New Mammalian Transforming Retrovirus: Demonstration of a Polyprotein Gene Product

ULF R. RAPP, FRED H. REYNOLDS, JR., AND JOHN R. STEPHENSON*

Laboratory of Viral Carcinogenesis, National Cancer Institute, Frederick, Maryland 21701

Received 22 March 1982/Accepted 15 November 1982

A new acute transforming type C retrovirus was isolated from mice inoculated with a virus stock obtained by iododeoxyuridine induction of methylcholanthrene-transformed C3H/10T1/2 mouse cells. This virus, designated 3611-MSV, transforms embryo fibroblasts and epithelial cells in culture and induces fibrosarcomas in vivo. 3611-MSV is replication defective, requiring a type C helper virus for propagation both in vitro and in vivo. By using endpoint transmission of 3611-MSV to MMCE C17 mouse and FRE 3A rat cells, several nonproductively transformed clonal cell lines have been derived. Pseudotype virus stocks obtained from such clones transform cells in vitro, are highly oncogenic in vivo, and exhibit host range and serological properties that are characteristic of their helper virus component. Analysis of viral antigen expression in 3611-MSV-transformed cells has led to the demonstration of a 90,000-molecular-weight (M_r) polyprotein and a 75,000- M_r probable cleavage product, both containing the amino-terminal murine leukemia virus *gag* gene proteins p15 and p12. In contrast to gene products of many previously described mammalian transforming viruses, 3611-MSV-encoded polyproteins lack detectable protein kinase activity, and 3611-MSV-transformed cells resemble chemically transformed cell line C3H/MCA-5, from which 3611-MuLV was originally derived, in that they do not exhibit elevated levels of phosphotyrosine. By using molecular hybridization the 3611-MSV transforming gene was found to be distinct from previously described mammalian cellular oncogenic sequences, including *c-ras*, *c-abl*, *c-fes*, *c-fms*, *c-sis*, and *c-mos*.

An important approach to studies of cellular regulatory pathways relevant to expression of the transformed phenotype involves the molecular cloning of cellular genetic sequences with transforming function. Type C viruses represent unique vectors for this purpose and in a number of instances have been used successfully for characterization of cellular transforming genes (11; G. Klein, ed., *Viral Oncology*, in press). This has been accomplished through studies of acute transforming viruses that arise naturally as a consequence of genetic recombination between the genomic RNAs of nontransforming retroviruses and transformation-specific cellular sequences (11; Klein, in press). Because of substitution of essential viral genes by host cellular sequences, such recombinant virus isolates are generally replication defective, requiring a helper type C virus for propagation both in vitro and in vivo. With a few possible exceptions, acquired cellular transforming genes, which are represented within the genomic RNAs of acute transforming retroviruses, are expressed as a result of translational readthrough initiated within preexisting viral genes. As a consequence, the products of such sequences

are in the form of polyproteins consisting of type C viral structural components covalently linked to acquired sequence-encoded transforming proteins (2, 3, 5, 20, 32, 33, 37). This finding has greatly facilitated purification and analysis of virus-encoded transforming proteins due to the immunological recognition of these proteins by antisera with specificities for virus structural components.

In the past, isolation of replication-defective transforming viruses of mammalian origin has been sporadic, occurring only in rare instances and generally from animals which developed spontaneous tumors or in which nontransforming retroviruses were maintained in continuous passage (31; Klein, in press). This has represented a major limitation to generalized use of type C viruses as vectors for molecular cloning of cellular genes. Recently, an improved, more systematic approach for the isolation of new transforming retroviruses from mouse cells has been described (18, 19). The system used for these tumor gene rescue experiments involves viruses obtained by iododeoxyuridine induction of chemically transformed C3H/10T1/2 cells (17). In this report we describe a new virus isolate

derived by this approach; this virus, designated 3611-MSV, encodes a 90,000-molecular-weight (M_r) polyprotein as its major translational product, transforms fibroblastic and epithelial cells in culture, and induces fibrosarcomas in newborn mice. C3H/MCA-5, the presumptive cell line of origin for 3611-MSV, resembles 3611-MSV-transformed cells with respect to both in vitro and in vivo properties, which is consistent with the possibility that the transforming sequences within the 3611-MSV genome may correspond to those responsible for the initial transformation of C3H/10T1/2 cells by methylcholanthrene.

MATERIALS AND METHODS

Cells and virus. The cells used were grown in the Dulbecco modification of Eagle medium supplemented with 10% calf serum (Colorado Serum Co., Denver, Colo.) and included a clonal line of methylcholanthrene-transformed C3H/10T1/2 cells (designated C3H/MCA-5) (17), a wild mouse line (MMCE C17) (16), a Fisher rat embryo line (FRE 3A) (33), and the highly contact-inhibited NIH/3T3 mouse embryo line (13). Snyder-Theilen and Gardner feline sarcoma virus (FeSV) nonproductively transformed FRE 3A clones (designated ST-FeSV FRE Cl10 and GA-FeSV FRE Cl14, respectively), a McDonough FeSV-transformed FRE 3A clone (McD-FeSV FRE Cl 3), an Abelson murine leukemia virus (MuLV)-transformed FRE 3A clone (AbLV-FRE Cl 2-9) (20), and murine leukemia virus (MuLV) stocks obtained by iododeoxyuridine activation of C3H/MCA-5 cells have been described previously (17). Additional nonproductively transformed rat cells used included simian sarcoma virus-transformed normal rat kidney cells (SSV NRK cells) (22) and Harvey sarcoma virus-transformed normal rat kidney cells (HaSV NRK cells) (10). Tumors induced in NFS/N mice were dispersed with 0.1% trypsin, and the cells were cocultivated with cell line NIH/3T3 by using previously described procedures (19). Viruses produced by these cultures were assayed for oncogenicity by intraperitoneal inoculation of newborn NFS/N mice and transmission to epithelial MMCE C17 and fibroblastic FRE 3A cells in culture (19).

Competition immunoassays. Competition immunoassays for MuLV structural proteins, including p15, p12, p30, p10, reverse transcriptase, and envelope glycoprotein (gp70), were performed by previously described methods (20).

Metabolic labeling, immunoprecipitation and SDS-PAGE. Cells were radioactively labeled by incubation for 2 h in medium containing 100 μ Ci of either [35 S]methionine (1,075 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) or 32 P_i (carrier-free; New England Nuclear Corp., Boston, Mass.) per ml. Immunoprecipitation and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) with 5 to 20% gradient gels were performed as previously described (34).

Protein kinase assay. Tissue culture cells (1.0×10^7 cells) were disrupted by repeated aspiration through a 25-gauge needle in 5 ml of 20 mM Tris-hydrochloride buffer (pH 7.2) containing 200 mM NaCl and 1.0% Triton X-100 (TBST buffer). Lysates were preabsorbed by adding 25 μ l of normal goat serum and 1.0

ml of a 10% suspension of protein A-Sepharose, incubating the preparation overnight at 4°C, and clarifying it by centrifugation at $100,000 \times g$ for 1 h. After 5 μ l of specific antiserum was added, lysates (1 ml) were incubated for 18 h at 4°C, 50 μ l of a 10% suspension of protein A-Sepharose was added, and incubation was continued for 2 h at 4°C. Immunocomplexes were collected by centrifugation for 10 min at $2,000 \times g$ and washed in TBST buffer. Protein kinase activity was assayed by suspending immunoprecipitates in TBST buffer containing 2 μ Ci of [γ - 32 P]ATP (2,000 Ci/mmol; New England Nuclear Corp.) adjusted to either 5 mM Mn²⁺ or 5 mM Mg²⁺ and incubating the preparation for 10 min at 30°C. Reactions were terminated by adding 3 ml of a solution containing ice-cold 10 mM sodium phosphate (pH 7.2), 100 mM NaCl, 1.0% Triton X-100, 0.5% deoxycholate, and 0.1% SDS (PBSTDS), and non-protein-bound 32 P was removed by repeated centrifugation in excess PBSTDS. After the final wash, immunoprecipitates were analyzed by SDS-PAGE as described above.

Phosphoamino acid analysis. Virus-encoded proteins were labeled by incubation in medium containing 32 P_i, purified by immunoprecipitation and SDS-PAGE, and subjected to phosphoamino acid analysis as previously described (21). In addition, the phosphotyrosine content of total 32 P_i-labeled cellular proteins was measured and expressed as a percentage of total phosphoamino acids by using previously described procedures (21).

Isolation of cellular DNA. Cells suspended in $1 \times$ SSC ($1 \times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) were lysed by incubation for 2 h at 37°C in $1 \times$ SSC containing 0.5% SDS and 100 μ g of proteinase K (E. Merck AG, Darmstadt, Germany) per ml. The DNA was then purified as previously described (15).

Gel electrophoresis, blotting, and hybridization of blots. DNA was electrophoresed in 0.8 or 1.0% agarose gels (Seakem; ME grade) by using a Tris-acetate buffer (pH 7.8) (26). Restriction fragments of λ and ϕ X174 DNAs were used as size markers. 32 P-labeled markers were made by using avian myeloblastosis virus DNA polymerase to fill in the ends of *Hind*III fragments of λ and *Taq*I fragments of ϕ X174 (36). After electrophoresis, DNA was transferred from gels onto genescreen (New England Nuclear Corp.) as previously described (15). Nick-translated probes were prepared with cloned DNA and hybridized to Genescreen membranes containing the blotted DNA (15).

RESULTS

Isolation of 3611-MSV: a new mouse transforming virus. A previously developed regime was used in an effort to obtain new acute mammalian transforming virus isolates (19). Newborn NFS/N mice were inoculated intraperitoneally with a stock of MuLV obtained by iododeoxyuridine activation of methylcholanthrene-transformed C3H/10T1/2 cell line C3H/MCA-5 (17), and 6 weeks later these mice were treated with a single dose of butylnitrosourea (3 mg/mouse). When they were 3 to 4 months old, several of the inoculated animals developed lung adenocarcinomas and histiocytic lymphomas.

TABLE 1. Biological properties of 3611-MSV^a

Cell line	Type C helper virus	Virus titer (log ₁₀ /ml)		Fibrosarcoma induction in vivo	
		PIU	FFU	Incidence	Latency (weeks)
FRE 3A	4070-A	6.0	<0.5	0/4	>36
3611-FRE Ac1		<0.5	<0.5	NT ^b	
	4070-A	6.0	4.8	16/16	4
3611-FRE Ac12	4070-A	5.0	3.7	NT	
MMCE C17	4070-A	<0.5	4.2	0/20	>36
3611-MMCE Ac2		<0.5	<0.5	NT	
	4070-A	6.0	4.7	12/12	3
3611-MMCE Ac8	4070-A	5.0	3.1	6/6	4

^a Cell lines were infected with 4070-A MuLV helper virus at a high multiplicity, and 2 weeks later culture fluids were filtered and assayed for transmission to FRE 3A rat cells, as measured by polymerase-inducing units (PIU) per milliliter, focus-forming units (FFU) per milliliter, and tumorigenicity in newborn NFS/N mice, using previously described procedures (19).

^b NT, Not tested.

After three sequential cycles of transplantation of one such tumor in weanling mice, cell-free tumor extracts were prepared; these extracts induced histiocytomas, erythroleukemias, and fibrosarcomas in newborn mice at efficiencies of 100% and with latency periods of less than 4 weeks. Infiltrates of these tumors were observed in spleens, kidneys, lungs, and, occasionally, lymph nodes. In the enlarged spleens the infiltrating cells consisted primarily of collagen-producing fibroblasts, histiocytes, erythroblasts, and granulocytes.

To characterize further the filterable agent associated with induction of the tumors described above, a cell-free extract of one tumor was prepared and cocultivated with in vitro propagated NIH/3T3 mouse embryo fibroblasts. After 2 weeks of incubation, culture fluids were harvested and assayed for biological activity. In vivo inoculation of filtered culture fluids into newborn NFS/N mice resulted in 100% frequency of fibrosarcoma induction, and after in vitro transmission to NIH/3T3 assay cells, 4 to 5 logs of focus-forming activity were observed. By using endpoint dilution, transmission of filtered culture fluids to MMCE C17 mouse (epithelial) and FRE 3A rat (fibroblastic) cells, and subsequent cloning in soft agar, we obtained several nonproductively transformed cell lines. Superinfection of such clones with 4070-A helper virus resulted in the generation of pseudotype virus stocks which transformed FRE 3A rat and MMCE C17 mouse cells in culture and induced fibrosarcomas upon intraperitoneal inoculation in vivo (Table 1).

Expression of MuLV p15 and p12 in 3611-MSV-transformed cells. To identify translational products of the newly isolated acute transforming virus, nonproductively transformed MMCE C17 mouse cells and FRE 3A rat cells were

analyzed in competition immunoassays for individual type C viral *gag*, *pol*, and *env* gene translational products. As Table 2 shows, each of two independently derived 3611-MSV-transformed MMCE C17 mouse clones expressed readily detectable levels of the amino-terminal MuLV *gag* gene proteins p15 and p12. In contrast, MuLV p30, p10, and reverse transcriptase were undetectable, and gp70 envelope glycoprotein activity was relatively low. Similarly, a large number of independently derived 3611-MSV-transformed FRE C13 rat clones expressed p15 and p12 and low levels of gp70 in the absence of detectable p30, p10, or reverse transcriptase reactivity. Moloney MuLV-infected MMCE C17 cells were highly reactive in immunoassays for each of the four *gag* gene-encoded proteins, reverse transcriptase, and gp70, whereas uninfected control MMCE C17 and FRE 3A cells lacked detectable reactivity in all six assays. These findings establish that the two amino-terminal MuLV *gag* gene products, p15 and p12, are translational products of the replication-defective 3611-MSV genome.

MuLV p15 and p12 in 3611-MSV-transformed cells are associated with a polyprotein. We undertook studies to characterize further the MuLV cross-reactive antigenic determinants in 3611-MSV-transformed cells and, in particular, to examine the possible association of these determinants with high-molecular-weight polyproteins, such as those expressed in cells transformed by many other retroviruses (31). To do this, extracts of 3611-MSV nonproductively transformed MMCE C17 mouse cells were subjected to molecular size analysis under denaturing conditions (6 M guanidine hydrochloride), and individual column fractions were tested for MuLV p12 reactivity. More than 60% of the p12 reactivity chromatographed at a molecular

TABLE 2. Expression of type C virus structural proteins in cells nonproductively transformed by 3611-MSV

Cells		Viral protein expression (ng of viral protein per mg of total cellular protein) ^a					
Type	Cell line	p15	p12	p30	p10	Reverse transcriptase	gp70
Fisher rat embryo							
Control	FRE 3A	<10	<2	<2	<10	<20	<50
3611-MSV transformed	3611-FRE Ac1	560	620	<2	<10	<20	110
	3611-FRE Ac3	620	670	<2	<10	<20	180
	3611-FRE Ac12	490	580	<2	<10	<20	130
Wild mouse							
Control	MMCE C17	<20	<10	<10	<20	<50	<50
3611-MSV transformed	3611-MMCE Ac8	1,860	1,670	<10	<20	<50	110
	3611-MMCE Ac2	1,910	1,812	<10	<20	<50	90
Moloney MuLV infected	MMCE C17 (M-MuLV)	3,620	4,920	5,620	2,900	350	6,200

^a Cellular extracts were tested at serial twofold dilutions in heterologous immunoassays by using goat antisera against detergent-disrupted Moloney MuLV for precipitation of ¹²⁵I-labeled Rauscher MuLV p15, p12, p30, p10, reverse transcriptase, and gp70 (20). The results are based on extent of displacement compared with known protein standards and represent mean values from three separate determinations. Positive values are enclosed in boxes.

weight of around 70,000 to 90,000, with smaller amounts of reactivity at a molecular weight of 25,000 (Fig. 1A). In other experiments, extracts were prepared in buffer containing 1% Triton X-100 and incubated for 30 min at 37°C before 6 M guanidine hydrochloride was added. When these disruption conditions were used, only minor peaks of reactivity were observed at 70,000 to 90,000 and 25,000 M_r , and there was a major p12 peak at 12,000 M_r (Fig. 1B). An analysis of the same column fractions for MuLV p15 revealed peaks of reactivity at 70,000 to 90,000, 25,000, and 15,000 M_r (data not shown). Thus, the MuLV p15 and p12 reactivities in 3611-MSV-transformed cells appeared to be associated with one or more polypeptides in the 70,000- to 90,000- M_r range; these polypeptides were susceptible to cleavage in extracts, giving rise to a 25,000- M_r intermediate cleavage product containing p15 and p12 and finally to p15 and p12 in their fully processed forms.

To define more accurately the 3611-MSV-encoded polypeptides described above, cells were metabolically labeled by overnight incubation in [³⁵S]methionine-containing medium and subjected to an immunoprecipitation-SDS-PAGE analysis. As shown in Fig. 2, lane B, immunoprecipitation of 3611-MSV nonproductively transformed FRE 3A cells with a high-titer goat anti-Moloney MuLV serum and subsequent analysis by SDS-PAGE revealed major protein bands at molecular weights of approximately 90,000 and 75,000. Both of these polypeptides were immunoprecipitable by goat antisera with specificity for MuLV p12 (Fig. 2, lane A), p15, and, to a lesser degree, p30 (data not shown), but neither was immunoprecipitable by control goat serum (Fig. 2, lane C) or by goat antiserum with specificity for MuLV p10. Analogous pro-

teins were observed in 3611-MSV-transformed mouse cells after immunoprecipitation by goat anti-Moloney MuLV (Fig. 2, lane F) but not after immunoprecipitation by control goat serum (Fig. 2, lane G). Combined with the absence of detectable levels of these polypeptides in immunoprecipitates of uninfected control FRE 3A rat cells (Fig. 2, lanes D and E) and MMCE C17 mouse cells (Fig. 2, lanes H and I), these findings established the viral specificity of these proteins. Each of the 3611-MSV-transformed clones also exhibited low levels of envelope protein, gp70, and P15 E expression (data not shown).

Lack of P90 and P75 expression in morphological revertants of 3611-MSV-transformed cells. To determine whether expression of the above-described 3611-MSV-encoded polypeptides is associated with expression of the transformed phenotype, we attempted to isolate spontaneous morphological revertants of 3611-MSV-transformed cells. Upon continuous in vitro passage of several transformed clones, nontransformed variants appeared at a low frequency. A total of six morphological revertants of one such clone, 3611-FRE Ac5, were isolated by endpoint dilution in microtiter plates and analyzed for viral antigen expression. As Fig. 3 shows, none of the six clones exhibited detectable P90 or P75 expression (Fig. 3, lanes B through F), in contrast to a control 3611-MSV clone, which expressed both virus-encoded polypeptides at high levels (lane A). The possibility that the lack of detectable P90 and P75 expression reflected loss of the complete 3611-MSV genome rather than a specific block to expression of these viral gene products was excluded by the rescue of 3611-MSV transforming pseudotype virus from such cells after superinfection with 4070-A helper

FIG. 1. Molecular size analysis of MuLV gag gene-encoded proteins expressed in 3611-MSV nonproductively transformed mouse cell clone 3611-MMCE Ac8. Cell extracts (10 mg) were prepared and either immediately (A) or after 30 min of incubation at 37°C (B) subjected to agarose gel filtration (Bio-Gel A-15 m) in the presence of 6 M guanidine hydrochloride (20). Individual column fractions were tested in a heterologous competition immunoassay for MuLV p12 as described in Table 1, footnote *a*. The Molecular weight markers used included tracer amounts of ^{125}I -labeled bovine serum albumin (molecular weight, 69,000), Rauscher MuLV p30 (30,000), Rauscher MuLV p12 (12,000), and free iodine (V_e). V_o , Void volume.

virus. Moreover, several of the clones continued to exhibit significant levels of envelope glycoprotein expression.

3611-MSV polyprotein lacks detectable tyrosine-specific protein kinase activity. Polyprotein gene products of many previously described acute transforming viruses are highly phosphorylated and exhibit either associated or intrinsic protein kinase activities with specificity for tyrosine residues (Klein, in press). Cells transformed

by such viruses have significantly (8 to 10-fold) elevated overall levels of phosphotyrosine. In contrast, when analyzed under similar conditions, none of six independently derived chemically transformed clones of C3H/10T1/2 cells, including C3H/MCA-5 (Table 3), exhibited elevated phosphotyrosine levels. Similarly, phosphotyrosine levels in cells transformed by 3611-MSV were not significantly greater than phosphotyrosine levels in nontransformed control cells. Gardner FeSV-transformed FRE 3A cells included as positive controls showed the expected 8- to 10-fold elevation of phosphotyrosine levels (Table 3).

To test more directly 3611-MSV polyproteins for protein kinase activity, P90 and P75 were immunoprecipitated from 3611-MSV-transformed rat cells and tested for $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ phosphotransferase activity in vitro. As a positive control, Snyder-Theilen FeSV P85^{gag-fes}, a known tyrosine-specific protein kinase (2, 21, 34), was analyzed in parallel. As Fig. 3 shows, when conditions resulting in efficient phosphorylation of Snyder Theilen P85^{gag-fes} were used, no ^{32}P labeling of either 3611-MSV P90 or 3611-MSV P75 was detected. In other experiments Mg^{2+} was substituted for Mn^{2+} or $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ was used in place of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. None of these conditions resulted in in vitro phosphorylation of the 3611-MSV gene products. Finally, to determine whether 3611-MSV gene products are phosphorylated in vivo, cells were metabolically labeled by incubation for 2 h in $^{32}\text{P}_i$ -containing medium and subjected to immunoprecipitation-SDS-PAGE analysis. A weakly phosphorylated protein band at 75,000 M_r immunoprecipitable by anti-MuLV p15 and p12 but not by normal goat serum was observed (Fig. 4). A phosphoamino acid analysis of this protein indicated that it consisted predominantly of phosphoserine and a minor amount of phosphothreonine but lacked detectable phosphotyrosine (Fig. 5).

Immunological type specificity of 3611-MSV p12 corresponds to that of an amphotropic MuLV strain, 4070-A. The findings described above demonstrate that 3611-MSV represents a genetic recombinant between MuLV and transformation-specific sequences which, by analogy to other acute transforming viruses, are of probable mouse cellular origin. For further characterization of this new transforming virus, it was important to identify the parental strain of MuLV involved in the original recombinational event that led to generation of 3611-MSV. Because of their high degree of type specificity, competition immunoassays for the p12 component of the 3611-MSV-encoded polyproteins provided a means of resolving this problem. Extracts of 3611-MSV nonproductively transformed mouse and rat cells were tested and

FIG. 2. Immunoprecipitation and SDS-PAGE analysis of high-molecular-weight polypeptides expressed in MMCE C17 mouse cells and FRE 3A rat cells nonproductively transformed by 3611-MSV. Cell clones, including 3611-FRE Ac1 (lanes A through C), FRE 3A (lanes D and E), 3611-MMCE Ac8 (lanes F and G), and MMCE C17 (lanes H and I), were metabolically labeled in [35 S]methionine-containing medium (100 μ Ci/ml) for 30 min. immunoprecipitated by using hyperimmune goat antiserum directed against Moloney MuLV p15 (lanes A, D, F, and H), or Moloney MuLV p12 (lane B) or by using control goat serum (lanes C, E, G, and I), and analyzed by SDS-PAGE. The molecular weight standards used included 14 C-labeled myosin (molecular weight, 200,000 [200K]), phosphorylase B (98,000), ovalbumin (46,000), carbonic anhydrase (30,000), and cytochrome c (12,000).

shown to react efficiently in a homologous immunoassay for 4070-A MuLV p12 (Fig. 6) but to lack detectable competing activity in immunoassays with specificity for the p12 structural components of xenotropic and ecotropic type C viruses (data not shown). In contrast to 4070-A amphotropic MuLV, which reacted efficiently and at high titer in the homologous 4070-A MuLV p12 assay, representative ecotropic and xenotropic MuLV isolates were only weakly reactive.

Hybridization of 3611-MSV proviral DNA in nonproducer rat cells with mammalian oncogene probes. DNA from nonproducer-transformed rat cells was digested with several restriction endonucleases, and Southern blots of digested cellular DNA were hybridized with each of six representative viral oncogene DNA probes. Figure 7 shows the hybridization of cellular DNA doubly digested with *Eco*RI and *Bam*HI (Fig. 7A through E) or *Bam*HI alone (Fig. 7F) and shows that only the endogenous homologs of *v-ras*, *v-abl*, *v-fes*, *v-fms*, *v-sis*, and *v-mos* were present in 3611-MSV-transformed and control FRE 3A cells. In contrast, cellular DNAs from FRE 3A cells transformed by five of the six different retroviruses showed one or more additional DNA restriction fragments which hybridized with their respective homologous oncogene probes. In the case of *v-mos*, nonproducer-

FIG. 3. Analysis of P90 and P75 expression in 3611-FRE Ac5 morphological revertants. Cell lines, including 3611-FRE Ac5 (lane A) and each of six independently isolated morphological revertants (lanes B through F), were metabolically labeled in [35 S]methionine-containing medium, immunoprecipitated by using hyperimmune goat antiserum to MuLV p12 and analyzed by SDS-PAGE as described in the legend to Fig. 2.

TABLE 3. Phosphotyrosine levels in 3611-MSV-transformed cells and in C3H/MCA-5, the cell line from which 3611-MSV was derived^a

Cell line	Transforming agent	Growth in soft agar (%)	Phosphotyrosine level (% of total phosphoamino acids)
FRE 3A		<0.01	<0.2
GA-FeSV FRE C14	GA-FeSV	16	1.8
AbLV-FRE C12	Abelson MuLV	22	1.6
3611-FRE Ac1	3611-MSV	21	<0.2
3611-FRE Ac8	3611-MSV	19	<0.2
C3H/10T1/2		<0.01	<0.2
C3H/MCA-5		21	<0.2

^a Growth of colonies in soft agar and phosphotyrosine levels (expressed as percentages of total cellular phosphoamino acids) were determined as previously described (21).

transformed FRE 3A cells were not available as a positive control; however, this probe did readily detect the viral genome in addition to *c-mos* homologs in DNA from nonproducer-transformed mink cells (data not shown). The relatedness between 3611-MSV and the *ras* family of viral oncogenes was tested by using a subgenomic restriction fragment of Harvey-*ras* (BS9)

as a probe; this fragment has previously been shown to share sequence homology with Kirsten-*ras*, the Kirsten isolate of murine sarcoma virus (10). More recently, we have confirmed and extended the experiments described here by hybridizing molecularly cloned 3611-MSV DNA to the same panel of mammalian retroviral oncogenes (data not shown).

DISCUSSION

The mouse system represents an important model for studies of malignant transformation. This is primarily due to the availability of well-characterized inbred strains and the amenability of mice to in vivo experimentation. In addition, several well-characterized in vitro carcinogen transformation systems in which mouse cell lines are used have been developed (22, 23). In the past, however, studies of acute transforming viruses of mouse origin have been restricted to a relatively limited number of isolates. In this study, we describe the isolation and characterization of a new acute transforming virus. This newly generated virus, 3611-MSV, represents a genetic recombinant between a mouse type C virus analogous to 4070-A (Moloney MuLV p12 specificity) and as-yet-undefined transformation specific sequences of probable mouse cellular origin. 3611-MSV resembles previously described mammalian transforming viruses in that

FIG. 4. Comparison of Snyder-Theilen FeSV- and 3611-MSV-transformed cells with respect to phosphorylation and associated protein kinase activity. Cell lines, including 3611-FRE Ac1 (lanes A through C) and ST-FeSV C110 (lanes E through G), were metabolically labeled in either [³⁵S]methionine-containing medium (lanes A, B, E, and F) or ³²P_i-containing medium (lanes C and G), immunoprecipitated by using goat antiserum directed against MuLV p12 (lanes A, C, E, and G) or control goat serum (lanes B and F), and analyzed by SDS-PAGE as described in the legend to Fig. 2. In addition, unlabeled 3611-FRE Ac1 (lane D) and ST-FeSV FRE C110 (lane H) extracts were immunoprecipitated by using either goat antiserum against MuLV (lane D) or anti-feline leukemia virus serum (lane H). Protein kinase reactions were performed as described in the text and were analyzed by SDS-PAGE. The molecular weight standards used were the ones described in the legend to Fig. 2.

FIG. 5. Phosphoamino acid analysis of 3611-MSV P75 phosphorylated in vivo. 3611-MSV P75 was labeled with $^{32}\text{P}_i$, immunoprecipitated, and purified by SDS-PAGE as described in the legend to Fig. 4. Phosphoamino acid analysis was performed as described in the text. The positions of unlabeled phosphoamino acid standards, including phosphothreonine (p-thr), phosphotyrosine (p-tyr), and phosphoserine (p-ser), are indicated.

it is replication defective, requiring an appropriate type C helper virus for its propagation. In vitro, 3611-MSV transforms embryo fibroblast, whereas upon intraperitoneal in vivo inoculation it induces fibrosarcomas.

The major 3611-MSV translational product is identified as a 90,000- M_r polypeptide containing p15 and p12 structural components and a non-structural, acquired, sequence-encoded component. The identification of this viral gene product was possible due to the recognition of its virus structural (p15 and p12) components by available serological reagents. A second immunologically related polypeptide of approximately 75,000 M_r represents a probable posttranslational cleavage product of P90. Of the two proteins, P75 is weakly phosphorylated predominantly in serine residues in 3611-MSV-transformed cells. Thus, if P75 is a cleavage product of P90, posttranslational phosphorylation must occur after cleavage of P90 in the generation of P75. Although 3611-MSV P90 has not been directly analyzed for transforming function, structurally analogous polypeptide gene products of other mammalian viruses are known to be directly involved in expression of the transformed phenotype. Moreover, morphological reversion of 3611-MSV-transformed cells to a non-transformed phenotype is associated with specific loss of expression of both P90 and P75.

Although the rate of isolation of new transforming retrovirus isolates is rapidly increasing,

many of these share analogous cell-derived oncogenic sequences (6). For instance, the Harvey murine sarcoma virus *v-ras* gene and the mouse cell-derived *v-bas* gene correspond to analogous genetic loci within their respective species of origin (1), as do the *v-fes* genes common to Gardner FeSV and Snyder-Theilen FeSV and the *v-fps* gene, which is presented within the genomes of several avian type C virus isolates (27). Similarly, many avian type C viral oncogenes other than those of the *v-fps* family have been shown to fall into distinct groups and to represent multiple isolates of the same cellular gene (6). More recently, two new transforming retroviruses of cat origin have been shown to contain oncogenic sequences corresponding to *v-abl* (P. Besmer, personal communication) and *v-sis* (P. Besmer, W. D. Hardy, Jr., and E. E. Zuckerman, personal communication). Finally,

FIG. 6. Analysis of 3611-MSV polypeptides in a homologous type-specific immunoassay for 4070-A MuLV p12. Unlabeled detergent-disrupted viruses (50 $\mu\text{g}/\text{ml}$) and cell extracts (3 mg/ml) were tested at serial twofold dilutions for the ability to compete with ^{125}I -labeled 4070-A MuLV p12 for binding limiting amounts of antibody to detergent-disrupted 4070-A MuLV. Results are expressed as mean values from two separate determinations and are normalized to 100% maximal binding at infinite antigen dilution. The viruses tested as competing antigens included 4070-A MuLV ($\square$), AKR-MuLV (Δ), and NZB-MuLV (∇). The cell extracts used included 3611-FRE Ac1 ($\bullet$), 3611-MMCE Ac8 ($\circ$), and FRE 3A ($\otimes$).

FIG. 7. Southern blot analysis of 3611-MSV nonproducer cellular DNAs (lanes 2). The negative control DNA used was FRE 3A DNA (lanes 1), and positive controls (lanes 3) were included in the blots. (A) HaSV NRK cells (10). (B) AbLV-FRE Cl 2-9 cells (20). (C) ST-FeSV FRE Cl 3 cells (33). (D) McD-FeSV FRE Cl 3 cells (33). (E) SSV NRK cells (24). Note that DNAs from nonproductively transformed FRE 3A cells were used as positive controls, with the exception of *v-mos*, for which we used DNA from mink $S^+ L^-$ cells. After sequential digestion with restriction endonucleases *Eco*RI and *Bam*HI (A through E) or *Bam*HI alone (F), DNAs were electrophoresed in 1% agarose. The DNA was transferred to Genescreen (New England Nuclear Corp.) and hybridized with the following nick-translated oncogene-specific probes purified by gel electrophoresis: *v-ras*, a 0.6-kilobase *Eco*RI restriction fragment of Harvey murine sarcoma virus DNA (BS9) (10) (A); *v-abl*, a 0.8-kilobase restriction fragment of Abelson MuLV DNA (K5) (30) (B); *v-fes*, a 0.5-kilobase *Pst*I restriction fragment of Snyder-Theilen FeSV DNA prepared by digestion of the pBR322 subclone with *Pst*I (S_L) (12) (C); *v-fms*, a 0.45-kilobase *Pst*I restriction fragment of McDonough FeSV DNA (PSM 7c) (9) (D); *v-sis*, a 1.0-kilobase *Pst*I-*Xba*I restriction fragment of simian sarcoma virus DNA (24) (E); *v-mos*, a 1.25-base pair *Pst*I fragment of Moloney murine sarcoma virus DNA (pHT8) (35) (F).

transforming genes directly isolated from human bladder and lung carcinoma cell lines have been shown to correspond to the human Harvey-*ras* and Kirsten-*ras* genes, respectively (8, 14, 25). In contrast, 3611-MSV contains oncogenic sequences which do not appear to correspond to sequences of previously described mammalian oncogenes, including *v-fes*, *v-fms*, *v-abl*, *v-sis*, *v-ras*, and *v-mos*. Although possible relatedness to *v-fos* or any of the avian oncogenes has not been examined yet, it appears very probable that

3611-MSV contains a new mammalian oncogene. Thus, although the number of cellular oncogenes may be more limited than initially realized, there probably are additional such genes which have not been identified yet.

There is accumulating evidence indicating that transformation of C3H/10T1/2 cells by chemical carcinogens involves activation of a single set of cellular genomic sequences (7, 28, 29). This hypothesis is based on the finding that the transforming sequences of independent chemi-

cally transformed C3H/10T1/2 cell lines, including C3H/MCA-5, exhibit similar patterns of susceptibility and resistance to various restriction enzymes. As shown in this study, C3H/MCA-5, the cell line from which the virus stock giving rise to 3611-MSV was originally derived, does not have the elevated levels of phosphotyrosine characteristic of many virus-transformed cell lines (J. R. Stephenson and G. J. Todaro, *Adv. Viral Oncol.*, in press). Similarly, 3611-MSV-induced transformation is not associated with an overall elevation of cellular phosphotyrosine levels, and the major polyprotein gene product of this virus lacks detectable protein kinase activity and is not phosphorylated in tyrosine in vivo. Combined with the similar morphology and in vivo tumorigenicity of C3H/MCA-5 cells compared with 3611-MSV-transformed cells, these findings suggest that the 3611-MSV transforming gene may correspond to sequences initially responsible for C3H/MCA-5 transformation. It will be important to test this possibility and to determine whether the transforming gene represented within the 3611-MSV genome corresponds to transforming sequences identified by transfection of C3H/MCA-5 DNA.

ACKNOWLEDGMENTS

We thank the following individuals for kindly providing molecular clones of v-onc DNA: C. J. Sherr (*v-fes* and *v-fms*), P. Reddy (*v-abl*), K. C. Robbins (*v-sis*), E. H. Chang (Kirsten-*ras*), R. W. Ellis (Harvey-*ras*), and T. Wood (*v-mos*). We also acknowledge the excellent technical assistance of P. Borchert and Mark Gunnell.

This work was supported by Public Health Service contract NOI-CO-75380 from the National Cancer Institute.

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