Generation of a Recombinant Moloney Murine Leukemia Virus Carrying the v-src Gene of Avian Sarcoma Virus: Transformation In Vitro and Pathogenesis In Vivo

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A Moloney murine leukemia virus (M-MuLV) recombinant carrying the v-src gene of avian sarcoma virus was generated by the introduction of a cloned portion of v-src from Schmidt-Ruppin A avian sarcoma virus into a molecular clone of M-MuLV provirus at the recombinant DNA level. The v-src sequences (lacking a portion of the 5' end of v-src) were inserted into the p30 region of the M-MuLV gag gene so that M-MuLV gag and v-src were in the same reading frame. Transfection of this chimeric clone, pMLV(src), into NIH 3T3 cells which were constitutively producing M-MuLV gag and pol protein resulted in the formation of foci of transformed cells. Infectious and transforming virus could be recovered from the transformed cells. This virus was designated M-MuLV(src). M-MuLV(src)-transformed cells contained two novel proteins of 78 and 90 kilodaltons. The 78-kilodalton protein, p78^{gag-src}, contained both gag and src determinants, exhibited kinase activity in an immune kinase assay, and is probably a fusion of Pr65^{gag} and src. The 90-kilodalton protein, which is of the appropriate size to be the gPr80^{gag} fused to src, contained gag determinants as well as a V8 protease cleavage fragment typical of the carboxy terminus of avian sarcoma virus pp60^{src}. However, it could not be immunoprecipitated with an anti-v-src serum. M-MuLV(src)-transformed cells showed elevated levels of intracellular phosphotyrosine in proteins, although the elevation was intermediate compared with cells transformed with wild-type v-src. M-MuLV and amphotropic murine leukemia virus pseudotypes of M-MuLV(src) were inoculated into newborn NIH Swiss mice. Inoculated mice developed solid tumors at the site of inoculation after 3 to 6 weeks, with most animals dying by 14 weeks. Histopathological analysis indicated that the solid tumors were mesenchymally derived fibrosarcomas that were both invasive and metastatic.

Retroviruses cause a variety of neoplasms in infected animals. These viruses can be divided into two classes: acutely transforming viruses and slowly transforming viruses. Acutely transforming viruses cause diseases with short latency periods, can transform cells in tissue culture, and are frequently replication defective (55). More importantly, they carry oncogenes which are responsible for their oncogenic potential and which are derived from normal cellular DNA (4). Different acutely transforming viruses cause diseases ranging from sarcomas to rapid leukemias and carcinomas. Slowly transforming viruses generally cause leukemias with long latency periods, are replication competent, do not transform cells in tissue culture, and lack oncogenes (55).

The specific neoplasm produced by an acutely transforming retrovirus could result from several possible determinants. One possibility is that the viral oncogene itself could be the primary factor. For example, several independently isolated avian retroviruses carry the oncogene *fps*, and all of them cause fibrosarcomas (4). On the other hand, the *abl* oncogene has been identified in Abelson murine leukemia virus and Hardy-Zucker feline sarcoma virus. The former virus causes B-cell lymphomas in mice (59), whereas the latter causes sarcomas in cats (3). This raises the possibility that the viral context in which an oncogene is found may be an important factor in determining the disease potential (42). A third possible control point for pathogenic potential could

be the tissue specificity of viral expression. Transcriptional enhancer elements in retroviral long terminal repeats (LTRs) have recently been implicated in both pathogenic potential and tissue tropism for expression (8, 17, 34).

To study the relationship between pathogenic potential and retrovirus structure, a new acutely transforming retrovirus was constructed by molecular cloning. The v-src gene from avian sarcoma virus (ASV) was inserted into the genome of Moloney murine leukemia virus (M-MuLV), a slow leukemia virus. This combination was chosen since both ASV and M-MuLV have been extensively characterized molecularly and biologically. In addition, both viral genomes have been molecularly cloned and sequenced, which facilitated the construction of the new recombinant transforming virus. Furthermore, v-src is the most extensively characterized retroviral oncogene. The v-src gene in ASV is expressed as a 60-kilodalton (kDa) phosphoprotein, pp60^{src} (7, 43), which is a tyrosine-specific protein kinase (12, 26, 35). ASV-infected cells show elevated levels of protein phosphorylation on tyrosine residues (52), and several substrates for phosphorylation have been identified (15, 26, 28). Recent results also suggest that pp60^{src}, as well as the *ros* oncogene transforming protein can also phosphorylate cellular lipid (37, 54a).

Insertion of v-src into the M-MuLV genome was chosen so that the resultant retrovirus could be propagated in mouse cells. Such a construct would allow studies of the pathogenicity of an src-carrying retrovirus in mice, which are well-characterized experimental animals (40). The pathoge-

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FIG. 1. Construction of a recombinant *gag*⁺ *pol*⁺ *env*⁻ M-MuLV provirus. p63-2 plasmid (36) DNA (carrying a complete integrated M-MuLV provirus) was digested with *Hpa*I and religated, deleting 1.5 kb of the M-MuLV *env* sequences and also 0.25 kb of the *pol* sequences. The *pol* gene was then reconstituted by reinsertion of the 260-base-pair *Hpa*I-to-*Sma*I restriction fragment. This procedure resulted in a provirus that contained complete *gag* and *pol* genes but which was deficient for *env*. The arrow in the provirus indicates the direction of transcription. The other arrow indicates the standard orientation of plasmid pBR322. Abbreviations for restriction enzymes: H, *Hpa*I; Sm, *Sma*I.

nicity of such a virus would be interesting since the only naturally occurring *src*-containing retroviruses isolated thus far have been avian retroviruses which cause sarcomas.

MATERIALS AND METHODS

Tissue culture cells. The cells used in this study were as follows: mouse NIH 3T3 cells (57); SR3T3, NIH 3T3 cells transformed by Schmidt-Ruppin Rous sarcoma virus (RSV) and provided by Bart Sefton, The Salk Institute, San Diego, Calif.; and A9 cells (21), a clone of NIH 3T3 infected with M-MuLV. Rat XC cells were used in the UV-XC syncytial plaque assay for titrating M-MuLV (49). All cell lines were grown as monolayer cultures in Dulbecco modified Eagle medium (GIBCO Laboratories) supplemented with 10% calf serum.

Derivation of 14-11 cells. For these experiments, a line of NIH 3T3 cells was isolated which produced M-MuLV *gag* and *pol* protein but not *env* protein. The construction of a molecular clone of an M-MuLV provirus with the *env* region deleted but nondefective for *gag* and *pol* is diagrammed in Fig. 1. DNA from this clone was cotransfected into NIH 3T3 cells together with a bacterial kanamycin resistance expression plasmid, pSHL72 (10, 58). NIH 3T3 cell colonies expressing kanamycin resistance were selected by growth in the antibiotic G418 (GIBCO) and isolated by selective trypsinization in cloning cylinders. Individual clones were then screened for the synthesis of M-MuLV *gag* and *pol* protein

by immunoprecipitation of radioactively labeled cells and by gel electrophoresis as described below. One such *gag* and *pol* positive clone was designated 14-11.

Transfections. Transfections were performed by calcium phosphate precipitation (23) with modifications as described previously (20, 32). Briefly, 10⁶ 14-11 cells were transfected as a calcium phosphate precipitate containing 1 µg of viral DNA, and after 4 h they were treated with 25% dimethyl sulfoxide for 45 s and allowed to recover overnight. The next day the cells were transferred at a 1:5 dilution and allowed to grow to confluency.

Isolation of transformed cells. Cells from transformed foci were isolated by selective trypsinization in cloning cylinders. Populations enriched in transformed cells were then plated at a low density to allow the isolation of colonies originating from single transformed cells. One such line was designated M-MuLV(*src*)B1. In addition, a noncloned population of transformed cells was isolated by selecting for cells that were less adherent. Tissue culture dishes containing a population enriched in transformed cells were gently shaken without the addition of trypsin. The nonadherent cells in the culture media were then transferred to a new plate. This resulted in a homogeneous population of transformed cells, designated M-MuLV(*src*)B.

Recombinant cloning. The starting recombinant clones were *psrc* and p63-2 (Fig. 2a). *psrc* contains the *Eco*RI B fragment of Schmidt-Ruppin A (SR-A) ASV subcloned into pBR322 and was kindly provided by J. Michael Bishop (16). The SR-A ASV *Eco*RI B fragment contains the entire protein coding sequence of v-*src* as well as some ASV *env* and LTR sequences. p63-2 is a pBR322 plasmid clone containing a complete M-MuLV provirus integration with both LTRs, which are bounded on either side by cellular sequences (36). Recombinant DNA cloning procedures were performed by standard protocols (24, 27, 38).

Labeling and immunoprecipitation of tissue culture cells. Tissue culture cells were biosynthetically labeled with [³⁵S]methionine (Amersham Corp.) in methionine-free media, and cell extracts were prepared and immunoprecipitated with different antisera according to the method of Sefton et al. (50). The antisera used were an anti-v-*src* tumor-bearing rabbit serum (kindly provided by Bart Sefton) and a rabbit anti-M-MuLV p30^{gag} serum (39). Immunoprecipitates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (31) followed by autoradiography or fluorography (33) on Kodak XAR-5 film.

Immune kinase assay. Unlabeled cellular lysates were prepared, immunoprecipitated, incubated with [γ-³²P]ATP, and then processed by SDS-PAGE according to the method of Sefton et al. (51).

Phosphoamino acid analysis. Cells in tissue culture dishes (35-mm diameter) were labeled overnight with 1.2 mCi of inorganic ³²P (ICN Pharmaceuticals, Inc.) and subjected to phosphoamino acid analysis as described by Hunter and Sefton (26).

V8 protease assay. [³⁵S]methionine-labeled and immunoprecipitated cell extracts were prepared as described above and subjected to SDS-PAGE and autoradiography. Bands containing labeled proteins of interest were removed and subjected to gel proteolysis with staphylococcal V8 protease (Worthington Diagnostics) as described by Cleveland et al. (9).

Animal inoculations and histopathology. Neonatal NIH Swiss mice (1 to 2 days old) were inoculated intraperitoneally with 200 µl of cell-free tissue culture supernatants from cells productively infected with transforming virus. Mori-

bund mice were sacrificed, and tissue samples were prepared for pathologic examination as described previously (40).

RESULTS

Construction of pMLV(*src*). For these experiments, a recombinant clone, pMLV(*src*), containing SR-A ASV *v-src*

FIG. 2. Construction of pMLV(*src*). (a) Restriction maps of the genome of M-MuLV (36) and the 3' portion of Schmidt-Ruppin ASV (SRA-2). Viral LTRs are indicated by open boxes, and the regions encoding each of the viral proteins are indicated. The SRA-2 region encoding *v-src* is indicated by cross-hatching. We used a pBR322 subclone of the *v-src*-specific *Eco*RI fragment, p*src* (*Eco*RI-B). Abbreviations for restriction enzymes: Ps, *Pst*I; X, *Xho*I; Bg, *Bgl*II; S, *Sal*I; HD, *Hind*III; C, *Cla*I; R, *Eco*RI; HC, *Hinc*II. (b) Nucleic acid sequences of M-MuLV *gag* around the *Xho*I site (55) and *v-src* around the *Hinc*II site (55). Relationships of restriction fragment ends to the protein reading frames are indicated. (c) Construction of pMLV(*src*). The starting plasmid was M-MuLV p63-2, a pBR322 plasmid clone containing a complete M-MuLV provirus inserted at

inserted into an M-MuLV proviral organization was first constructed. The cloning was designed so that the *v-src* sequences would be inserted into the M-MuLV *gag* gene, resulting in the production of a M-MuLV *gag*-ASV *src* fusion protein.

The construction of pMLV(*src*) is diagrammed in Fig. 2c. First, the *Eco*RI site in p63-2, the plasmid containing the M-MuLV proviral organization, was destroyed by the digestion of p63-2 with *Eco*RI, followed by filling of the staggered ends and religation (p63-2[R⁻]). This allowed conversion of the unique *Sal*I site at 4.2 kilobases (kb) on the M-MuLV proviral map to a unique *Eco*RI site (p63-2[S⁻ R⁺]). p63-2 (S⁻ R⁺) was then cleaved with *Xho*I. The staggered ends were then partially filled by the Klenow fragment of *Escherichia coli* DNA polymerase in the presence of dATP and dGTP, and the remaining two-nucleotide overhang was removed by digestion with mung bean nuclease. This was done to allow joining of the M-MuLV *gag* and *v-src* genes in the same reading frame (Fig. 2b). After cleavage and modification of the *Xho*I site, the plasmid DNA was then digested with *Eco*RI.

p*src* DNA was digested with *Eco*RI and *Hinc*II, and the 1.8-kb fragment containing the 3' 1,249 nucleotides of *v-src* as well as a portion of the ASV LTR (not including the promoter) was recovered. This fragment was then ligated with the *Eco*RI- and *Xho*I-digested p63-2(S⁻ R⁺) and transformed into *E. coli* C600. The *Eco*RI cohesive ends on both p63-2(S⁻ R⁺) and the *v-src* fragment ensured the insertion of the *v-src* fragment at high efficiency and in the correct orientation. Bacterial colonies containing plasmids with the *v-src* insertion were identified by colony hybridization, and plasmids were mapped by restriction endonuclease digestion. Several of the desired clones were obtained, and one (pMLV(*src*)) was used for further experiments.

A derivative of pMLV(*src*) lacking the M-MuLV *env* region was also isolated. pMLV(*src*) was digested with *Hpa*I, and the plasmid was religated. This resulted in the deletion of a 1.4-kb fragment from the M-MuLV *env* region. This clone was designated pMLV(*src*⁺ *env*⁻).

Transfection of mouse cells with pMLV(*src*) and focus formation. pMLV(*src*) was transfected into mouse cells to determine the transforming potential of the virus. The construction of pMLV(*src*) resulted in a deletion in the M-MuLV *gag*-*pol* region, rendering the viral genome defective for replication. To complement the mutation, pMLV(*src*) was transfected into a derivative of NIH 3T3 cells, 14-11. This cell line, producing M-MuLV *gag* and *pol* proteins constitutively, was generated to support the replication of murine leukemia viruses (MuLVs) defective in *gag* and *pol*. The *env*-negative state of 14-11 cells should render them susceptible to infection or transfection by *env*-containing MuLVs. In principle, when the defective *env* gene of the 14-11 provirus is complemented by an *env*-positive MuLV

the *Hind*III site. Before insertion, the *Sal*I site in pBR322 was deleted. M-MuLV p63-2 was then modified by the removal of the *Eco*RI site in pBR322 and the substitution of an *Eco*RI site for the *Sal*I site, yielding M-MuLV p63-2(S⁻ R⁺). This plasmid was then linearized at the *Xho*I site, and the end was filled with DNA polymerase in the presence of dTTP and dCTP, followed by the removal of the two remaining unpaired nucleotides by digestion with mung bean nuclease (38). The plasmid was then digested with *Eco*RI, and the 1.5-kb *Hinc*II-to-*Eco*RI fragment from p*src* (*Eco*RI-B) was cloned into the plasmid. Ligation at the *Eco*RI site was done at cohesive ends and at blunt ends at the modified *Xho*I site.

FIG. 3. Transformation of cells by pMLV(*src*). (A) Strain 14-11 cells (10^6) were transfected with 1 μ g of pMLV(*src*) DNA. Foci of morphologically transformed cells were visible approximately 10 days after transfection. A typical focus is shown. (B) Untransformed 14-11 cells. (C) pMLV(*src*)-transformed 14-11 cells isolated after cloning from single cells (M-MuLV[*src*]B1). The transformed characteristics of the cells were increased compared with the original foci, with numerous round cells present. (D) Infection of fresh 14-11 cells with M-MuLV(*src*)B1 tissue culture supernatants. The culture, infected with a 10^{-1} dilution of supernatant, is shown 10 days postinfection.

genome such as pMLV(*src*), infectious virus should be produced. In the absence of recombination, the result would be a mixed viral stock containing particles with both the *gag*⁺ *pol*⁺ *env*⁻ genome resident in the 14-11 cells and the transfected *env*-positive genome.

pMLV(*src*) DNA was transfected into 14-11 cells by calcium phosphate precipitation, and the following day the cells were transferred and allowed to grow to confluency. Foci of morphologically transformed cells appeared after 1 to 2 weeks. The foci were evident as small areas of round growth containing elongated, spindle-shaped, refractile cells (Fig. 3A). The quantification of focus-forming activity is shown in Table 1. Transfection of pMLV(*src*) onto 14-11 cells resulted in 30 to 100 foci per μ g of DNA. When NIH 3T3 cells were transfected instead of 14-11 cells, no foci were detected. This supports the hypothesis that upon transfection of 14-11 cells by pMLV(*src*), the spread of infectious virus is instrumental in focus formation.

To study the presumed requirement of *env* for the establishment of pMLV(*src*) foci in 14-11 cells, pMLV(*src*⁺ *env*⁻) DNA was transfected into 14-11 cells. In theory, no infectious virus should have been produced due to the lack of *env* protein, and therefore no foci should be detected. However, foci did appear (Table 1), but at fivefold-lower levels than for

pMLV(*src*), and the cells produced no detectable infectious virus. This suggests that although the synthesis of *gag* and *pol* protein is essential for high-efficiency focus formation (i.e., transfection of 14-11 versus NIH 3T3 cells), *env* protein is not absolutely required. Nevertheless, *env* protein did increase the efficiency fivefold. Alternatively, an endogenous MuLV provirus could have contributed complementing *env* protein, although NIH 3T3 cells (from which the 14-11 cells were derived) express little or no endogenous *env* glycoprotein.

Cells from transformed foci were then selectively transferred into secondary plates by trypsinization in cloning cylinders. The cells were seeded at low densities, and colonies originating from single transformed cells were isolated. Two such cell lines obtained from transfections of pMLV(*src*) and pMLV(*src*⁺ *env*⁻) were designated M-MuLV(*src*)B1 and M-MuLV(*src*⁺ *env*⁻), respectively. In addition, a culture of noncloned M-MuLV(*src*)-transformed cells was obtained by continuous transfer of loosely adherent cells from a transformed focus without single-cell cloning. This culture was designated M-MuLV(*src*)B. All analyses so far have shown no differences between M-MuLV(*src*)B and M-MuLV(*src*)B1.

M-MuLV(*src*)B cell lines were more spindle shaped and

TABLE 1. Focus-forming activity after transfection of cells with pMLV(*src*)

Expt no.	Transfecting DNA	No. of foci ^a :	
		14-11	NIH 3T3
1	pMLV(<i>src</i>)	30 (4)	0 (4)
	p63-2	0 (3)	0 (3)
2	pMLV(<i>src</i>)	106 (4)	0 (4)
	pMLV(<i>src</i> ⁺ <i>env</i> ⁻)	24 (4)	0 (4)
	p63-2	0 (4)	0 (3)

^a 10⁶ NIH 3T3 or *gag*⁺ *pol*⁺ *env*⁻ 14-11 cells were transfected with 1 µg of plasmid DNA according to the calcium phosphate procedure (20, 23, 32). One day after transfection the cells were transferred into five tissue culture dishes and grown to confluency. Foci were evident 8 to 10 days posttransfection. The cumulative numbers of foci (scored microscopically) are indicated; the numbers in parentheses indicate the number of dishes scored. The efficiencies of focus formation for pMLV(*src*) in the first and second experiments were 36 and 132 per µg, respectively.

more refractile than nontransformed cells (Fig. 3C). Their morphology was fusiform, as opposed to the rounded shapes of highly transformed cells. They generally grew faster and to a higher density than normal cells, but further characterization of the transformed state has not yet been performed.

Expression of M-MuLV(*src*) in transformed cells and production of transforming virus. If the transformed M-MuLV(*src*)B cells resulted from the expression of transfected pMLV(*src*), it would be expected that they would express *src*-specific RNA. Therefore, the M-MuLV(*src*)B cells were tested for *src*-specific RNA by dot blot analysis (56). As expected, M-MuLV(*src*)B cytoplasmic RNA spotted onto nitrocellulose showed detectable hybridization to nick-translated *psrc* and p63-2 probes, whereas no *src* hybridization was detected in the parental 14-11 cells or uninfected NIH 3T3 cells (data not shown).

M-MuLV(*src*)B transformed cells were also tested for the production of infectious virus (Table 2). The presence of replicating *env*-positive M-MuLV was assayed by infecting NIH 3T3 or 14-11 cells and then measuring UV-XC syncytial plaque formation (49). Infectious transforming virus was assayed by the infection of NIH 3T3 or 14-11 cells and the observation of foci. The M-MuLV(*src*)B cells produced both XC-positive and infectious transforming virus (Table 2).

TABLE 2. Infectivity of virus from M-MuLV(*src*)-transformed cells^a

Transformed cells	Titer	
	FFU/ml	XC PFU/ml
M-MuLV(<i>src</i>)B	2.9 × 10 ⁴	3.6 × 10 ⁴
M-MuLV(<i>src</i>)B1	1.1 × 10 ⁴	6.1 × 10 ⁴
	9.2 × 10 ^{3b}	7.7 × 10 ^{4b}
M-MuLV(<i>src</i> ⁺ <i>env</i> ⁻) ^c	1.1 × 10 ⁵	1.7 × 10 ⁶
	1.3 × 10 ^{5b}	1.0 × 10 ^{6b}

^a Virus released in 24 h from tissue culture supernatants of M-MuLV(*src*)-transformed cells was assayed on NIH 3T3 or 14-11 cells by standard infectivity procedures (52). Different dilutions of the virus were tested, each in duplicate. One infected culture was assayed for the presence of infectious M-MuLV after 5 days by UV irradiation and XC cell overlay (49). The other culture was scored for formation of foci 10 days after infection. Titers for XC plaque-forming activity and focus-forming activity are given in units per milliliter (PFU and FFU, respectively). M-MuLV(*src*)B cells were not cloned. M-MuLV(*src*)B1 is a single-cell clone out of the M-MuLV(*src*)B-transformed cells.

^b Titration was performed on 14-11 cells.

^c Cell line was superinfected with wild-type virus.

Transformed foci resulting from the infection of M-MuLV(*src*)B cell supernatant into 14-11 cells had the same morphology as foci arising in 14-11 cells by DNA transfection with pMLV(*src*) (Fig. 3D).

The M-MuLV(*src*)B cells produced a 10-fold excess of XC plaque-forming activity over focus-forming activity. This suggested that wild-type recombinant M-MuLV was present. M-MuLV could be generated by recombination between the M-MuLV(*src*) genome and the *gag*⁺ *pol*⁺ *env*⁻ genome resident in 14-11 cells. Once generated, the replication-competent virus could easily spread through the culture, increasing the titer of *env*⁺ particles. In support of this explanation, Southern blot analysis (53) of M-MuLV(*src*)B cell DNA revealed an internal M-MuLV-specific 3-kb *Bam*HI fragment characteristic of wild-type M-MuLV but absent from both M-MuLV(*src*) and 14-11 M-MuLV DNA (data not shown). Southern blot analyses with other diagnostic restriction enzymes in conjunction with labeled M-MuLV or *v-src* probes confirmed the presence of the expected M-MuLV(*src*) proviral organization in M-MuLV(*src*)B and M-MuLV(*src*)B1 cells.

M-MuLV(*src*⁺ *env*⁻) cells did not produce XC-positive virus, which is consistent with the inability of the virus in these cells to produce *env* glycoprotein. However, when these cells were superinfected with replication-competent

FIG. 4. Viral proteins from M-MuLV(*src*)-transformed cells. Transformed 14-11 cells were labeled with [³⁵S]methionine for 1 h and immunoprecipitated with rabbit anti-M-MuLV p30^{gag} (39). The immunoprecipitated material was then analyzed by electrophoresis on a 5-to-18% gradient SDS-polyacrylamide gel (31) and fluorography (33). For comparison, NIH 3T3 cells productively infected with wild-type M-MuLV (A9) are also shown. Lanes: A, immunoprecipitation of 14-11 cells; B, M-MuLV(*src*)B; C, A9; D, M-MuLV(*src*)B1. Mobilities of Pr65^{gag}, gPr80^{gag}, and mature p30 protein are indicated.

FIG. 5. Viral proteins from M-MuLV(*src*)-transformed cells. M-MuLV(*src*)B, NIH 3T3, and SR3T3 (NIH 3T3 cells transformed with RSV) cells were labeled with [35 S]methionine for 4 h and immunoprecipitated with rabbit anti-M-MuLV p30^{gag} or anti-*v-src*. The immunoprecipitated material was then analyzed by electrophoresis on a 10% SDS-polyacrylamide gel. Lanes: A and B, SR3T3 precipitated with anti-M-MuLV p30^{gag} and anti-*v-src*, respectively; C and D, M-MuLV(*src*)B precipitated with anti-*v-src* and anti-M-MuLV p30^{gag}, respectively; E and F, NIH 3T3 cells precipitated with anti-*v-src* and anti-M-MuLV p30^{gag}, respectively.

M-MuLV or amphotropic MuLV, an infectious transforming viral stock was produced at high titers (Table 2).

Characterization of virus-related proteins in transformed cells. The nature of virus-related proteins in M-MuLV(*src*)-transformed cells was analyzed by the immunoprecipitation of extracts from cells labeled with [35 S]methionine followed by SDS-PAGE. A *gag-src* fusion protein would be expected from the design of the clone. Since M-MuLV encodes two different *gag* polypeptide precursors, Pr65^{gag} and gPr80^{gag}, which differ by the presence of carbohydrate residues and also additional amino-terminal peptides on gPr80^{gag} (19), two *gag-src* fusion proteins might be expected in M-MuLV(*src*)-transformed cells. M-MuLV(*src*)B cells labeled for 1 h and immunoprecipitated with an anti-M-MuLV p30^{gag} serum indicated the presence of two novel *gag*-containing proteins of 78 and 90 kDa in addition to the *gag*-specific proteins characteristic of wild-type M-MuLV (Fig. 4). The 78-kDa protein would be an appropriate size for a fusion protein of the amino-terminal portion of M-MuLV Pr65^{gag} and the *src* sequences in pMLV(*src*). The 90-kDa protein was of an appropriate size to be a fusion of the amino terminus of M-MuLV gPr80^{gag} to *v-src*. The 90-kDa protein appeared to be unstable, since it was not detectable in experiments in which cells were labeled for longer periods of time.

The M-MuLV(*src*)B cells were also tested for the presence of *v-src*-related protein. Immunoprecipitation with an anti-*v-src* serum also identified a protein of 78 kDa that comigrated with the 78-kDa protein identified with anti-p30 serum (Fig. 5). This supports the conclusion that the 78-kDa

protein was a *gag-src* fusion product of the pMLV(*src*) genome. However, the 90-kDa *gag*-containing protein in M-MuLV(*src*)-transformed cells could not be immunoprecipitated with anti-*src* serum.

The nature of the 90-kDa *gag*-related protein was further investigated by protease analysis. [35 S]methionine-labeled 90-kDa protein was prepared from M-MuLV(*src*)B cells and partially digested with staphylococcal V8 protease, and the products were analyzed by SDS-PAGE. For comparison, labeled pp60^{src} from RSV-transformed NIH 3T3 cells was also analyzed. A major V8 peptide of 26 kDa was released from the 90-kDa protein, which comigrated with a 26-kDa V8 peptide from pp60^{src} (Fig. 6). The 26-kDa V8 peptide from pp60^{src}, characteristic of pp60^{src}, has been shown to originate from the carboxyl terminus of the protein (6, 11, 13). The V8 cleavage analysis therefore supports the hypothesis that the 90-kDa protein in M-MuLV(*src*)-transformed cells contains *src* as well as *gag* sequences.

Immune kinase and phosphoamino acid analysis of M-MuLV(*src*) transformed cells. As mentioned above, M-MuLV(*src*) was designed to contain the tyrosine-specific protein kinase activity of *v-src*, since the tyrosine kinase activity is necessary for transformation by RSV (52). M-

FIG. 6. V8 protease analysis of M-MuLV(*src*) 90-kDa protein. *gag*-containing 90-kDa protein from M-MuLV(*src*)B cells was isolated by preparative gel electrophoresis and subjected to gel staphylococcal V8 protease digestion (9). Lanes A through D: pp60^{src} digested with 0, 0.5, 0.05, and 0.005 μ g of V8 protease, respectively. Lanes E through H: 90-kDa protein digested with 0, 0.5, 0.05, and 0.005 μ g of V8 protease, respectively. Asterisks indicate the 26-kDa V8 peptide characteristic of the carboxy terminus of pp60^{src}.

FIG. 7. Assay for immune kinase activity. Unlabeled cellular extracts were immunoprecipitated with anti-*v-src* and anti-M-MuLV p30^{gag} sera according to the method of Sefton et al. (51). After incubation with [γ -³²P]ATP, the precipitates were subjected to SDS-PAGE and analyzed by autoradiography. For comparison of phosphorylated proteins with viral products, lanes A through C show standard immunoprecipitations of cells labeled for 4 h with [³⁵S]methionine as follows: A, SR3T3 immunoprecipitated with anti-*v-src* serum; B, M-MuLV(*src*)B immunoprecipitated with anti-*v-src* serum; C, M-MuLV(*src*)B immunoprecipitated with anti-M-MuLV p30^{gag} serum. Lanes D and E: immune kinase assays of M-MuLV(*src*)B reacted with anti-*v-src* and anti-M-MuLV p30^{gag} sera, respectively. Lanes F and G: immune kinase assays of M-MuLV(*src*)B1 reacted with anti-M-MuLV p30^{gag} and anti-*v-src* sera, respectively. Lanes H and I: immune kinase assays performed on SR3T3 cells precipitated with anti-*v-src* and anti-M-MuLV p30^{gag} sera, respectively. When M-MuLV(*src*)B and M-MuLV(*src*)B1 were precipitated with anti-*v-src* serum, phosphorylation occurred preferentially on the immunoglobulin G heavy chain. When the same two cell lines were reacted with anti-M-MuLV p30^{gag} serum, phosphorylation occurred preferentially on p78^{gag-src}, with low levels of phosphorylation on a 90-kDa protein.

MuLV(*src*)-transformed cells were tested for the presence of protein kinase activity by immune kinase assay. Cell extracts from M-MuLV(*src*)B cells were immunoprecipitated with anti-M-MuLV p30^{gag} or anti-*v-src* serum, the bound immunoprecipitates were incubated with [γ -³²P]ATP, and the labeled protein was analyzed by SDS-PAGE (Fig. 7). When anti-*v-src* serum was used as the antibody in the immune kinase assay, the immunoglobulin G heavy chain of the antibody was extensively labeled, and p78^{gag-src} was labeled to a minor degree (Fig. 7). The amount of kinase activity detected was considerable, especially in comparison with a parallel immune kinase assay on SR3T3 cells which contain wild-type RSV pp60^{src}. When anti-p30^{gag} serum was used in the immune kinase assay, phosphorylation was also observed. There was extensive phosphorylation of p78^{gag-src} in addition to the phosphorylation of the immunoglobulin G. There was also low but detectable phosphorylation of minor proteins, including the 90-kDa protein. These results strongly suggest that p78^{gag-src} contains the protein kinase activity expected of a *gag-src* fusion protein. Furthermore, the 90-kDa protein may be a substrate for phosphorylation or possibly a kinase with low activity. The immune kinase assay with anti-p30^{gag} serum suggested that immunoprecipitated M-MuLV Pr65^{gag} may also be a substrate for the kinase activity. The relatively greater extent of p78^{gag-src} phosphorylation observed when anti-p30^{gag} serum was used in the immune kinase assay instead of anti-*v-src* serum might reflect the physical distance between the kinase catalytic site in the *v-src* domain and the antibody binding site in the *gag*

sequences. Alternatively, the two antibodies may have contained different relative concentrations of immunoglobulin G subclasses which were available as kinase substrates.

Phosphoamino acid analysis was also performed on M-MuLV(*src*)-transformed cells, since cells transformed with wild-type pp60^{src} show increased levels of phosphotyrosine in protein (52). M-MuLV(*src*)B cells were labeled overnight with ³²P, the proteins were extracted and hydrolyzed to single amino acids, and the extract was analyzed by two-dimensional thin-layer electrophoresis. For comparison, M-MuLV-infected NIH 3T3 cells (A9) and wild-type RSV-transformed NIH 3T3 cells (SR3T3) were also analyzed. SR3T3 gave phosphotyrosine levels 10 times higher than those found in A9 cells (Fig. 8 and Table 3). M-MuLV(*src*)B cells showed a fourfold elevation in phosphotyrosine levels. Although this elevation was not as great as for cells transformed with wild-type pp60^{src}, it was still consistent with the notion of M-MuLV(*src*) encoding an active tyrosine kinase.

Inoculation of M-MuLV(*src*) into mice and tumor formation. M-MuLV(*src*) viruses, carried as pseudotypes with either M-MuLV or amphotropic MuLV helper, were inoculated intraperitoneally into neonatal NIH Swiss mice (1 to 2 days old) to test for tumorigenicity. The viral stocks were obtained from the M-MuLV(*src*⁺ *env*⁻) cells superinfected with either wild-type M-MuLV or amphotropic MuLV. These stocks were chosen for two reasons. These cell lines gave the highest titers of infectious focus-forming virus and allowed variation of the helper virus (Table 2). Amphotropic MuLV (1504A) has recently been reported to cause a mod-

FIG. 8. Phosphoamino acid analysis of M-MuLV(*src*)B. Cells were labeled overnight with [32 P]orthophosphate in phosphate-free medium. Cellular lysates were prepared and subjected to acid hydrolysis as described previously (51). The mixture was then subjected to two-dimensional electrophoresis on cellulose plates. Panels: A, M-MuLV(*src*)B; B, M-MuLV(*src*)B1; C, SR3T3; D, A9. Autoradiography indicated the presence of phosphoserine (pser), phosphothreonine (pthr), and phosphotyrosine (ptyr). The corresponding areas of the plate were removed and quantified by scintillation counting.

erate incidence of null and B-cell lymphomas as well as occasional hind-limb paralysis after 6 to 12 months (44); M-MuLV causes thymic lymphomas after 3 months (55). Inoculations were performed in neonatal mice since this procedure has been most effective in retroviral leukemogenesis, presumably because neonatal animals are not immunocompetent (55). Animals inoculated with M-MuLV(*src*) pseudotyped with either of these helper virus types developed palpable tumors after a latency period of approximately 3 to 6 weeks. In contrast, control animals inoculated either with M-MuLV or with amphotropic MuLV alone showed no outward signs of disease during the same time period. Mice injected with M-MuLV(*src*) pseudotyped with either helper virus began to die at approximately 6 weeks; by 16 weeks more than 90% of these animals had died, with approximately half of them dying between weeks 10 and 14 postinjection (Fig. 9). In contrast, mice injected with amphotropic MuLV alone showed no signs of overt disease at 6 months postinjection (Fig. 9A). Mice injected with M-MuLV alone showed no signs of overt disease until

10 weeks postinjection, after which all animals died during the next 8 weeks (Fig. 9B). Although all animals inoculated with M-MuLV alone eventually died, animals inoculated with M-MuLV(*src*) as an M-MuLV pseudotype died 2 weeks earlier.

As summarized in Table 4, individual animals inoculated with M-MuLV(*src*) in the amphotropic MuLV pseudotype demonstrated solid soft-tissue tumors at the site of injection. Solid tumors were observed in all 15 mice examined. The tumors were histopathologically diagnosed as malignant neoplasms of mesenchymal cells (sarcomas), with the predominant cell type in all 15 animals being fibroblastic (fibrosarcoma) (Fig. 10A and B). It was interesting that in addition to fibroblastic morphology, sarcomas from several animals exhibited myoblastic morphology in some areas (Fig. 10C and D). In most of the animals, extensive tumor metastases to other internal organs including liver, lungs (Fig. 10E), and spleen had occurred. No other neoplasms were observed in this group. Similar to animals inoculated with M-MuLV(*src*) pseudotyped with amphotropic M-MuLV, all 14 mice which had been inoculated with M-MuLV(*src*) pseudotyped with wild-type M-MuLV and which were available for pathologic examination also developed histologically identical solid soft-tissue sarcomas with distant metastases (as described above; Fig. 10A through E). In addition, 10 of these 14 animals also developed thymic lymphoblastic lymphoma (Fig. 10F) at the time of death. Lymphoblastic lymphoma is the characteristic disease caused by wild-type M-MuLV, and it is likely that the M-MuLV in the M-MuLV(*src*) stock caused this additional pathology. This hypothesis is supported by the fact that all 6 mice which were examined that had been inoculated with

TABLE 3. Relative abundance of phosphoamino acids

Cell line	Phosphoamino acid (% of total) ^a :		
	Phosphotyrosine	Phosphothreonine	Phosphoserine
A9	0.025	10.09	89.88
M-MuLV(<i>src</i>)B	0.110	10.29	89.60
M-MuLV(<i>src</i>)B1	0.097	9.37	90.52
SR3T3	0.250	10.06	89.76

^a Radioactivity is presented as a percentage of the total radioactivity recovered as phosphoamino acids.

FIG. 9. Cumulative time course of animal mortality (morbidity). (A) Solid circles indicate the time of death for individual mice inoculated with M-MuLV(*src*) (amphotropic MuLV helper). The dashed horizontal line indicates that none of the mice injected with amphotropic MuLV alone died during the same time period. (B) Solid circles indicate the time of death for individual mice inoculated with M-MuLV(*src*) (M-MuLV helper). Open circles indicate individual animals injected with M-MuLV alone.

M-MuLV alone developed histologically identical lymphoblastic lymphomas arising in and involving the thymus (T lymphocytes) (Fig. 10F).

DISCUSSION

These experiments report the generation of a new transforming retrovirus containing a portion of the *v-src* gene of ASV inserted into the genome of M-MuLV. Construction of this virus, M-MuLV(*src*), was accomplished by recombinant DNA cloning followed by transfection into M-MuLV *gag*- and *pol*-positive NIH 3T3 cells. Foci arose on the initially transfected cells, and infectious, transforming M-MuLV(*src*) could be rescued from cell lines isolated from these foci.

The design of pMLV(*src*) predicted a virus encoding an M-MuLV *gag*-ASV *src* fusion protein, with the *v-src* sequence containing the tyrosine kinase domain of *v-src*. Cells transformed by M-MuLV(*src*) produced two novel *gag*-containing proteins of 78 and 90 kDa. The 78-kDa protein (p78^{*gag-src*}) was the major protein and could also be immunoprecipitated with anti-*v-src* serum. p78^{*gag-src*} was of appropriate size to represent a fusion of the M-MuLV *gag* precursor polyprotein Pr65^{*gag*} with *v-src*. Immune kinase assays with either anti-*v-src* or anti-M-MuLV *gag* sera demonstrated the phosphorylation of p78^{*gag-src*} as well as the phosphorylation of immunoglobulin. This is consistent with p78^{*gag-src*} containing kinase activity, and it seems likely that this protein is the transforming protein of the virus.

The minor 90-kDa *gag*-containing protein in M-MuLV(*src*)-transformed cells was somewhat more difficult to characterize, since it was not immunoprecipitable with anti-*v-src* serum. However, the size of the 90-kDa *gag*-containing proteins was of appropriate size to represent a fusion between M-MuLV gPr80^{*gag*} and *v-src*. Indeed, partial V8 cleavage of the 90-kDa protein released a 26-kDa peptide which comigrated with the 26-kDa carboxy-terminal peptide of ASV pp60^{*src*}, identifying the presence of *src* sequences in this protein. The 90-kDa protein could be detected in M-MuLV(*src*)-transformed cells labeled for relatively short periods of time (1 h) but not in cells labeled for longer periods of time. This behavior is similar to that observed for the glycosylated M-MuLV *gag* polyprotein precursor gPr80^{*gag*} (19).

As described above, the design of M-MuLV(*src*) predicted a *gag-src* fusion protein, most likely p78^{*gag-src*}. This construction eliminated the amino-terminal 13-kDa region of ASV pp60^{*src*} while maintaining the catalytic kinase domain. Previous reports have shown that the amino-terminal membrane-binding portion of pp60^{*src*} is required for transformation and that small deletions or mutations result in a less-transformed phenotype. The cells transformed by altered forms of *src* are frequently fusiform (22, 47) and may show phosphotyrosine levels that are less elevated than in RSV-transformed cells (29). These and other parameters examined in these studies indicate an intermediate level of transformation induced by the mutant genes. It seems likely that for M-MuLV(*src*) the M-MuLV *gag* sequences in the *gag-src* fusion protein may provide a membrane-binding function, although not as effectively as the native *src* sequences. Indeed, the amino-termi-

TABLE 4. Pathology of malignant neoplasms induced in neonatal NIH Swiss mice inoculated with recombinant retrovirus

Virus ^a	Histopathologic type ^b	No. of animals with neoplasms (total no. of animals examined)
M-MuLV(<i>src</i>) with Am-MuLV	Fibrosarcoma	15 (15)
	Lymphoblastic lymphoma	0 (15)
M-MuLV(<i>src</i>) with M-MuLV	Fibrosarcoma	14 (14)
	Lymphoblastic lymphoma	10 (14)
M-MuLV alone	Fibrosarcoma	0 (6)
	Lymphoblastic lymphoma	6 (6)
Am-MuLV alone	NE ^c	NE

^a M-MuLV(*src*) was injected with either amphotropic (Am-MuLV) or Moloney (M-MuLV) helper.

^b Several animals in each group of mice exhibiting fibrosarcoma also exhibited myoblastic morphology in some areas of the sarcoma (see the text and Fig. 10C and D).

^c Not examined (NE) because animals in this group were still alive and well as of this writing (see the text).

FIG. 10. Histopathology of malignant neoplasms induced in NIH Swiss mice. (A and B) Fibrosarcoma appearing in an animal inoculated with M-MuLV(*src*) (amphotropic MuLV helper). Low-power view (A) shows the characteristic spindle-shaped appearance of malignant fibroblasts, which at a higher power (B) exhibit nuclear atypia. (C and D) Myosarcoma variant appearing in an animal inoculated with M-MuLV(*src*) (amphotropic MuLV helper). Low-power (C) and high-power (D) views show that the neoplastic mesenchymal cells have a considerable amount of cytoplasm characteristic of primitive myoblasts. This animal had other areas of tumor which were histologically identical to those seen in panels A and B (i.e., fibroblastic and myoblastic variants in the same animal). (E) Fibrosarcoma metastatic to lung in an animal inoculated with M-MuLV(*src*) (amphotropic MuLV helper). (F) Lymphoblastic lymphoma arising in and involving the thymus of an animal inoculated with M-MuLV alone. Note primitive chromatin and high mitotic rate. (All samples were stained with hematoxylin and eosin. A through D, soft tissue; E, lung; F, thymus. Bars: A, 33 μ m; B, 17 μ m; C, 33 μ m; D, 17 μ m; E, 170 μ m; F, 14 μ m. Magnifications: A, $\times 270$; B, $\times 540$; C, $\times 270$; D, $\times 540$; E, $\times 54$; F, $\times 630$.)

nal domain of Pr65^{gag}, the viral p15 protein, has been shown to associate with viral and cellular membranes (18).

It should be noted that M-MuLV(*src*)-transformed cells were fusiform, suggesting an intermediate level of transformation. Consistent with this, phosphotyrosine levels were elevated but not to the same extent as cells transformed by wild-type pp60^{src}. This might reflect a decreased transformation efficiency of the *gag-src* fusion protein compared with wild-type pp60^{src}, although the immune kinase assays showed high activity. It is also possible that p78^{gag-src} would not phosphorylate the same cellular substrates of pp60^{src}.

Transfection of pMLV(*src*) DNA into the *gag*⁺ *pol*⁺ 14-11 cells was essential in these experiments, since no foci were observed when the DNA was transfected into standard NIH 3T3 cells. The 14-11 cells were generated since it has been shown that high-efficiency transfection results from the cell-to-cell spread of infectious retrovirus (14). Strain 14-11 cells could provide the transacting *gag* and *pol* gene products deleted from pMLV(*src*), resulting in the production of infectious virus. The high transformation efficiency of the 14-11 cells upon transfection as opposed to NIH 3T3 cells suggests that focus formation by pMLV(*src*) in 14-11 cells involves the cell-to-cell spread of infectious transforming virus. The finding that pMLV(*src*⁺ *env*⁻) also formed foci on 14-11 cells (although at reduced frequency) was initially somewhat surprising, since virus particles released by the transfected cells would lack *env* glycoprotein. However, it is possible that the cell-to-cell spread of *env*-negative virus occurred when the cells were in close contact, as was true for the transfections described above. In fact, cells infected with retroviruses defective for *env* proteins can produce viral particles (2). Furthermore, infection of retroviruses in the absence of the appropriate *env* receptor can be mediated by cell fusion (25).

Inoculation of M-MuLV(*src*) into neonatal mice resulted in the development of solid soft-tissue sarcomas at or near the site of injection independent of the type of helper virus used. When amphotropic MuLV was used as the helper, the sarcomas were the only neoplasms observed. The tumors appeared after 3 weeks in all mice injected with M-MuLV(*src*) and were progressively lethal after 6 weeks. The short latency period is similar to those in other acutely transforming viruses (those carrying oncogenes). These tumors were the only neoplasms attributable to M-MuLV(*src*). A possible target cell for transformation in neonatally inoculated mice could be primitive mesenchymal myofibroblasts, which are capable of differentiation into myoblasts and fibroblasts (45) and are the most plentiful cells in neonatal mice. However, when wild-type M-MuLV was used as a helper, thymus-derived lymphoblastic lymphomas were also observed in 10 of the 14 mice examined. These data suggest that the helper and M-MuLV(*src*) acted independently to cause neoplasia.

The apparent failure of the recombinant M-MuLV(*src*) alone to cause lymphoid malignancies in inoculated mice was noteworthy. Recent experiments have implicated the enhancer sequences in retroviral LTRs as important determinants in disease specificity. Thus, one possibility is that M-MuLV(*src*), which contained a standard M-MuLV LTR, would cause thymic leukemia similar to that caused by wild-type M-MuLV. Another possibility is that M-MuLV(*src*) would cause B-cell lymphoma similar to that caused by Abelson MuLV (55). Abelson MuLV has several structural similarities to M-MuLV(*src*). It is derived from M-MuLV, and its transforming protein is a fusion between M-MuLV *gag* sequences and the *abl* oncogene

(e.g., P120^{gag-abl}). Furthermore, *v-abl* has considerable nucleic acid sequence homology and even some immunological cross-reactivity with *v-src* (30, 46, 54). Like pp60^{src}, P120^{gag-abl} is a tyrosine protein kinase (48). Nevertheless, no B-cell lymphomas were observed in M-MuLV(*src*)-infected animals. This was most evident in mice inoculated with the amphotropic MuLV pseudotype, since amphotropic MuLV has a very long latency period and a low frequency of pathogenicity.

Anderson and Scolnick (1) have also described the generation of an MuLV carrying the RSV *v-src* oncogene. Their virus differs from the M-MuLV(*src*) described here in several ways. The *v-src* gene was inserted into the *env* region of an amphotropic MuLV in such a fashion that wild-type pp60^{src} unattached to any MuLV protein was produced. In contrast to the intermediate level of transformation induced by M-MuLV(*src*) (i.e., cells with fusiform morphology, intermediate elevation of phosphotyrosine level, and failure to grow in agar suspension), the virus described by Anderson and Scolnick transformed cells to a high degree as evidenced by a highly rounded refractile morphology as well as growth in soft agar. The difference in the degree of transformation induced by these two viruses may reflect the nature of the transforming proteins (*gag-src* versus wild-type *src*).

The Anderson and Scolnick virus was initially reported to cause hematopoietic cell proliferation and splenomegaly upon its inoculation into 6-week-old NIH Swiss mice, although the mice did not develop clinical leukemia (5). The M-MuLV(*src*) described here did not cause any detectable splenomegaly or other disease when injected into 6-week-old mice. Recent experiments have indicated that the Anderson and Scolnick virus can infect and increase the growth potential of several different lineages of hematopoietic cells (including erythroid, myeloid, and lymphoid cells) upon in vitro infection of mouse bone marrow cultures (5, 41). It is very interesting that the inoculation of this virus into neonatal mice also results in the development of fibrosarcomas (41), which is similar to the results described here for M-MuLV(*src*). This suggests that the nature of the *src* oncogene, not the viral context in which it is found, is the primary determinant for the pathogenic potential of retroviruses carrying this oncogene.

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