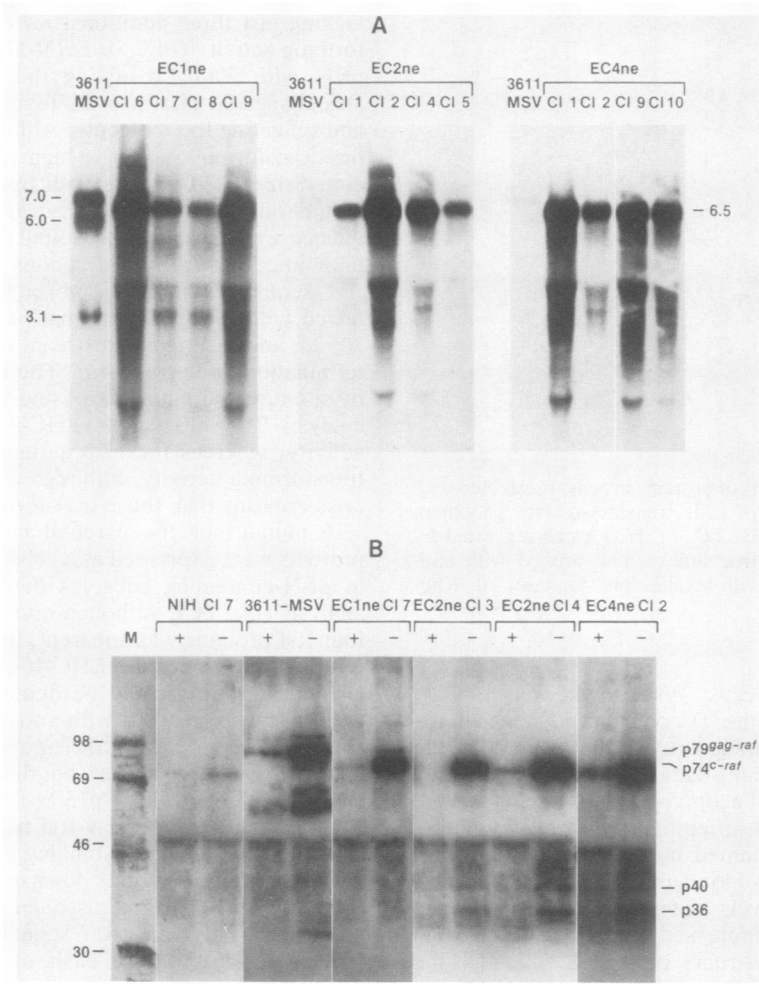

FIG. 4. Expression of c-*raf* in cells transfected with linker insertion mutants. Several independent neomycin-resistant clones were expanded for each construct, and RNA and protein were extracted. (A) Northern blot analysis. RNAs from four independent clones of each linker insertion mutant were compared with the RNA in cells transfected with pSV2neo alone and with 3611-MSV plus pSV2neo by using human c-*raf* probe. The positions of endogenous c-*raf* (3.1 kilobases) and the full-length and spliced viral RNAs (7 and 6 kilobases for 3611-MSV and 6.5 kilobases for EC constructs) are indicated. The left panel was exposed five times longer than the middle and right panels. (B) Western blot analysis of immunoprecipitates obtained with anti-SP63 antiserum from extracts of cells transfected with pSV2neo (NIH 3T3 Cl 7), 3611-MSV and pSV2neo, EC1neo, EC2neo, and EC4neo. In the case of EC2neo, two clones are shown. The low-molecular-weight bands visible below the consistently visualized immunoglobulin heavy-chain band are breakdown products either of Raf or of the antibody. Their intensity varied between experiments and depended on the antibody used.

contemporary work by others (54) who generated deletion mutants of c-*raf*-1 clone 627 (5) and found that the removal of about 320 codons resulted in maximal oncogenic activation. The data are also consistent with the fact that the v-*raf* sequences can be replaced with A-*raf* CR3 (2, 22). Here we have carried this point further by showing that the *gag* fusion and the accompanying myristylation are not necessary for oncogenic activation, since the truncated version of c-*raf*-1 in EC12 and the *gag*-c-*raf* fusion construct TCG, which both lack the myristylation site, have transforming activity. Independence from a membrane-anchoring site provided by the fatty-acid modification also reaffirms that the Raf kinase acts below the cell membrane (34, 47) and distinguishes Raf protein kinase function from that of many tyrosine kinases encoded by oncogenes such as v-*src* (6, 39, 49) and v-*abl* (26, 40) in which myristylation is critical for transformation. These results are also supported by our previous finding of a long terminal repeat (LTR)-activated transforming version of c-*raf*-1 which lacks these sequences and modifications (34). These findings suggest that the location of the v-*raf* sequence within the 3611-MSV genome is not the result of selection for a specific transforming *gag-raf* recombinant out of a pool of nontransforming recombinants but rather that the homology between p30 and c-*raf* (5) determines the position of v-*raf* in the virus.

Although our data suggest that the amino-terminal half of the protein is not necessary for kinase activity and substrate recognition, at least for substrates involved in mitogen signal relay, one has to keep in mind that all these experiments were done with cells that express normal amounts of wild-type c-*raf*.

The v-Raf polypeptide consists of only 329 amino acids (31) and as such is the smallest member of the *src* family of viral oncogenes, with an average of 41% homology to its relatives and to bovine cAMP-dependent protein kinase (1) occurring exclusively in the kinase domain (32). Through deletions induced by restriction enzymes or site-directed mutagenesis of v-*raf*, we have established the 5′ and 3′

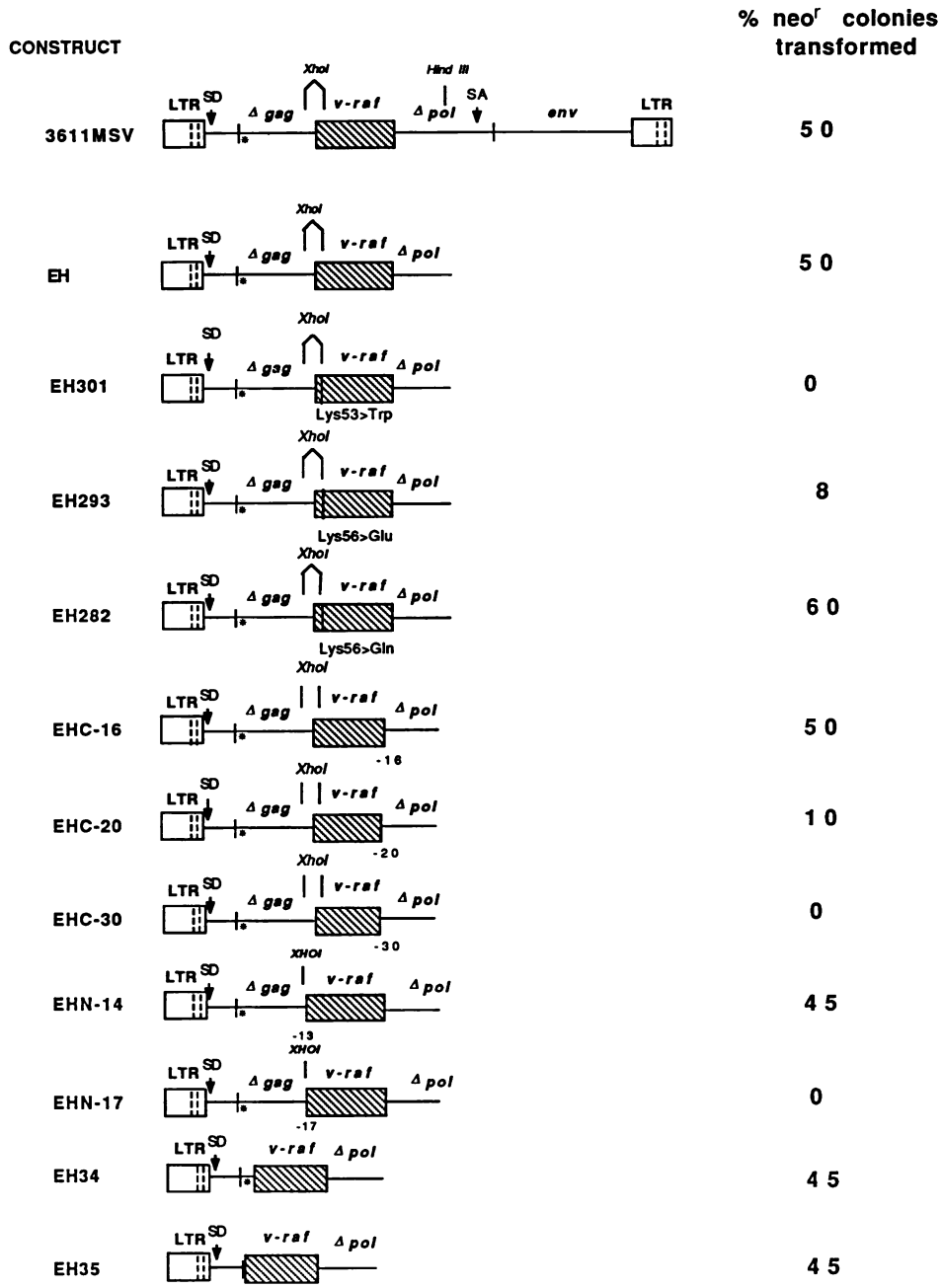

FIG. 5. Genomic organization of EH constructs. See the legend to Fig. 1 for explanation and abbreviations. All constructs were cotransfected with pSV2neo DNA.

borders defining the minimal sequence for transforming activity. The removal of 17 amino-terminal residues and 20 amino acids from the carboxy terminus of v-Raf abolishes its transforming activity; however, the 14 N-terminal residues and the 16 C-terminal residues of v-Raf are not required for transforming activity. It has been suggested for the protein kinase C family of Ser-Thr protein kinases that the carboxy-terminal amino acids confer substrate specificity, since they are not conserved between protein kinase C isozymes (28, 38). Our results show, however, that the equivalent region of the Raf protein is not required for recognition of substrate(s) involved in the mitogenic signal pathway.

The N-terminal deletion of 14 amino acid residues in v-Raf does not affect the apparent nucleotide-binding site (the canonical Gly-X-Gly-X-X-Gly sequence, amino acids 35 to 40, and Lys-53) shared with *src* family oncogene products, cAMP- and cGMP-dependent protein kinases, lactate dehydrogenase, and the products of the *ras* family of oncogenes (1, 17, 26, 58). The sequence requirements extend to a region N-terminal of this consensus sequence, as EC13, which retains 12 residues in front of it, does not have transforming activity.

Lys-53 is indeed essential for Raf activity, since changing this residue to Trp completely abolishes transformation. This agrees with data which show that changing the Lys of v-Mil at position 622 to Arg also destroys its transforming

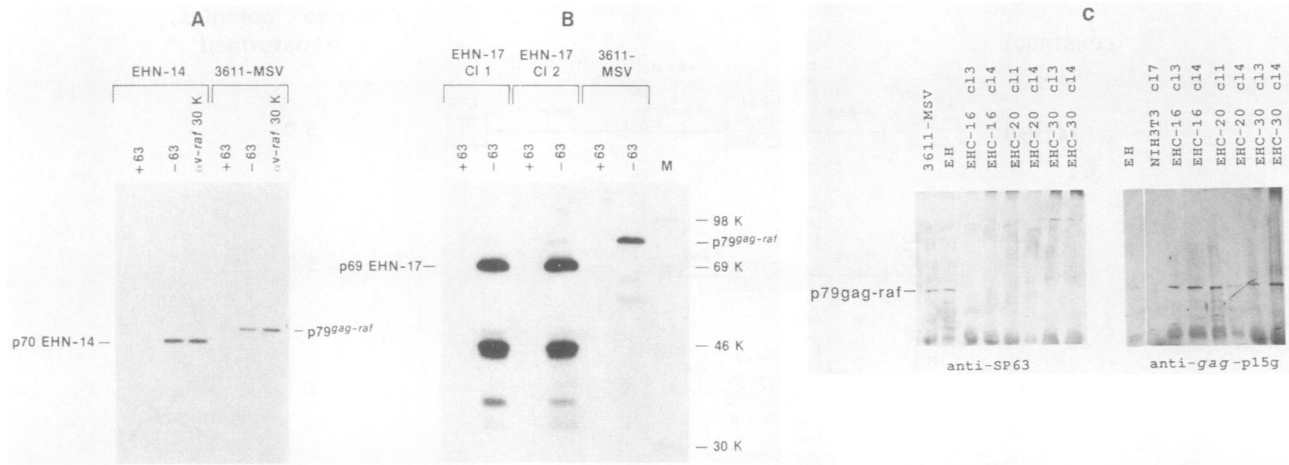

FIG. 6. Characterization of Raf proteins transfected with EH constructs. (A) Autoradiograph of a polyacrylamide gel with [^{35}S]methionine-labeled proteins precipitated from EHN-14- and 3611-MSV-transfected cells with anti-SP63 antiserum in the presence and absence of SP63 or with anti-v-*raf*30K antiserum (29). The sizes of the proteins are indicated in kilodaltons (K). (B) Western blot results obtained with extract from two clones transfected with EHN-17. (C) Western blot analyses with cell extracts obtained from cells transfected with pSV2neo and EH (clone 6), EHC-20 (clone 3), EHC-16 (clone 3), 3611-MSV, and EHC-30. Immunoprecipitates of anti-p15^{gag} antiserum (Anti-*gag*-p15g) were split in two, separated by gel electrophoresis, and transferred to DMBA paper. The blot on the right was probed with anti-p15^{gag} antibody, and the blot on the left was probed with anti-SP63 antiserum.

activity (9). In contrast, altering Lys-56 to either Glu or Gln did not inactivate v-Raf. Instead, the Glu substitution (EH293) led only to a 10-fold reduction, while the Gln exchange (EH282) resulted in an even more active form, inducing a more pronounced morphological transformation.

The inability of the full-length Raf protein to induce acute transformation is not surprising, since NIH 3T3 cells normally express c-Raf protein. Activation of normal c-Raf kinase activity has been demonstrated in response to various mitogenic signals (35), and it is possible that cells carrying

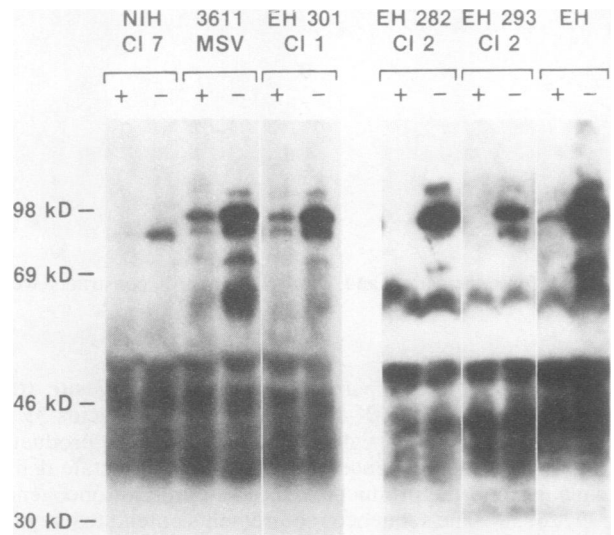

FIG. 7. Western blot analysis of ATP-binding-site mutants. Single-cell-derived clones of cells transfected with the different mutants and pSV2neo were expanded, and protein was extracted. v-*raf* proteins were precipitated in the presence or absence of competing SP63 peptide with anti-SP63 antiserum. After SDS-PAGE, the proteins were transferred to DMBA paper and probed with the same antiserum followed by ^{125}I-protein A. kD, Kilodaltons.

any of the EC virus constructs respond with reversible transformation when the appropriate ligand is provided. The importance of the amino-terminal half in the regulation of Raf activity is underscored by the fact that the only point mutant tested that resulted in oncogenic activation is located in this domain. EC2 carries a linker insertion into CR2, a region high in serine and threonine that is highly conserved between *raf* family members. In addition to the induction of transforming activity, this mutation also resulted in increased activity when EC2 was assayed for its ability to enhance transcription from an AP1-dependent promoter (56). The function of CR2 is as yet unknown; however, it might be speculated that it serves as a substrate for its own or other activating kinases. In addition to a possible distortion of the protein structure, the linker insertion causes the duplication of a sequence similar to a consensus kinase substrate site which might increase the affinity of this region as a substrate for phosphorylation. In light of results by Ishikawa et al. (24), who found that a deletion of CR2 induced transforming activity, it seems more likely that a distortion of the protein is the major reason. It will be interesting to see whether the tissue tropism and histogenesis of tumors induced by any of these mutants are altered in comparison with the studies we have done before (13, 27).

The results of this study allow us to formulate a working hypothesis for the structure of the c-Raf protein. The kinase domain is defined by the minimal transforming sequence mutants and consists of CR3. The kinase activity is regulated by the amino-terminal half of the Raf protein, which is activated by phosphorylation and/or by ligand binding. As a consequence of these stimuli, the molecule, which in its uninduced state presents a closed structure, unfolds, and the kinase domain becomes accessible to its substrates. The *gag*-c-*raf*-1 fusion constructs suggest that steric hindrance can prevent the formation of the closed structure and results in constitutive activation.

We are currently testing this hypothesis by generating point mutations in specific segments of both the regulatory and kinase domains.

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