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Transforming Activity of Retroviral Genomes Encoding Gag-Axl Fusion Proteins

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Retroviral genomes encoding a portion of the Moloney murine leukemia virus Gag protein fused to portions of the murine *axl* cDNA were constructed so as to mimic naturally occurring transforming viruses. Virus MA1 retained 5 amino acids of the extracellular domain and the complete transmembrane and intracellular domains of Axl; virus MA2 retained only the intracellular Axl sequences beginning 33 amino acids downstream of the transmembrane region. Although both viruses could transform NIH 3T3 cells, they induced different morphological changes. MA1 transformants became elongated and assumed a cross-hatched pattern, while MA2 transformants were round and very refractile and grew to high density. Gag-Axl and Glyco-Gag-Axl proteins were detected in both types of transformed cells and were predominantly localized to the cytoplasmic compartment. When cell-free v-*axl* virus supernatants were introduced into wild-type BALB/c neonates, Rag-2-deficient mice, or c-*myc* transgenic mice, they did not cause tumors in a 3-month period. However, MA2-transformed NIH 3T3 cells, but not MA1 or control cells, could establish sarcomas by subcutaneous or intraperitoneal injection into BALB/c neonates. These results show that the transforming potential of the *axl* gene can be activated by truncation of the extracellular domain of the receptor and fusion of the remaining sequence to the *gag* gene.

Receptor tyrosine kinases (RTKs) play a key role in regulating normal cellular growth and differentiation (12). For many receptors, ligand binding induces conformational changes that stabilize dimerization of adjacent receptors, leading to activation of the kinases (36). RTKs can be aberrantly activated and can express an oncogenic transforming potential by several other mechanisms: retroviral transduction resulting in mutation and overexpression of a receptor (11), chromosomal translocation producing inappropriate expression of a chimeric protein (15), and point mutation (e.g., see reference 40). Studying these mutant forms of a receptor can help us to understand the mechanism of signal initiation through the receptor, characterize the downstream signaling cascade, and evaluate the transforming potentials of specific signal transduction pathways mediated by the receptor.

Axl is an RTK with a unique extracellular structure characterized by the juxtaposition of two immunoglobulin-like and two fibronectin type III repeats (28). Axl was originally identified by a DNA transfection-tumorigenesis assay of DNA from two patients with chronic myelogenous leukemia (CML), a hematopoietic disorder involving increased myelopoiesis and attributable to the *bcr-abl* fusion formed at the translocation breakpoint of the Philadelphia chromosome. Analysis of the transforming DNA revealed it to be identical to a wild-type gene termed c-*axl*. Overexpression of c-*axl* in NIH 3T3 cells also resulted in neoplastic transformation (28).

Axl expression has been detected in most tissues and in several tumor types, but its precise role in oncogenesis has been difficult to elucidate (18, 28). Since *axl* is expressed in the peripheral blood of patients with CML, it has been speculated that the transforming potential of *axl* may be involved in the progression of CML from chronic phase to blast crisis (28). However, the subsequent finding of *axl* expression in normal early myeloid lineages may simply indicate a role in early hematopoietic differentiation rather than a direct contribution to leukemogenesis (26). The human *axl* gene has been mapped to chromosome 19q13.1-13.2, a locus involved in nonrandom chromosomal abnormalities associated with malignant glioma (28). This raises the possibility that *axl* may be a candidate locus for glioblastoma-associated translocation. In addition, two other Axl subfamily members, Sky/Tyro3 and Eyk/Mer, have been implicated in tumorigenesis. Sky mRNA is abundant in mammary tumors isolated from Wnt-1 and Fgf-3 transgenic mice, while being nearly undetectable in normal and preneoplastic mammary glands (44). Eyk is found in an acute transforming avian retrovirus, RPL30 (19). These findings support the hypothesis that Axl-related RTKs are transforming agents in natural tumorigenic processes.

Recently, the product of growth arrest-specific gene 6 (*gas6*), a homolog of the blood-clotting inhibitor protein S, was identified as a ligand for the Axl receptor (43, 47) and shown to stimulate Axl phosphorylation. This secreted protein may stimulate other members of this receptor family (29). The action of Gas6 on Axl may be related to that of another coagulation-related ligand, thrombin, which binds and sends mitogenic signals via a G protein-coupled cell surface receptor. The activation event occurs by a novel mechanism in which thrombin recognizes and cleaves the N-terminal exodomain of its receptor, leading to self-activation of the truncated receptor (48). So far, it is not known whether Gas6 resembles thrombin in induction of its receptor, or what signals are propagated in response to the Gas6-Axl interaction (14).

To study signal transduction by Axl in a new setting, we have sought to activate its transforming potential by constructing genomes mimicking naturally existing acute transforming retroviruses (21, 51). The advantage of such an approach has been shown in the study of the insulin-like growth factor receptor (30, 49). Analysis of a viral active form of Axl may help us understand the mechanism of activation and transformation by

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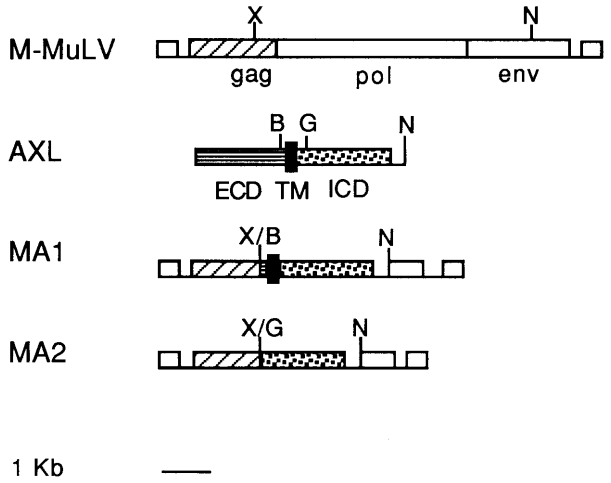

FIG. 1. Recombinant retroviral constructs containing the *axl* gene. In construct MA1, part of *gag*, all of *pol*, and most of *env* of M-MuLV are replaced by *axl* cDNA sequences encoding 5 aa of its extracellular domain (ECD) and the entire TM region and intracellular domain (ICD), including the tyrosine kinase. MA2 differs from MA1 in excluding the region encoding the ECD, the TM region, and 33 aa of the juxtamembrane region of the ICD of Axl from the recombinant genome. X, *Xho*I; N, *Nco*I; B, *Bst*UI; G, *Ngu*MI.

the receptor. Furthermore, replication-defective *axl*-containing viruses complemented by helper Moloney murine leukemia virus (M-MuLV) should be capable of spreading to different tissues in the animal. Analysis of tumors induced by such viruses can provide information about the tissue tropism of *axl*-mediated transformation.

Here we report that the fusion of Axl sequences to the viral Gag protein in a viral genome causes activation of the receptor. The recombinant viruses can transform NIH 3T3 cells, and this oncogenic potential is enhanced by deletion of the transmembrane (TM) region and part of the juxtamembrane region of the receptor. The v-*axl* oncoproteins are shown to be phosphorylated and mostly sequestered in the cytoplasmic compartment. While v-*axl* virus supernatants are apparently not tumorigenic in BALB/c mice, Rag-2-deficient mice, or c-*myc* transgenic mice, cells transformed by one of the v-*axl* viruses can induce solid tumors in mice.

MATERIALS AND METHODS

Recombinant-DNA construction. Plasmid pNCA contains the complete M-MuLV genome (9). Plasmid pAXL contains 3.2 kb of C-terminal sequence of mouse *axl* cDNA. pNCA was digested with *Xho*I, treated with Klenow fragment to blunt the termini, and cleaved with *Nco*I. Plasmid pAXL was cleaved with *Ngu*MI, blunted with Klenow fragment, and then digested with *Nco*I. The released 1.55-kb *axl* insert was ligated to the larger pNCA fragment to form pMA1. The resulting genome encodes a fusion protein consisting of residues 1 to 313 of M-MuLV Gag (39), a single aspartic acid residue created at the junction, and residues 438 to 888 of c-Axl (13). The site of joining to the Gag protein is very similar to that in a number of natural virus isolates: the junction in v-abl is 78 amino acids (aa) upstream (33), that in v-akt is 52 aa upstream (6), that in v-fos is 10 aa downstream (46), and that in v-raf is 70 aa downstream (23). To construct pMA2, a 1.5-kb *Bst*UI-*Nco*I insert from pAXL was ligated to the large pNCA fragment (Fig. 1). The protein encoded by this construct contains the same Gag and junction sequences and residues 501 to 888 of c-Axl.

Cell culture, DNA transfection, virus infection, and focus assay. NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum. Cotransfections of NIH 3T3 cells (5×10^6 cells per 100-mm-diameter dish) were performed by the DEAE-dextran method (24) with 0.5 μg of replication-defective virus DNA and 0.5 μg of pNCA per dish. After splitting once, transfected cells were maintained in DMEM with 5% calf serum for 2 weeks and colonies were scored and cloned.

To assay the transforming activity of virus, culture supernatants from the cloned cells were passed through 0.45-μm-pore-size filters, mixed with 8 μg of Polybrene per ml, and used to infect fresh NIH 3T3 cells for 2 h at 37°C. Foci were counted after 2 weeks.

Analysis of DNA and protein. The preparation of total cellular DNA, restriction enzyme digestion, gel electrophoresis, and transfer to nitrocellulose have been described elsewhere (42). To analyze the structure of the proviruses in tumors, DNA was prepared from solid tumors and used as a substrate for PCRs with one primer specific for *gag* sequences (P121-F, 5′-GCGGGATCCCAGCC CTCACTCC-3′) and one specific for *axl* sequences (Axl-B, 5′-GCAATTTTCA TGGTCTTCACAGCGACCTTG-3′). Reactions were carried out for 30 cycles (1 min at 95°C, 2 min at 65°C, 3 min at 72°C), and the products were analyzed by gel electrophoresis and ethidium staining.

For protein analysis, cells were lysed in a solution containing 1% Triton X-100, 50 mM NaCl, 50 mM Tris (pH 7.5), 100 mM EDTA, 5 mM glycerol-PP_i, and 50 mM sodium fluoride. Cell lysates were immunoprecipitated, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotted as described previously (45).

The rabbit anti-Axl antibody was generated against a Gst-Axl fusion protein bearing 86 aa of the C-terminal peptide sequence of Axl. The fusion protein was expressed in bacteria, isolated from bacterial extracts with glutathione-agarose beads, and purified by SDS-PAGE. Rabbits were immunized with Gst-Axl protein at Cocalico Biologicals (Reamstown, Pa.). Antibodies against phosphotyrosine (RC20) and viral Gag (79S-804) were obtained from Transduction Laboratories and the National Cancer Institute, respectively.

Subcellular localization. Cells were grown on poly-D-lysine-coated Lab Tek chamber slides (Sigma). After 2 days, the cells were rinsed briefly with phosphate-buffered saline (PBS) and fixed with acid ethanol (90% ethanol, 5% glacial acetic acid, 5% H_2O) for 30 min at room temperature. Fixed cells were rinsed and blocked in 2% bovine serum albumin (enzyme-linked immunosorbent assay grade; Sigma)–0.1% Triton X-100 in PBS at 4°C overnight. Rabbit anti-Axl antiserum was added at various dilutions (1:50, 1:100, and 1:500) and incubated for 2 h at 4°C. Cells were washed three times with PBS containing 0.1% Triton X-100 and reblocked for 1 h as described above. Affinity-purified rhodamine-conjugated goat anti-rabbit immunoglobulin antibody (Tago-Biosource) at a 1:100 dilution was added. After incubation at 4°C for 2 h, cells were washed with PBS.

Nuclei were counterstained with 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI) (Boehringer Mannheim Biochemicals) at a final concentration of 1.5 μg/ml in PBS for 30 min at room temperature and visualized by fluorescence microscopy (Nikon microscope).

Tumorigenicity assays. Newborn BALB/c mice (Charles River) and c-*myc* transgenic mice (Eμ-myc mice [1]; Charles River) and 4-week-old Rag-2-deficient mice (38) (generous gift from Fred Alt, Harvard University) were injected with 5×10^3 virus-transfected cells or 100 μl of supernatant containing 1×10^3 to 1×10^4 focus-forming units of viruses. The mice were examined for palpable tumor masses daily. Autopsies were performed after death or after sacrifice of animals suffering from severe disease. Pathological analyses were done by the service in the Department of Comparative Medicine at Columbia University.

In attempts to derive cell cultures, solid tumors were explanted from tumor-bearing animals, dispersed with trypsin, and placed in culture.

RESULTS

Construction of v-*axl* genomes. To create viruses mimicking naturally occurring transforming RTK mutants, we constructed M-MuLV-based, replication-defective retroviral genomes containing 5′-truncated portions of the murine *axl* cDNA (Fig. 1). The *axl* sequence was fused to the viral *gag* gene of M-MuLV truncated at the *Xho*I site. MA1 resembles TM-containing RTK mutants observed in nature, either as viral oncogenes such as v-*fms*, v-*ros*, and most variants of v-*erbB* or as gene fusions generated by chromosomal translocation such as Tel-PDGFRβ (15) and Tropo-Trk (10). MA1 lacks almost the entire extracellular domain but retains 5 aa of the extracellular domain and the complete TM and intracellular domains. Another recombinant virus, MA2, resembles mutants such as v-*kit*, some variants of v-*erbB* (5), Tpr-Trk (16), and Tpr-Met (41) in that the TM region is not retained. MA2 includes *axl* sequences beginning 33 aa downstream of the TM, and the tyrosine kinase domain remains intact.

Transforming activity of recombinant viral DNA in NIH 3T3 cells. We first tested the transforming potential of these recombinant *axl*-containing viral genomes. MA1 and MA2 DNAs were each cotransfected into NIH 3T3 cells with pNCA DNA, containing a complete copy of the M-MuLV proviral genome and providing necessary replication functions. The transfected cells were passaged once, allowing any potentially

TABLE 1. Transforming activities of v-*axl* constructs

DNA	No. of foci from expt:		
	1	2	3
Control	0	0	0
M-MuLV	0	0	0
MA1 + M-MuLV	30	12	18
MA2 + M-MuLV	8	0	2

formed viruses to spread throughout the cultures. The cultures were grown to confluence and held in medium containing 5% serum for 2 to 3 weeks, and then the numbers of transformed foci were counted (Table 1). Parental NIH 3T3 cells and cells transfected with helper virus DNA alone did not produce any foci. In contrast, both MA1 and MA2 caused focus formation. MA1 produced 20 to 30 foci per 100-mm-diameter dish, with cells becoming elongated and assuming a cross-hatched pattern of growth. MA2 yielded, per dish, two to eight foci of highly refractile, rounded cells that piled up to high density and were readily dispersed by pipetting without trypsin. Transfected cells, cloned by limiting dilution and grown in monolayer culture, retained their distinct morphologies (Fig. 2).

Transforming activity is transmissible through *axl*-containing viruses in vitro. The transforming DNAs expressing the Axl kinase should produce RNA genomes that can be efficiently packaged and transmitted by helper virus. To test for the efficiency of viral transduction, supernatants from MA-NCA-transfected cells were collected and passed through 0.45-μm-pore-size filters. NIH 3T3 cells were infected by these filtered supernatants in the presence of Polybrene. After one passage, the cells were maintained in DMEM with 5% calf serum for 2 weeks and foci were scored. The results revealed that supernatants from both MA1-NCA and MA2-NCA transfectants could cause focus formation while control supernatants from NIH 3T3 cells and NCA transfectants could not (Table 2). Titers of the transforming supernatants were determined to be 10^3 to 10^4 focus-forming units/ml. Thus, both MA1 and MA2 were relatively potent focus-forming agents for NIH 3T3 cells and were capable of helper virus-mediated transfer.

Transmission of viral genome. To confirm that foci recovered from transfection and infection experiments actually contain the proviral constructs and to assess the stability of the proviruses, cellular DNA from the transformants was assayed for the presence of v-*axl* sequences by hybridization. Total cellular DNA was digested with several restriction enzymes and subjected to Southern blot analysis by using an *axl* cDNA probe (Fig. 3). NIH 3T3 cells and NCA-transfected cells gave rise to similar patterns of endogenous fragments, while digestion of DNA from MA-transformed cells revealed novel fragments; the intensity of these bands suggests that multiple copies of these DNAs were present. For both MA1 and MA2 viruses, these additional bands correspond in size to the predicted fragments from the construct. Therefore, the foci obtained by DNA transfection and by infection with virus supernatants are true v-*axl* transformants. Furthermore, in comparison with the original recombinant viral constructs, no obvious changes in the proviral genomes were detected by Southern blot analysis, suggesting that both recombinant genomes were genetically stable.

Expression of Gag-Axl oncoproducts. The MA1 and MA2 transforming viruses should both encode Gag-Axl fusion proteins required for their oncogenic activity. To test whether the MA-transformed cells expressed v-*axl* gene products, cell lysates of the transformants and control cells were subjected to Western blot (immunoblot) analysis using antiserum against the C terminus of Axl. Endogenous Axl protein was not detected in uninfected NIH 3T3 cells. MA1 transformants produced two new proteins, dubbed p100 and p84; MA2 transformants also exhibited two new products, p93 and p77 (Fig. 4A).

We then determined whether these Axl-antigenic proteins contained viral Gag determinants. Cell lysates were immunoprecipitated with anti-Axl antiserum, and the immunoprecipitated proteins were analyzed by Western blot analysis, probing with anti-Gag antiserum (Fig. 4B). The appearance of bands corresponding to species p100 and p84 in MA1 and p93 and p77 in MA2 suggested that v-*axl* products were indeed Gag-Axl fusion proteins. It should also be noted that several smaller bands were detected in MA1 and MA2 transformants with anti-Gag antiserum. These could represent Gag-Axl breakdown products or different Gag products coprecipitated with Gag-Axl through Gag-Gag protein interactions (3).

p100 and p93 are glycoproteins. While p84 and p77 have the size predicted for Gag-Axl proteins, p100 and p93 are larger than expected. M-MuLV is known to direct the synthesis of two Gag-related proteins: $Pr65^{gag}$, the precursor to the virion core structural protein, and $Pr75^{glyco\text{-}gag}$, a glycosylated Gag protein found on the surface of MuLV-infected cells (Glyco-Gag was originally identified as gross cell surface antigen [GCSA]). Translation of $Pr65^{gag}$ starts at an AUG, while translation of $Pr75^{glyco\text{-}gag}$ initiates at a CUG located within the 5′ leader sequence and in frame with the AUG of Gag (7). We tested whether the p100 and p93 proteins represented Glyco-Gag-Axl products. Cell lysates from MA transfectants were treated with either endoglycosidase H (endo H) or endo F at 37°C for 16 h. Products from this reaction were subjected to Western blot analysis with anti-Axl antiserum. This analysis demonstrated that p100 and p93 were sensitive to both endo H and endo F and shifted in mobility by approximately 6 kDa on an SDS-PAGE gel; the products of the reaction were still larger than the p84 and p77 proteins. p84 and p77 exhibited no change in mobility, suggesting that these smaller proteins were not glycosylated (Fig. 5). These results suggest that the molecular weights of p100 and p93 are higher than those of p84 and p77 because of both additional N-terminal peptide sequences of Gag sequences and glycosylation of the fusion proteins. It is unclear whether the sugar addition sites lie in the Gag or the Axl portion of the fusion protein. However, these fusion proteins both contain the regions of Gag that are glycosylated normally in the parental Glyco-Gag, making it likely that the Gag region contains at least some of the modifications.

V-Axl oncoproteins are phosphorylated at tyrosine residues. Many viral oncoproteins identified in transformed cells contain phosphotyrosine. Cell lysates from MA1 and MA2 transfectants and control cells were immunoprecipitated with an antiphosphotyrosine antibody. The precipitates were then assayed for v-Axl products by Western blot analysis with anti-Axl antiserum. While NIH 3T3 cells and NCA-transfected cells showed no major bands, the $p100^{glyco\text{-}gag\text{-}axl}$ and $p84^{gag\text{-}axl}$ products were detected in MA1 and the $p77^{gag\text{-}axl}$ protein was detected in MA2, suggesting that these three v-Axl oncoproteins were phosphorylated on tyrosine residues (Fig. 6). The $p93^{glyco\text{-}gag\text{-}axl}$ protein was apparently not phosphorylated. A protein with a molecular mass of about 55 kDa in MA1 transformants was immunoprecipitated by an antiphosphotyrosine antibody and detected by anti-Axl antiserum in Western blots. This protein could be a v-*axl* or c-*axl* breakdown product or another activated protein in MA1-transformed cells.

Subcellular localization of v-Axl oncoproteins. To further characterize the v-*axl* oncoproteins, we analyzed the subcellular distribution of v-*axl* products. Immunofluorescent staining

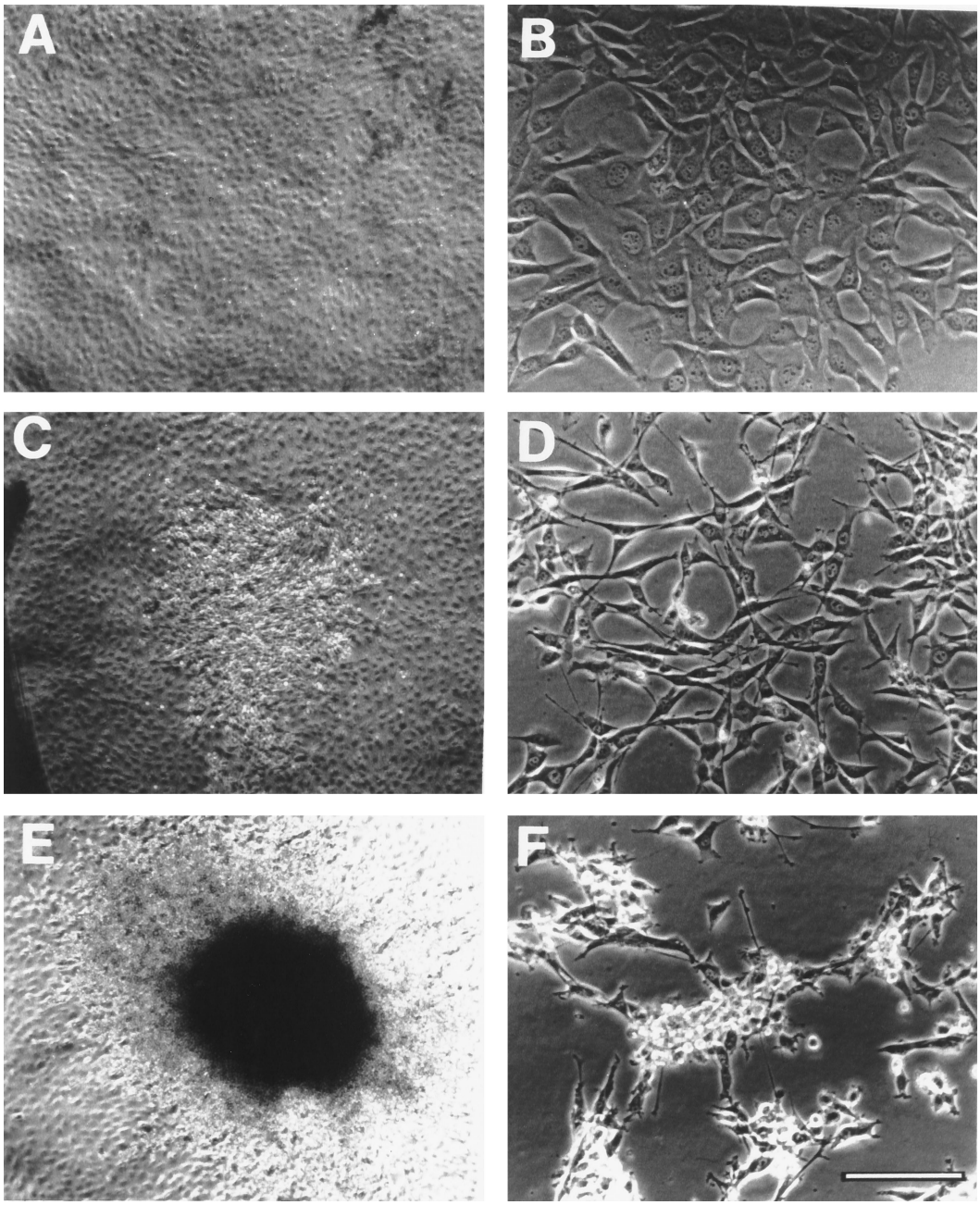

FIG. 2. Transforming activity of v-*axl* genomes in NIH 3T3 cells. (A, C, and E) NIH 3T3 cells were transformed with 0.5 μg of recombinant viral DNA and 0.5 μg of M-MuLV helper virus DNA (pNCA). Transfectants were passaged once, and foci were counted 14 days later. (B, D, and F) Morphology of transformants in monolayer culture after subcloning. NIH 3T3 cells infected by supernatants collected from the transformants described above showed similar phenotypes. (A and B) Cells infected with pNCA alone; (C and D) cells infected with pMA1 and pNCA; (E and F) cells infected with pMA2 and pNCA. Bar, 500 μm (A, C, and E) and 100 μm (B, D, and F).

was performed on fixed, permeabilized MA-transformed cells. Intense cytoplasmic fluorescence of MA-transformed cells was seen with anti-Axl antiserum under conditions in which wild-type NIH 3T3 cells and NCA-transfected cells were minimally labelled (Fig. 7). Staining was most dramatic in the perinuclear region and radiated to the cell periphery. Some weak staining of the cell borders was also observed.

Tumorigenicity of v-*axl* viruses in mice. To evaluate the ability of the two v-*axl* virus genomes to initiate tumors, newborn BALB/c mice were injected intraperitoneally with filtered

TABLE 2. Transforming activities of v-*axl* viruses

Supernatant	No. of foci with supernatant dilution:			
	1:10	$1:10^2$	$1:10^3$	$1:10^4$
Control	0	0	0	0
M-MuLV	0	0	0	0
MA1 + M-MuLV	+++[a]	+++	+++	16, 20, 30
MA2 + M-MuLV	+++	+++	+++	23, 74, 105

[a] +++, too many to count.

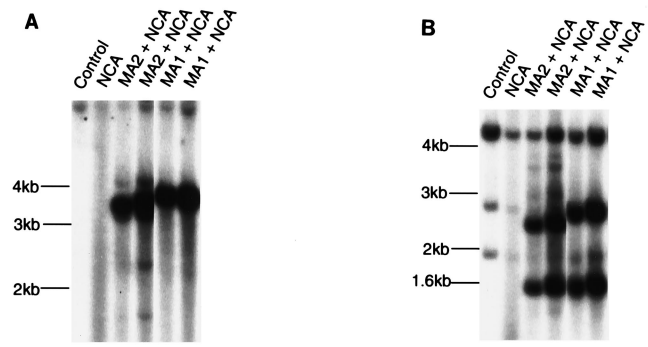

FIG. 3. Characterization by Southern blot analysis of viral genomes in v-*axl* transformants. The genomic DNA of v-*axl* transformants was digested and analyzed by electrophoresis and blot hybridization with a cDNA probe containing the cytoplasmic portion of Axl. (A) DNAs were cleaved with *Eco*RV in both long terminal repeats, and a fragment spanning the entire viral genome was released. (B) DNAs were digested with *Sac*I, releasing the viral genome and separating it into two parts. Lanes: Control, NIH 3T3 cells; NCA, cells transfected with pNCA; MA1 + NCA, cells transfected with pMA1 and pNCA; MA2 + NCA, cells transfected with pMA2 and pNCA. Two transformants are shown for each virus.

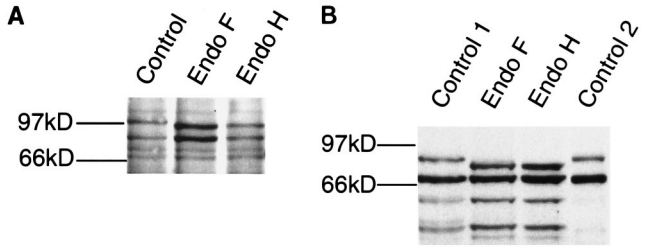

FIG. 5. Glycosylation of v-Axl products. Proteins in cell lysates were denatured and digested with endo H or endo F at 37°C overnight. Reaction products were analyzed by immunoblotting with anti-Axl antiserum. (A) MA1-transfected cells; (B) MA2-transfected cells. Two untreated control lanes are shown for aligning with experimental lanes.

culture supernatants from MA-transformed cells. Control animals were injected with supernatants from NIH 3T3 cells or helper-M-MuLV-infected cells. While animals inoculated with NIH 3T3 cell supernatants remained healthy, without any evidence of tumor induction, mice receiving either M-MuLV or v-*axl* virus developed T-cell lymphoma after 3 to 4 months, with no significant difference in the latency (Table 3). Leukemia cells were explanted, and genomic DNAs were prepared. Southern blot analysis of these DNAs did not reveal any novel sequences detected by an *axl* hybridization probe, suggesting that the leukemia cells were induced by the helper virus, M-MuLV. We have also introduced v-*axl* viruses into mice genetically predisposed to the development of tumors. Mice expressing c-*myc* under the control of the immunoglobulin heavy chain enhancer element as a transgene (Eμ-myc mice) frequently develop B-cell lymphomas (1, 17); further, the transgene can synergize with exogenous leukemia viruses (8) and with acute transforming retroviruses (2, 20) to accelerate the formation of tumors. A total of 68 mice were injected at birth with MA1 and MA2 virus supernatants, but no v-*axl*-induced acute tumors have been found to date (data not shown). To test whether the immune systems of the host animals were responsible for rejection of tumors, the pathogenicity of the viruses in genetically immunodeficient animals was tested. A total of 12 mice homozygous for the *rag-2* gene (38) were similarly injected with the MA supernatants. As before, no v-*axl* tumors were observed.

To test the tumorigenesis of v-*axl*-transformed cells, we subcutaneously or intraperitoneally injected v-*axl*-transformed NIH 3T3 cells into BALB/c neonates. Mice implanted with NIH 3T3 cells, M-MuLV-transfected cells, or MA1 transformants did not produce any solid tumors, although the last two cell types led to the formation of M-MuLV-induced leukemia in about 4 months. In contrast, mice injected with MA2 transformants developed solid tumors 2 to 3 cm in diameter at the injection site within 1 month (Table 3). These tumors were classified as fibrosarcomas by pathological examination. To confirm the origin of the tumors and the presence of the virus, DNA was recovered from the primary explants and shown to contain v-*axl* sequences by PCR analysis. Primers hybridizing to the *gag* and *axl* sequences flanking the *gag-axl* junction were used to amplify genomic DNA; the resulting product was identical in size to that in the original MA2 virus (Fig. 8). There were no signs of gross rearrangements at the 5′ junction. We conclude that MA2-transformed cells, but not MA1-transformed cells, can be tumorigenic in vivo. Whether a subset of these tumor cells were induced in the host cells by v-*axl* viruses released from MA2 transformants is not known at this time. Efforts to culture the explanted tumor cells, and to recover transforming virus from the explanted tumor cells, have not been successful to date.

DISCUSSION

Activation of Axl RTK. The design of the *axl*-containing transforming viruses analyzed in this study was based on two long-standing observations: (i) the majority of naturally occurring constitutively active mutants of RTKs, generated by either viral transduction or chromosomal translocation, harbor trun-

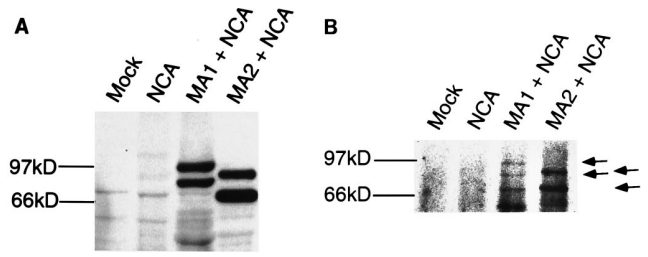

FIG. 4. Expression of v-Axl products in transformed cells. (A) Transfected cells were lysed, and proteins were separated on an SDS-PAGE gel, blotted to nitrocellulose membrane, and probed with anti-Axl antiserum. (B) Cell lysates were immunoprecipitated with anti-Axl antiserum. Immunoprecipitated proteins were analyzed by Western blot analysis, probing being done with anti-Gag antiserum. Arrows indicate the v-Axl proteins. For lanes, see the legend to Fig. 3.

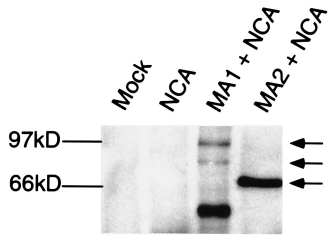

FIG. 6. Tyrosine autophosphorylation of v-Axl products. Cell lysates were immunoprecipitated by anti-phosphorylated tyrosine antibody. Immunoprecipitates were analyzed by Western blotting and probing with anti-Axl antiserum. Arrows show the v-Axl proteins. For lanes, see the legend to Fig. 3.

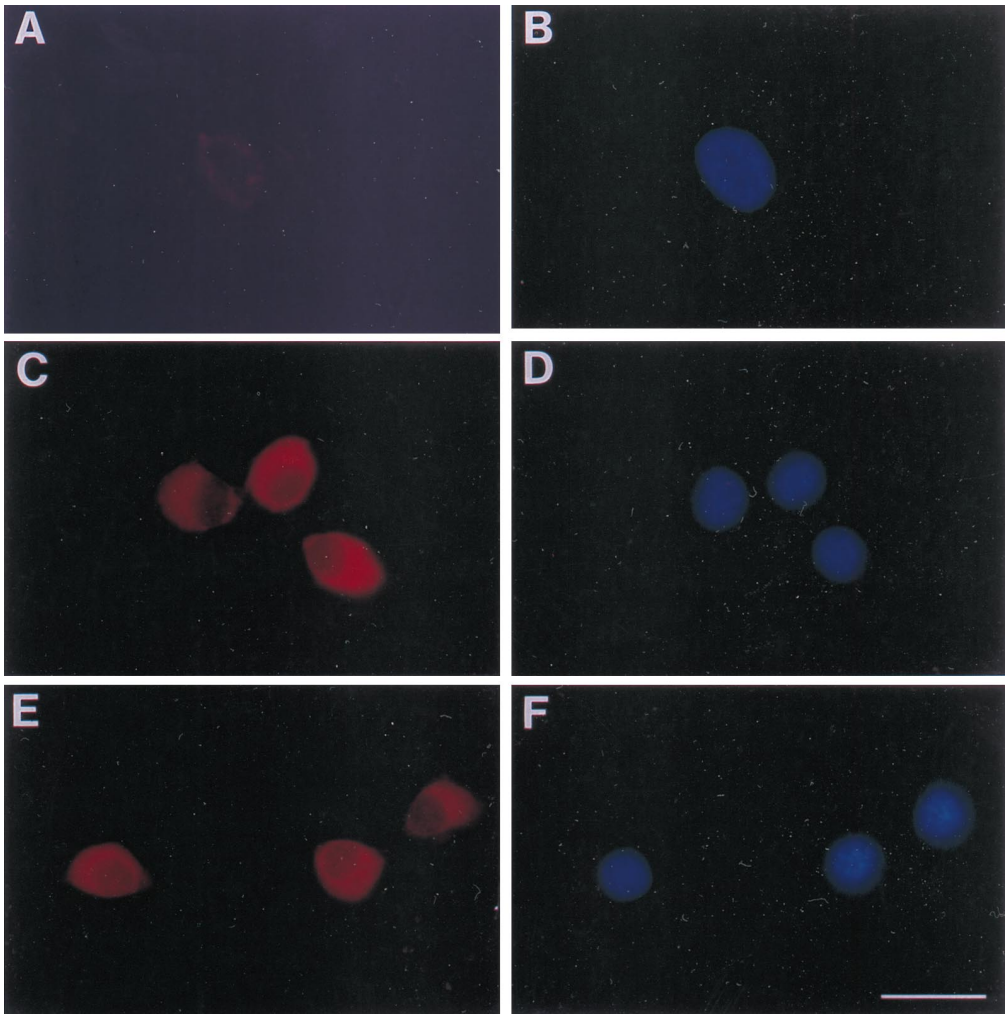

FIG. 7. Indirect immunofluorescence staining of v-Axl products in NIH 3T3 transformants. (A, C, and E) Cells were fixed with acid ethanol and stained with rabbit anti-Axl antiserum and rhodamine-conjugated goat anti-rabbit immunoglobulin antibody. (B, D, and F) Nuclei were counterstained with DAPI and visualized by fluorescence microscopy. (A and B) Cells transfected with NCA; (C and D) cells transfected with MA1 and NCA; (E and F) cells transfected with MA2 and NCA. Bar, 20 μm.

cations at the N terminus of the receptor, and (ii) many retroviral oncogenes are expressed as fusions with viral *gag* sequences (51). The results indicate that large N-terminal truncations and fusion of Axl can indeed activate the receptor. These viruses possess the ability to transform fibroblasts with considerable potency.

TABLE 3. Tumorigenicity of v-*axl* in mice

Virus	No. of mice with tumors/total no. injected with:			
	Supernatants		Cells	
	Acute tumor[a]	Slow tumor[b]	Acute tumor	Slow tumor
Control	0/10	0/10	0/16	0/16
M-MuLV	0/11	11/11	0/11	11/11
MA1 + M-MuLV	0/14	14/14	0/20	20/20
MA2 + M-MuLV	0/14	14/14	14/14	ND[c]

[a] Tumors induced within 3 months of injection.
[b] M-MuLV-induced T-cell lymphoma with a 3-month latency period.
[c] ND, not determined.

The viral Gag domain may contribute to the stability of the fusion protein and promote its oligomerization. In Abelson murine leukemia virus, Gag sequences present in the Gag–v-*abl* fusion protein are required for transforming activity and are known to contribute to protein stability (32). The Gag region retained in v-*axl* is sufficient to mediate dimerization as judged by using the yeast two-hybrid system (3). A potential role for Gag-induced dimerization in the activation of Gag-Axl as well as other Gag-RTK proteins is further suggested by the structures of activated RTKs formed in chromosomal translocation events. Interestingly, such fusion partners often include domains of proteins that normally oligomerize, and removal of these domains can abolish the oncogenic property of the fusion proteins (35). For example, the c-*abl* proto-oncogene becomes fused to the *bcr* gene in Philadelphia chromosome-positive human leukemia cells. Tetramerization of Bcr-Abl through Bcr domain 1 correlates with activation of the tyrosine kinase activity of Abl (25). For Gag-Axl, it is plausible that both events, truncation of the receptor and fusion with Gag, could contribute to its transforming activity.

MA2 virus was shown to have a transforming potential greater than that of MA1 virus in both the in vitro focus assay

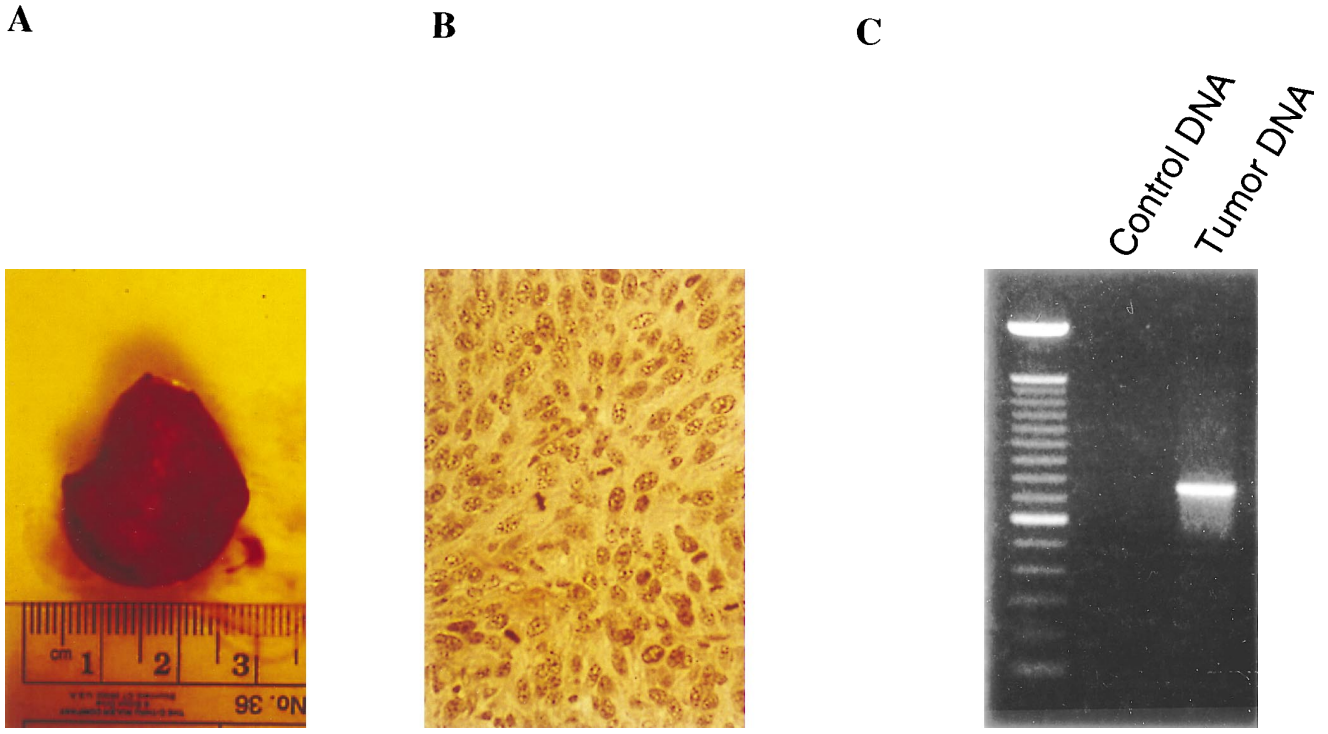

FIG. 8. Tumorigenicity of MA2-transformed cells in vivo. NIH 3T3 cells and NCA-MA1-, and MA2-transformed cells were injected subcutaneously into BALB/c neonates. (A) After 1 month, only MA2-transformed cells grew to yield 2- to 3-cm-diameter tumors. (B) Pathological examination revealed the tumors to be fibrosarcomas (hematoxylin and eosin staining). (C) A v-*axl*-specific signal was detected in the tumor DNA by PCR analysis.

and the in vivo tumorigenicity assay. The viral genome of MA2 differs from that of MA1 in lacking a stretch of Axl sequences that include 5 aa of the extracellular domain, the TM region, and 33 aa of the intracellular domain. Since the subcellular localizations of the viral oncoprotein in the two v-*axl* transformants are similar, we propose that the TM region and the juxtamembrane region may negatively influence protein stability or conformation. Alternatively, other proteins may bind to these domains in MA1 and exert negatively regulatory effects.

Phosphorylation and glycosylation of v-Axl oncoproteins. Regardless of the mechanism of activation, a requisite feature of many activated RTKs is autophosphorylation of the receptor. This is believed to occur by cross-phosphorylation between dimerized receptors (36). Our results show that v-*axl* oncoproteins are phosphorylated on tyrosine residues in transformed cells, suggesting that these molecules are in a constitutively active state. The c-*axl* product has 14 tyrosine residues in its cytoplasmic domain: 9 within the conserved tyrosine kinase domain and 5 outside the kinase domain. Studies of other receptors have shown that tyrosine residues located inside the kinase domain can serve as allosteric sites regulating the V_{max} of the kinase function. Phosphorylation of these sites occurs prior to phosphorylation of other sites and leads to an increase in kinase activity. Phosphorylation of tyrosine residues outside the kinase domain of many receptors mainly creates docking sites for downstream SH2-containing molecules and is the crucial step for signal transduction mediated by RTKs.

The v-*axl* proteins encoded by our viruses exist in two forms: Glyco-Gag-Axl and Gag-Axl. Glyco-Gag-onc fusion proteins have been identified in other acute transforming retroviruses. For example, v-*erbB* produces two proteins, a cytoplasmic form and a minor population of a glycosylated membrane form which has been shown to extend its ErbB domain outside the cell (31). Treatment of v-*erbB* transformants with glycoprotein-processing inhibitors blocks normal Glyco-Gag-ErbB synthesis but does not alter the transformed phenotype, suggesting that carbohydrate processing is not required for transforming activity of v-*erbB* (37). The glycosylated form of the v-Abl oncoprotein has also been reported. It cannot be phosphorylated and is not required for the transforming activity (50). However, the issue is a matter of controversy for other oncogenic retroviruses (22, 27, 34). The relative functionality of the two viral proteins Gag-Axl and Glyco-Gag-Axl has yet to be determined. In MA1, both forms were heavily phosphorylated; in MA2, only the cytoplasmic form was phosphorylated, suggesting that the glycosylated form is not required in this case.

Subcellular localization of v-Axl oncoproteins. Indirect immunohistochemistry revealed that in both MA1- and MA2-transformed cells, v-*axl* oncoproteins were expressed mainly in the cytoplasmic compartment, with staining strongest in the perinuclear region and radiating to the periphery. This compartmentalization is to be distinguished from the membrane localization of the normal TM receptor and is consistent with findings obtained with some RTK-containing transforming viruses. In v-*erbB*-transformed cells, the bulk of the oncoprotein remains associated with dense membranes after synthesis, although a small quantity may slowly become associated with the plasma membrane (52). In cells transformed by v-*fms*, the majority of v-*fms*-encoded antigen was also internally sequestered, with only a minor population, gp140fms, detected at the plasma membrane (4).

Transforming activity. v-*axl* viral supernatants were able to induce morphologically transformed foci in NIH 3T3 cells in vitro but could not induce tumors in BALB/c neonates in a 3-month latency period before the appearance of M-MuLV helper-virus-induced leukemia. The fundamental reasons for

the limited pathogenicity of the v-*axl* genomes are unclear. Results obtained with immunodeficient mice suggest that the nonpathogenicity of v-*axl* viruses cannot be entirely explained by host rejection of nascent tumors. It is known that for some oncogenes in some settings, such as *ras* and c-*myc*, an additive effect of two genes is required for in vivo tumorigenesis. Nevertheless, cooperative oncogenicity was not observed when v-*axl* viruses were introduced into c-*myc* transgenic mice. It is also possible that the 3-month latency period for M-MuLV-induced leukemia might not be long enough to allow observation of a novel tumor induced by v-*axl* viruses.

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