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# Structure and biological activity of v-*raf*, a unique oncogene transduced by a retrovirus

(malignant transformation/transduction/molecular cloning)

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**ABSTRACT** We have molecularly cloned a unique acutely transforming replication-defective mouse type C virus (3611-MSV) and characterized its acquired oncogene. The viral genome closely resembles Moloney (M) murine leukemia virus (MuLV), except for a substitution in M-MuLV in the middle of p30 and the middle of the polymerase gene (*pol*). Heteroduplex analysis revealed that 2.4 kilobases of M-MuLV DNA were replaced by 1.2 kilobases of cellular DNA. The junctions between viral and cellular sequences were determined by DNA sequence analysis to be 517 nucleotides into the p30 sequence and 1,920 nucleotides into the polymerase sequence. Comparison of the transforming gene from 3611-MSV, designated v-*raf*, with previously isolated retrovirus oncogenes either by direct hybridization or by comparison of restriction fragments of their cellular homologs shows it to be unique. Transfection of NIH 3T3 cells with cloned 3611-MSV proviral DNA leads to highly efficient transformation and the recovered virus elicits tumors in mice typical of the 3611-MSV virus. Transfected NIH 3T3 cells express two 3611-MSV-specific polyproteins (P75 and P90), both of which contain $NH_2$-terminal *gag* gene-encoded components linked to the acquired sequence (v-*raf*) translational product. The cellular homolog, c-*raf*, is present in one or two copies per haploid genome in mouse and human DNA.

Retroviruses act as natural vectors for the transduction of at least some cellular genes, designated proto-oncogenes (1, 2), which bring about malignant transformation of infected cells. The cellular origin of a retroviral transforming gene (v-*onc*) was first demonstrated for v-*src*, the oncogene of Rous sarcoma virus (3, 4). Since then, 14 additional v-*onc* genes present in different acutely transforming retroviruses of avian and mammalian origin have been described, each similarly derived from cellular genes (c-*onc*) (1, 2). At least six of the v-*onc* genes code for functionally related tyrosine-specific protein kinases (5, 6), several of which appear to have a common evolutionary origin (7–9). Although the v-*onc* genes that do not encode protein kinase make up an evolutionarily more diverse group, some, such as v-*kis* and v-*has*, also appear to be members of gene families (10).

The significance for human disease of c-*onc* genes had been purely hypothetical prior to the recent demonstration by DNA transfection that active forms of such genes may be associated with human cancers (11, 12). Furthermore, human c-*onc* genes are associated with specific translocations characteristic of certain types of human cancer (13–15). These findings emphasize the potential importance of *onc* genes for an understanding of human malignancy and point to a need for a more complete accounting of such genes in human DNA.

The isolation of retroviral oncogenes has in the past been sporadic and limited mainly to isolations from spontaneous tumors. More recently we have designed experiments to generate acutely transforming retroviruses by growth of IdUrd-induced endogenous type C viruses in chemically transformed mouse cells, followed by selection for tumor induction *in vivo* (16, 17). In the course of these experiments, we have isolated several transforming viruses (16–19). One of these, 3611-murine sarcoma virus (MSV), transforms fibroblasts and epithelial cells in culture and induces fibrosarcomas in newborn NFS/N mice within 4 wk of injection. The 3611-MSV translational products have been identified as $M_r$ 90,000 (P90) and $M_r$ 75,000 (P75) polyproteins (19) containing Moloney (M) murine leukemia virus (MuLV) *gag* gene proteins p15 and p12 but no tyrosine-specific protein kinase activity.

## MATERIALS AND METHODS

**Cells and Virus.** Cells were grown in Dulbecco's modification of Eagle's medium supplemented with 10% calf serum. Cell lines included the highly contact-inhibited NIH 3T3 mouse embryo line and a series of previously described subclones of FRE 3A Fisher rat cells nonproductively transformed by 3611-MSV (19). As a substitute for M-MuLV, we have used the Leuk 2 virus, which had been molecularly cloned by the addition of *Eco*RI linkers to a linear unintegrated viral DNA (unpublished data). Leuk 2 differs from M-MuLV at only 1 of 28 restriction enzyme sites.

**Cloning of Viral DNA.** Integrated viral DNA was enriched by velocity sedimentation of *Eco*RI-digested cellular DNA in a 5–20% sucrose gradient. The *Eco*RI-digested DNA corresponding to a size range of 8–12 kilobase pairs (kb) (0.45 μg) was ligated with λgt*WES*·λB *Eco*RI arms (20) and packaged into phage particles by using Pacagene (Biotec). Phage were propagated in *Escherichia coli* strain LE 392 and plaques were screened for viral DNA by using the Benton and Davis plaque-lift procedure (21) with viral DNA corresponding to the gp70 region of M-MuLV as a probe. Phage DNA containing the 3611-MSV viral DNA inserts were digested with *Eco*RI, and the inserts were purified by electrophoresis (22) and cloned into the *Eco*RI site of pBR322.

**Transfection of Viral DNA.** NIH 3T3 Cl 7 cells, a subline of NIH 3T3 cells selected for flat morphology, were transfected with viral DNA by using calcium phosphate according to a modification (unpublished data) of the procedure of Graham and Van der Eb (23). Foci of transformed cells were detected 8 days after transfection.

**Metabolic Labeling, Immunoprecipitation, and NaDod$SO_4$/Polyacrylamide Gel Electrophoresis.** Cells were radioactively labeled by incubation for 2 hr in medium containing

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Abbreviations: MuLV, murine leukemia virus; MSV, murine sarcoma virus; M, Moloney; kb, kilobase pair(s).

100 $\mu$Ci of [$^{35}$S]methionine per ml (1,075 Ci/mmol; 1 Ci = 3.7 × $10^{10}$ Bq; Amersham). Immunoprecipitation and $NaDodSO_4$/polyacrylamide gel electrophoresis, with 5–20% gradient gels, were performed as described (19).

## RESULTS

**Viral DNA in FRE 3A Cells Nonproductively Transformed by 3611-MSV.** On the basis of our previous characterization of the P90 and P75 polyproteins encoded by 3611-MSV, we assumed the parent of this virus to be closely related to M-MuLV (19). Therefore, we expected that some portion of M-MuLV could be used as a probe to detect 3611-MSV DNA in Southern blots of transformed FRE 3A cells. Most of these probes detected numerous bands in normal FRE 3A cellular DNA, making detection of the transforming provirus in transformed FRE 3A cells difficult. However, by using a 1.3-kb *Hpa* I fragment of Leuk 2 that is specific for the gp70 gene, it was possible to eliminate essentially all FRE 3A-specific bands and identify the 3611-MSV provirus. The integrated viral genome was detected as an 8.9-kb *Eco*RI fragment, present in DNA from a clone (ACl 10) of nonproductively transformed FRE 3A cells but absent in uninfected FRE 3A cell DNA (Fig. 1*A*). Digestion with *Sac* I generated a single 7-kb fragment, which was cleaved by *Hin*dIII to yield a fragment of 3.3 kb (not shown). In addition, an *Xba* I fragment of 2.3 kb was detected. The *Hin*dIII–*Sac* I 3.3-kb and the *Xba* I 2.3-kb bands are characteristic of the 3′ end of M-MuLV and suggested that the transforming virus contains these sequences.

**A Molecular Clone of 3611-MSV Proviral DNA.** The integrated provirus in ACl 10 cells was cloned as described in *Materials and Methods*. Comparison of its restriction map with that of M-MuLV (Fig. 2) shows that the 8.9 kb of cloned 3611-MSV proviral DNA contains the genome of a defective virus, which is 7.6 kb in length and contains long terminal repeat sequences at both ends. In addition, it includes a 1.3-kb piece of 3′ flanking cellular DNA but only about 0.05 kb of 5′ flanking DNA. It is also evident that a portion of the *gag* and *pol* genes of M-MuLV between the *Bal* I site at 2.1 kb and the *Bst*EII site at 4.7 kb has been replaced by another sequence. Note that the viral sequence excluding this substitution region is very closely related to M-MuLV based on a comparison of the restriction sites. The cloned 3611-MSV contains an extra *Sma* I site at 0.8 kb but is missing the *Cla* I and *Kpn* I sites at 5.5 and 6.2 kb in M-MuLV. The heteroduplex of cloned 3611-MSV

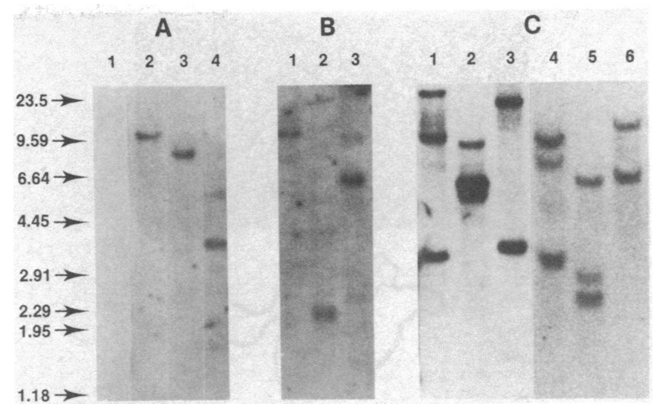


FIG. 1. Southern blot analysis of integrated 3611-MSV viral DNA from clones of nonproductively infected transformed FRE 3A cells (*A* and *B*) and uninfected mouse and human DNA (*C*). DNA digests were electrophoresed in a 0.8% agarose gel, transferred to nitrocellulose filters, and hybridized as described (17) with nick-translated *env* HH probe (*A* and *B*) or v-*raf*-specific XB probe (*C*). (*A*) DNA from uninfected FRE 3A cells after digestion with *Eco*RI (lane 1); DNA from ACl 10 cells digested with *Eco*RI, *Sac* I, or *Sac* I–*Hin*dIII (lanes 2, 3, and 4, respectively). (*B*) DNA from mutant ACl 3-1 cells digested with *Hin*dIII (lane 1), *Sac* I (lane 2), or *Sac* I–*Hin*dIII (lane 3). (*C*) Mouse DNA (lanes 1–3) or human DNA (lanes 4–6) digested with *Eco*RI (lanes 1 and 4), *Pst* I (lanes 2 and 5), or *Sph* I (lanes 3 and 6). The sizes of marker fragments (*Hin*dIII digest of phage λ *Taq* I digest of $\phi$X174) are indicated on the left in kb.

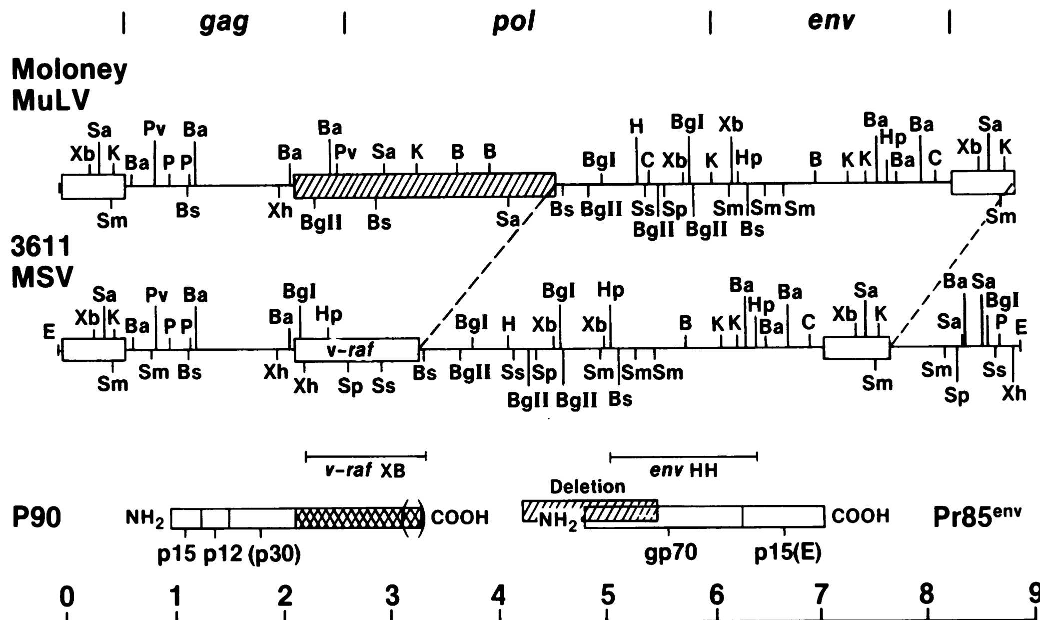


FIG. 2. Restriction endonuclease maps comparing 3611-MSV and M-MuLV. The maps are shown in 5′ to 3′ orientation. Boxes within the viral genomes define the areas of deletion (▨) and substitution (v-*raf*), as determined by heteroduplex mapping. The lines below the 3611-MSV map indicate the areas of the genome used as specific probes: *Xho* I–*Bst* II (v-*raf* XB 2.25–3.45 map units) and *Hpa* I–*Hpa* I (*env* HH 5.05–6.45 map units). Restriction enzymes include: *Bam*HI (B), *Hin*dIII (H), *Kpn* I (K), *Bgl* II (BgII), *Eco*RI (E), *Pst* I (P), *Bgl* I (BgI), *Xho* I (Xh), *Xba* I (Xb), *Bal* I (Ba), *Hpa* I (Hp), *Sst* II (Ss), *Sma* I (Sm), *Sph* I (Sp), *Pvu* I (Pv), *Cla* I (C), *Bst*EII (Bs), and *Sac* I (Sa). The presumed translational products of 3611-MSV are shown in the bottom line. The position of a deletion present in 3611-MSV ACl 3-1 is also indicated on this line (▨).

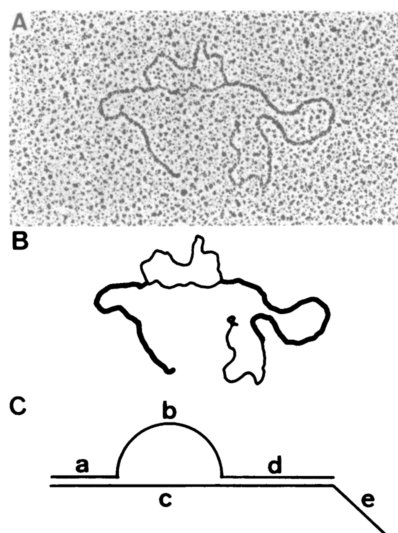


FIG. 3. DNA heteroduplex analysis of the genomic relationship of cloned Leuk-2 and 3611-MSV. Heteroduplexes were prepared as described (24) with viral DNAs separated from their cloning vectors. (*A*) Actual heteroduplex; (*B*) interpretive drawing; and (*C*) schematic representation. Contour lengths, measured from actual photographs of 25 molecules with a digital length calculator, were as follows (mean ± SEM; in kilobases): a = 2.12 ± 0.09; b = 2.18 ± 0.13; c = 1.20 ± 0.16; d = 3.94 ± 0.40; and e = 0.87 ± 0.12. The Leuk 2 strand is represented by the segments a, b, and d, and the 3611-MSV strand is represented by a, c, d, and e. The v-*raf* substitution in 3611-MSV is segment c.

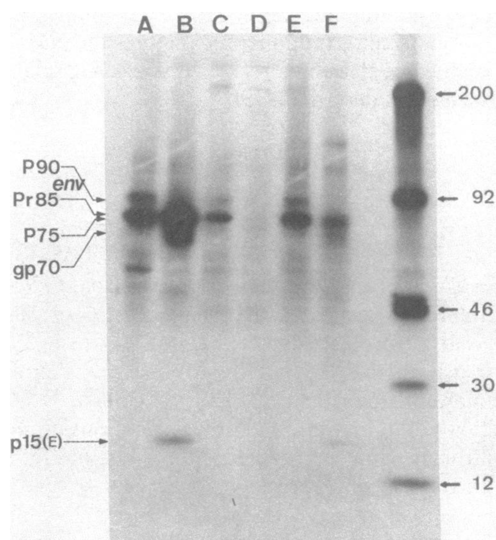


FIG. 5. Immunoprecipitation and NaDodSO$_4$/polyacrylamide gel electrophoresis analysis of viral-related proteins expressed in cells nonproductively transformed by wild-type 3611-MSV, ACl 10 (lanes A and B); a 3611-MSV deletion mutant, ACl 3-1-4 (lanes C and D); and molecularly cloned 3611-MSV transfected to NIH 3T3 cells (lanes E and F). Cells were metabolically labeled in [$^{35}$S]methionine-containing medium (100 $\mu$Ci/ml) for 2 hr, immunoprecipitated by goat antiserum against either M-MuLV p12 (lanes A, C, and E) or *env* products (lanes B, D, and F), and analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis. Molecular weight standards include $^{14}$C-labeled myosin ($M_r$ 200,000), phosphorylase B ($M_r$ 92,000), ovalbumin ($M_r$ 46,000), carbonic anhydrase ($M_r$ 30,000), and cytochrome *c* ($M_r$ 12,000).

and Leuk 2 DNAs (Fig. 3) revealed a single deletion-substitution region, indicating that 2.4 kb of Leuk 2 DNA was replaced by 1.2 kb of unrelated DNA. Alignment of the heteroduplex map with the restriction map indicated that the *Bal* I site at 2.1 kb and the *Bst*EII site at 3.5 kb in 3611-MSV are within 0.2 kb of the ends of the substitution loop.

To more precisely define the borders of nonviral DNA in 3611-MSV, the DNA sequence was determined in the region of these restriction sites. As shown in Fig. 4, the nonviral DNA sequence begins no later than nucleotide 1,782 of the M-MuLV sequence (26), corresponding to the carboxyl-terminal third of p30 (27), and terminates ≈1,140 nucleotides further upstream at or near nucleotide 4,165 of M-MuLV, which is within the *pol* gene. It appears that a single nucleotide, 10 nucleotides before the beginning of the substitution, was deleted, presumably allowing the inserted sequence to be read in the correct frame. The location of the substitution accounts for the presence of p15 and p12 in P90 and P75 polyproteins. Furthermore, if there is no introduced splice site, then the carboxyl terminus of the P90 protein is very near the 3′ end of the substitution region.

**Biological Activity of Cloned 3611-MSV Viral DNA.** 3611-MSV DNA from the pBR322 clone was transfected onto NIH 3T3 cells and foci of transformed cells were counted 8 days later. The specific activity of the cloned viral DNA was 4 focus-forming units/ng. Cells derived from transformed foci expressed both polyproteins (P90 and P75) characteristic of 3611-MSV (Fig. 5). These cells further resembled the original ACl 10 transformant, in that they exhibited low levels of Pr85$^{env}$, gp70, and p15(E) expression. To test the pathogenicity of cloned 3611-MSV, virus was rescued with M-MuLV from transfected NIH 3T3 cells and inoculated into newborn NFS/N mice. All 11 of these animals developed tumors within 4 wk with a pathology indistinguishable from that of 3611-MSV-induced tumors.

**Characterization of a 3611-MSV Deletion Mutant.** The map of 3611-MSV shown in Fig. 3 is consistent with the sizes of the fragments that were generated upon digestion of cellular DNA from ACl 10, as shown in Fig. 1*A*. The map also allows us to characterize a second nonproducer cell clone, 3611-MSV ACl 3-1, as containing an additional deletion. A *Hin*dIII–*Sac* I double-digest of this cellular DNA yielded a fragment of 1.75 kb (not shown), whereas *Xba* I and *Sac* I digests gave 5.5-kb fragments (Fig. 1*B*). These results suggest that there has been a 1.5-kb deletion that spans the *Xba* I sites at 4.6 and 5.0 kb of the F4 clone. This was verified by double-digestion experiments using *Sac* I in combination with either *Xba* I, *Bgl* III, or *Sst* II, which showed that the *Sst* II site at 4.1 kb was retained, whereas the *Bgl* II sites at 4.2 and 4.5 map units and the *Xba* I sites at 4.6 and 4.9 map units were absent. This places the 5′ end of the 1.5-kb deletion betweeen 4.1 and 4.2 map units and suggests that the 3′ end is between 5.6 and 5.7 kb on the map.

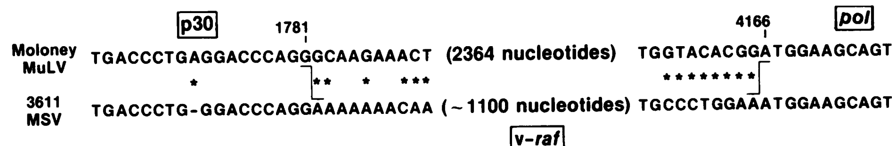


FIG. 4. Alignment of M-MuLV p30 and polymerase DNA sequences with those derived for 3611-MSV to display the DNA substitution junctions. The 3611-MSV sequence was determined by the chemical modification method (25) on the 3′ side of the *Bal* I site (map position, 2.1) and on the 5′ side of the *Bst*EII site (map position, 3.5). Nucleotide numbers refer to positions in the sequence of MuLV derived by Shinnick *et al.* (26). *, Denotes a nonhomologous nucleotide.

Comparison with the map of M-MuLV shows that the deleted DNA includes the 3′ end of the reverse transcriptase gene as well as the 5′ end of the *env* gene and its accompanying splice acceptor sequence. As shown in Fig. 5 (lane C), both of the major 3611-MSV polyproteins, P90 and P75, are expressed in ACl 3-1 cells. This result suggested that the carboxyl terminus of neither polyprotein P90 nor P75 is within the *env* gene and that both contain amino acids encoded by the v-*raf* insert. The absence of detectable $Pr85^{env}$, gp70, or p15(E) expression in lane D of Fig. 5 is consistent with the position of the 1.5-kb deletion and confirms that the polyproteins contain no *env* gene contribution.

**Identification of a Conserved Cellular Homolog of v-*raf*.** To obtain a probe specific for the substitution region, a subclone was generated, designated v-*raf* XB (Fig. 2), containing 1.3 kb of DNA from the *Xho* I to *Bst*EII site (2.2–3.4 map units). This subclone is 50 base pairs shorter than v-*raf* at its 5′ end and extends 48 base pairs into the M-MuLV sequence at its 3′ end. The v-*raf* XB probe was used to detect the cellular homolog of v-*raf*, in restriction digests of mouse and human DNAs (Fig. 1*C*). Because v-*raf* readily hybridizes to human DNA under moderately stringent conditions, these sequences are well conserved. Although most of the restriction fragments are different in human than in mouse DNA, it appears that two *Eco*RI bands and one *Pst* I band are conserved. Comparison of the intensities of the bands in mouse DNA with the intensity of the band in ACl 3-1 nonproducer FRE 3A cells, which contains a single copy of 3611-MSV per cell, indicates that the mouse contains one to four copies per cell—i.e., one or two copies per haploid genome. Two human homologs that account for all of the fragments seen in Fig. 1*C* have recently been cloned (unpublished data). One gene spans ≈6.5 kb and contains at least four introns, whereas the second gene contains only 0.45 kb of v-*raf*-related sequence, with no introns.

**Comparison of v-*raf* with Other Retrovirus-Transduced Oncogenes.** Oncogene-containing DNAs from seven different mammalian transforming viruses were hybridized at low stringency with nick-translated v-*raf* DNA. Because v-*raf* is sufficiently well conserved to hybridize at higher stringency to the human homolog, these conditions should allow the detection of its homolog from any mammalian species. However, none of the seven mammalian oncogenes tested, including v-*abl*, v-*fes*, v-*fms*, v-*kis*, v-*has*, v-*mos*, and v-*sis*, hybridized to the v-*raf* probe (Fig. 6). This list may be extended to include the v-*fps* and v-*bas* oncogenes because they have been found to be the avian counterpart of the v-*fes* oncogene (8) and the murine counterpart of the v-*has* oncogene (10), respectively. An additional mammalian oncogene, v-*fos*, of murine origin, appeared to be unrelated to v-*raf*, as judged by a comparison of their restriction enzyme maps (33). v-*raf* is distinct from the avian oncogenes v-*myc* (34) and v-*myb* (35) because the bands detected in human DNA by these oncogenes are different from those detected by the v-*raf* XB probe. Similarly, the sequences in chicken DNA that hybridize to this v-*raf* probe are distinguishable from those homologous to the v-*rel* oncogene (Nancy Rice, personal communication), the v-*ros* oncogene (36), or the v-*erb* oncogene(s) (37). Finally, the two remaining oncogenes v-*src* and v-*yes* are tyrosine-specific protein kinase genes, whereas v-*raf* is not. Furthermore, their DNA sequences show no significant similarity (unpublished data) to that of v-*raf*. Thus, we conclude that v-*raf* is a unique oncogene.

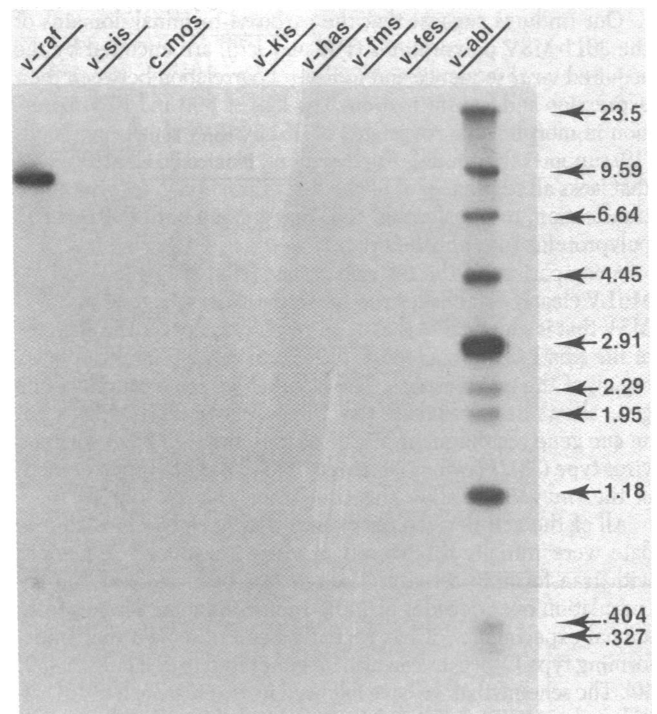


FIG. 6. Comparison of v-*raf* with oncogenes from other mammalian retroviruses by Southern blot analysis of cloned DNAs. v-*raf*, an *Eco*RI digest of cloned 3611-MSV; v-*sis*, a *Pst* I–*Xba* I double-digest of simian sarcoma virus DNA (28); c-*mos*, a *Hin*dIII–*Ava* I double-digest of c-*mos* from mouse DNA (29); v-*kis*, a *Pvu* II digest of Ki-MSV clone KBE-2 (10); v-*has*, an *Eco*RI digest of Harvey-MSV DNA clone BS 9 (10); v-*fms*, a *Pst* I digest of McDonough feline sarcoma virus DNA clone PSM 7c (30); v-*fes*, a *Pst* I digest of Snyder–Theilen feline sarcoma virus DNA clone SL (31); and v-*abl*, a *Bgl* II digest of the phage clone of Abelson-MuLV DNA (32). The oncogene-specific fragments in all lanes except v-*raf* ranged in size from 0.45–1.2 kb. DNA size markers, indicated by arrows, are as described in the legend to Fig. 1. Ten to 20 ng of cloned retroviral DNA containing oncogenes was digested with restriction enzymes, electrophoresed on 0.8% agarose gels, and blotted onto nitrocellulose membranes. Nick-translated v-*raf* XB DNA was hybridized in 0.45 M sodium chloride/0.045 M sodium citrate, pH 7.0, at 60°C and washed under the same conditions.

## DISCUSSION

In the present study, we describe the isolation and characterization of the proviral DNA of 3611-MSV, an acutely transforming replication-defective mouse type C virus. 3611-MSV has acquired 1.2 kb of a cell-derived oncogene, v-*raf*, and has lost 2.4 kb of *gag–pol* DNA in the process. v-*raf* and its cellular homologs are distinct from previously described retroviral oncogenes and appear to be well conserved in evolution. The molecularly cloned 3611-MSV DNA is biologically active, transforming NIH 3T3 cells in culture with an efficiency of 4 focus-forming units/ng.

The DNA sequence at the junctions between viral genes and v-*raf* shows that, as in other acutely transforming retroviruses (8, 9), cell-derived sequences were incorporated with apparent disregard for the integrity of viral genes. Similarly, it appears that the cell-derived sequence, v-*raf*, may not be a complete cellular gene because there is no promoter-like structure evident from its 5′ end nor transcription termination or poly(A) addition signal from its 3′ end (unpublished data). Because the 5′ junction of v-*raf* is not near a viral sequence involved in normal RNA splicing events, v-*raf* was apparently not acquired by a RNA splicing mechanism. The deletion of the adenosine nucleotide (Fig. 4, position 1,772) suggests that the translation of the acquired sequence v-*raf* did not occur after initial formation of the 3611-MSV recombinant virus but depended upon subsequent frame shift for its expression.

Our findings suggest that the carboxyl-terminal domains of the 3611-MSV polyproteins (P90 and P75) are encoded by the acquired v-*raf* sequence and indicate a correlation between their expression and transformation. The loss of P90 and P75 expression in morphologic revertants of 3611-MSV-transformed cells (19) supports this model. Furthermore, cloned 3611-MSV DNA that lacks all sequences 3′ of the v-*raf* insertion gives rise, upon transfection, to transformed cells that contain both P90 and P75 polyproteins (unpublished data).

A comparison of the restriction map from 3611-MSV and M-MuLV clearly establishes the presence of an *env* gene in 3611-MSV that is identical to that of M-MuLV except for the absence of the *Kpn* I site at nucleotide 5,576 (26) in the 5′ nontranslated region of the *env* message. The fact that we see a complete *env* gene allows us to conclude that the parent of 3611-MSV is not an *env* gene recombinant MuLV of the mink cell focus-forming virus type (38). Previous isolates of MSVs did not retain enough of the *env* gene to allow this conclusion (10, 29, 32, 33).

All of the cell-derived oncogenes that have been isolated to date were initially discovered as virus-transduced sequences with transforming activity. Though this had required the accumulation over decades of rapid tumor-inducing viruses from animal experiments, it has recently been observed that transforming type C viruses can also be generated in cell culture (39, 40). The scheme that we have followed involved growth of IdUrd-induced C3H/MuLV in chemically transformed cells in culture, followed by selection for tumor induction *in vivo*. Because the scheme selects only for biological activity it is not possible to predict how the virus acquires pathogenicity or at which step this event will take place. One of the products of such selections, a virus associated with the induction of lung carcinoma *in vivo*, has been characterized (18) as a virus–virus recombinant of the mink cell focus-forming virus class (38), which arose during the cell culture phase. In contrast, the virus that we have described here arose by recombination with a cell-derived oncogene. Although it is not known in this case whether the recombination events occurred during the cell culture phase or during the *in vivo* phase, transforming viruses isolated in this way should provide important tools for the study of human cancer.

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