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Gene Products of McDonough Feline Sarcoma Virus Have an In Vitro-Associated Protein Kinase That Phosphorylates Tyrosine Residues: Lack of Detection of This Enzymatic Activity In Vivo

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The primary translational product of the McDonough (SM) strain of feline sarcoma virus (FeSV) is a 180,000-dalton molecule, SM P180, that contains the p15-p12-p30 region of the FeLV *gag* gene-coded precursor protein and a sarcoma virus-specific polypeptide. In addition, cells transformed by SM-FeSV express a 120,000-dalton molecule, SM P120, that is highly related to the non-helper virus domain of SM P180. Both SM-FeSV gene products were found to be intimately associated with the membrane fraction of SM-FeSV-transformed cells. Immunoprecipitates containing SM P180 and SM P120 exhibited a protein kinase activity capable of phosphorylating tyrosine residues of both viral gene products but not immune immunoglobulin G molecules. By independently immunoprecipitating each of the two SM-FeSV proteins we found that most of the tyrosine-specific phosphorylating activity was associated with the SM P120 molecule. In vivo analysis of ^{32}P-labeled SM P180 and SM P120 revealed their phosphoprotein nature; however, both molecules exhibited low levels of phosphorylation and did not contain phosphotyrosine residues. Finally, we did not detect any significant elevation in the levels of phosphotyrosine in the protein fraction of SM-FeSV transformants. Thus, if SM-FeSV were to induce malignant transformation by a mechanism involving phosphorylation of tyrosine residues, the viral gene products must interact with a small subset of cellular proteins that do not represent a significant fraction of the total cellular protein content.

The McDonough strain (SM) of feline sarcoma virus (FeSV) was isolated in association with a feline leukemia virus (FeLV) from a pleomorphic fibrosarcoma of an 18-month-old female domestic cat (25). Like other feline sarcoma viruses (18, 29, 35), SM-FeSV is replication defective, requiring the presence of a helper type C virus for its propagation (29). Recently, the isolation of clonal cells nonproductively transformed by SM-FeSV (29) has allowed the characterization of the viral genome and its translational products (3, 13, 34, 40). The SM-FeSV genome is a recombinant between FeLV and a subset of cat cellular sequences that directs the synthesis of two high-molecular-weight proteins, designated SM P180 and SM P120. SM P180 is composed of most, if not all, of the FeLV *gag* gene-coded precursor protein and a sarcoma virus-specific polypeptide. In contrast, SM P120 does not exhibit any detectable FeLV-specific antigenic determinants (3). Structural studies have indicated that the sarcoma virus-specific domain of SM P180 possesses all the methionine-containing tryptic peptides of SM P120, suggesting that both polypeptides may be translated from the same subset of viral sequences (3).

Two other isolates of FeSV, the Snyder-Theilen (ST) (39) and Gardner-Arnstein (GA) (14) strains, have been recently characterized. Current evidence favors the concept that these two strains arose by recombination of different FeLV's with the same subset of cat cellular sequences (3, 4, 11a, 13, 33). In contrast, the sarcoma virus-specific sequences of SM-FeSV shared no detectable homology with those of either ST- or GA-FeSV (13). Moreover, neither SM P120 or the sarcoma virus-specific domain of SM P180 shared detectable antigenic cross-reactivity with the corresponding regions of the ST- and GA-FeSV gene products (3, 34). Thus, the transforming gene of SM-FeSV appears to have originated from an unrelated subset of cellular genetic information.

The gene products of acute transforming retroviruses, including the Rous, Fujinami, PRC II, and Y73 strains of avian sarcoma virus, the Abelson strain of murine leukemia virus, and the

ST and GA strains of FeSV, possess an in vitro-associated tyrosine-specific protein kinase that appears to be required for the malignant transformation induced by these viruses (1, 8, 12, 19, 21, 24, 27, 28, 41, 42). The present studies were undertaken to characterize the functional properties of the translational products of the SM-FeSV genome. Our results indicate that a protein kinase activity capable of phosphorylating tyrosine residues is present in immunoprecipitates containing SM P180 or SM P120 proteins or both. However, the SM-FeSV gene products exhibit certain structural properties, such as low levels of phosphorylation and absence of phosphorylated tyrosine residues, that are unique among known retroviral transforming proteins with in vitro-associated protein kinase activity.

MATERIALS AND METHODS

Cells and viruses. Cells were cultivated in Dulbecco's modification of Eagle minimum essential medium supplemented with 10% of heat-inactivated calf serum. Mink lung (Mv1Lu) (ATCC 64) cells nonproductively transformed by the SM, ST, and GA strains of FeSV (clones 116A, 61-1, and 65-5, respectively) have been described (29). Mouse NIH/3T3 fibroblasts nonproductively transformed by SM-FeSV (clone 21-2) were obtained by transfection of high-molecular-weight DNA isolated from 116A cells. Mv1Lu cells infected with cloned FeLV subgroup C were also utilized.

Antisera. Caprine antisera directed against autologous cells infected with ST- or GA-FeSV have been described (3). In the present study, a similar antiserum was prepared by immunizing a goat with autologous cells transformed by SM-FeSV. An antiserum elicited against partially purified SM P180 has been reported elsewhere (3). Finally, antisera directed against chromatographically purified FeLV p30 and isopycnically banded FeLV virions were provided by the Office of Resources and Logistics of the National Cancer Institute.

Analysis of viral proteins in infected cells. Subconfluent cultures (around 10^7 cells per 10-cm petri dish) were labeled for 3 h at 37°C with 4 ml of either methionine-free Dulbecco modified Eagle minimum essential medium containing 50 μCi of [^{35}S]methionine (1,200 Ci/mmol; Amersham) per ml or phosphate-free Dulbecco modified Eagle medium containing 1 mCi of $^{32}P_i$ (carrier free; New England Nuclear Corp.) per ml. Radiolabeled cells were lysed with 1 ml of lysing buffer (10 mM sodium phosphate, pH 7.5, 100 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1 mM phenylmethylsulfonyl fluoride) per petri dish, clarified at 100,000 × *g* for 30 min, and divided into four identical aliquots. Each aliquot was incubated with 0.01 ml of appropriate antisera for 60 min at 4°C. Immunoprecipitates were recovered with the aid of *Staphylococcus aureus* protein A bound to Sepharose beads (Pharmacia) and analyzed by electrophoresis in sodium dodecyl sulfate (SDS)-polyacrylamide gradient gels as previously described (3).

Assay for in vitro protein kinase activity. The standard assay for protein kinase activity involved the incubation of immunoprecipitates obtained from 2 × 10^6 SM-FeSV-transformed cells with 5 μCi of [γ-^{32}P]-ATP (3,000 Ci/mmol; Amersham) at 30°C for 10 min. Reactions were carried out by adding 0.025 ml of a buffer containing 20 mM Tris-hydrochloride (pH 7.2) and 12 mM manganese chloride to the *S. aureus* protein A-Sepharose 4B beads containing the corresponding immunoprecipitates (1). $^{32}P_i$-labeled proteins were analyzed by electrophoresis in SDS-polyacrylamide gels.

Phosphoamino acid analysis. Phosphoamino acid analysis of either purified viral proteins or total cellular proteins was performed as described elsewhere (19).

Subfractionation of SM-FeSV-transformed cells. A modification (9) of the procedure described by Hay (17) was utilized. Briefly, exponentially growing 116A transformed cells, either unlabeled or metabolically labeled with [^{35}S]methionine or $^{32}P_i$, were harvested with isotonic phosphate-buffered saline containing 1 mM EDTA, washed with the same buffer, and suspended in 10 mM Tris-hydrochloride (pH 7.5) containing 1 mM $MgCl_2$ at a final density of 10^7 cells per ml. Cells were incubated on ice for 15 min and disrupted with 20 to 25 strokes in a Dounce homogenizer. Undisrupted cells, nuclei, and large subcellular fragments were removed by centrifugation at 2,000 rpm for 5 min. The pellet resulting from this low-speed centrifugation was suspended in 0.5 volume of 20 mM Tris-hydrochloride (pH 7.5) containing 1 mM $MgCl_2$, 0.5% Triton X-100, and 30% sucrose with the aid of a Dounce homogenizer. Resuspended nuclei were applied on top of 10 ml of the above buffer containing 60% sucrose. The discontinuous gradient was centrifuged at 100,000 × *g* for 1 h, and the pellet containing the nuclei fraction was washed twice in the above buffer and either utilized immediately or stored at −70°C until used (no longer than 36 h). The supernatant obtained after the low-speed centrifugation step used to separate the nuclei fraction was centrifuged again for 90 min at 100,000 × *g* to separate the supernatant containing the soluble protein fraction (S-100) from the pelletable cellular membrane fraction (P-100). The latter was washed once by resuspension in 0.5 volume of 20 mM Tris-hydrochloride (pH 7.5) containing 1 mM EDTA and resedimentation at 100,000 × *g* for 60 min.

RESULTS

Immunoprecipitates containing SM-FeSV gene products possess protein kinase activity. Cell extracts from SM-FeSV transformants of both mink (clone 116A) and mouse (clone 21-2) origin were immunoprecipitated with a variety of different antisera. They included those directed against sucrose-banded FeLV, purified FeLV p30, and partially purified SM P180. In addition, we utilized a caprine antiserum prepared against autologous cells transformed by SM-FeSV that contains high-titered antibodies against SM P120. The resulting immunoprecipitates were assayed for protein

kinase activity in the presence of γ-^{32}P-labeled ATP and divalent cations. Immunocomplexes obtained with immune but not with normal goat serum were able to phosphorylate SM P180 molecules (Fig. 1). Phosphorylation of SM P120 could also be detected in immunoprecipitates obtained with the autologous caprine antiserum. Thus, the gene products of SM-FeSV, like those of an increasing number of acute transforming retroviruses, possess an in vitro-associated protein kinase activity. With SM-FeSV, however, no trans-phosphorylation of immune immunoglobulin G molecules was observed with any of the antisera used.

The SM-FeSV-associated protein kinase phosphorylates tyrosine residues. To determine the substrate specificity of the protein kinase activity associated with SM-FeSV gene products, we analyzed the phosphoamino acid content of in vitro-phosphorylated SM P180 and SM P120 molecules. Immunoprecipitates obtained by incubating cell extracts from SM-FeSV-transformed Mv1Lu cells with anti SM-FeSV autologous antiserum were assayed for protein kinase, and the resulting phosphorylated SM-FeSV gene products were isolated by preparative SDS-polyacrylamide gel electrophoresis (PAGE). Most of the $^{32}P_i$ incorporated into SM P120 corresponded to phosphotyrosine residues, although significant levels of [^{32}P]phosphoserine as well as traces of [^{32}P]phosphothreonine could be detected (Fig. 2B). In the case of in vitro-phosphorylated SM P180 molecules, comparable phosphorylation of tyrosine, serine, and threonine residues was observed (Fig. 2A). In contrast, phosphotyrosine could not be detected in several high-molecular-weight proteins that, at variable amounts, contaminated immunoprecipitates containing SM-FeSV gene products (Fig. 1).

Transformation-defective mutants of SM-FeSV have, as yet, not been isolated, making it difficult to establish the specificity of this in vitro phosphorylating activity. Nonetheless, we performed an experiment in which SM-FeSV-transformed (116A) cells and uninfected Mv1Lu mink cells were processed in parallel in an effort to determine whether the presence of phosphotyrosine was specifically associated with expression of SM-FeSV gene products. Cell extracts from both transformed and control cells were incubated with anti SM-FeSV autologous anti-

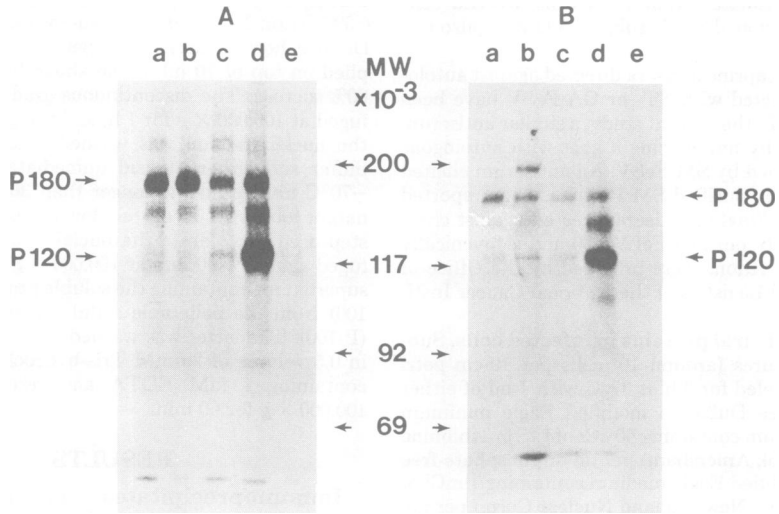

FIG. 1. *Protein kinase activity in immunoprecipitates containing SM-FeSV gene products. Postmicrosomal fractions from 4 × 10^7 SM-FeSV-transformed (A) Mv1Lu mink (clone 116A) and (B) NIH/3T3 mouse (clone 21-2) cells were immunoprecipitated with (a) anti-FeLV serum, (b) anti-FeLV p30 serum, (c) antiserum to partially purified SM P180, (d) anti-SM-FeSV autologous serum, or (e) normal goat serum, as described (3). A 0.025-ml volume of a buffer containing 20 mM Tris-hydrochloride (pH 7.2) and 12 mM $MnCl_2$ was added to the immunocomplexes isolated with the aid of S. aureus protein A bound to Sepharose 4B beads and incubated for 10 min at 30°C in the presence of 5 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol). ^{32}P-labeled proteins were analyzed by electrophoresis in 6 to 12% linear gradient polyacrylamide gels in the presence of SDS and detected by autoradiography. Dried polyacrylamide gels were exposed to Kodak XR-5 film for 36 h at −70°C in the presence of an intensifier screen. Migration of SM P180 and SM P120 is indicated by arrows. Molecular weight (MW) markers coelectrophoresed with the samples include myosin (200,000), β-galactosidase (117,000), phosphorylase b (92,000), and bovine serum albumin (69,000).*

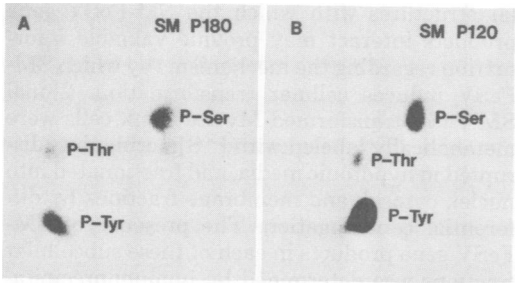

FIG. 2. *Phosphoamino acid analysis of SM-FeSV gene products labeled in vitro by their associated protein kinase activity. Immunoprecipitates obtained by incubating cell-free extracts from 10^8 SM-FeSV-transformed Mv1Lu cells with a 1:40 final dilution of anti-SM-FeSV autologous serum were incubated at 30°C for 10 min in a 2.5-ml reaction mixture containing 20 mM Tris-hydrochloride buffer (pH 7.2), 12 mM $MgCl_2$, and 0.25 mCi of [γ-^{32}P]ATP (3,000 Ci/mmol). ^{32}P-labeled (A) SM P180 and (B) SM P120 were purified by preparative SDS-PAGE, eluted from the gel, precipitated with 20% trichloracetic acid, and submitted to partial acid hydrolysis in the presence of 6 N HCl. The hydrolysate was lyophilized and suspended in 0.01 ml of a solution containing phosphoserine (P-Ser), phosphothreonine (P-Thr), and phosphotyrosine (P-Tyr) markers. A portion (5,000 cpm) of each sample was submitted to two-dimensional electrophoresis at pH 3.5 (bottom to top) and pH 1.9 (left to right) in 20-by-20-cm cellulose plates. The electrophoresed plates were stained with ninhydrin and exposed at −70°C to Kodak XR-5 film in the presence of intensifier screens for 3 days.*

serum, and the resulting immunoprecipitates were assayed for protein kinase activity. The in vitro-phosphorylated proteins were fractionated by preparative SDS-PAGE, and the gel areas containing SM P180 and SM P120 proteins were excised. Identical regions were cut out from the control gel in which the immunoprecipitate obtained from Mv1Lu cells had been fractionated. Proteins were eluted and submitted to phosphoamino acid analysis. One third of each sample was applied to a cellulose plate and electrophoresed at pH 3.5.

The SM-FeSV gene products exhibited levels of [^{32}P]phosphoserine and [^{32}P]phosphothreonine similar to those of control samples from uninfected mink cells (Fig. 3). However, [^{32}P]-phosphotyrosine was detected exclusively in SM P180 and SM P120 molecules. Finally, immunoprecipitates obtained by incubating anti-SM-FeSV autologous antiserum with SM-FeSV-transformed 116A cell extracts from which the SM-FeSV gene products had been removed by a previous immunoprecipitation failed to exhibit any detectable tyrosine-specific phosphorylation (data not shown). These results demonstrate that phosphorylation of tyrosine residues specifically occurs in those immunoprecipitates containing SM-FeSV gene products. In addition, they suggest that most, if not all, of the observed phosphoserine and phosphothreonine residues may be derived from contaminating cellular proteins.

The above results did not establish whether both, or only one of the known SM-FeSV gene products have associated tyrosine-specific protein kinase. Thus, we prepared immunoprecipitates containing either SM P180 or SM P120 alone. This was achieved by incubating SM-FeSV-transformed Mv1Lu cell extracts with anti-FeLV p30 antibodies and immunoprecipitating the resulting supernatant with anti-SM-FeSV autologous antiserum. Both immunoprecipitates were then assayed for protein kinase activity, and the corresponding ^{32}P-labeled SM-FeSV gene product was purified by preparative electrophoresis and subsequently submitted to

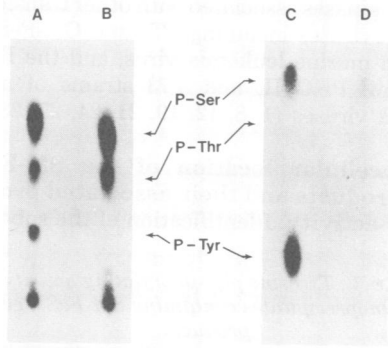

FIG. 3. *Specificity of tyrosine phosphorylation by immunoprecipitates containing SM-FeSV gene products. Immunocomplexes obtained by incubating 10^8 SM-FeSV-transformed Mv1Lu cells with a 1:40 final dilution of anti-SM-FeSV autologous serum were assayed for protein kinase activity as described in the legend to Fig. 2. ^{32}P-labeled immunoprecipitates were fractionated by preparative SDS-PAGE, and the gel areas containing (A) SM P180 and (C) SM P120 were excised with the aid of a scalpel. A control experiment in which uninfected Mv1Lu cells were used instead was performed in parallel. Gel areas corresponding to those of the SM-FeSV gel containing SM P180 (B) and SM P120 (D) were also excised from the control Mv1Lu preparative gel. Proteins were eluted and submitted to phosphoamino acid analysis. Samples were mixed with unlabeled phosphoserine (P-Ser), phosphothreonine (P-Thr), and phosphotyrosine (P-Tyr) markers and subjected to electrophoresis toward the anode (top) at pH 3.5. Electrophoresed plates were stained with ninhydrin and exposed at −70°C to Kodak XR-5 film in the presence of intensifier screens for 5 (samples A and B) or 2 (samples C and D) days.*

phosphoamino acid analysis. Immunoprecipitates containing SM P180 alone had a very reduced, although detectable, phosphorylating tyrosine activity (Table 1). On the other hand, the levels of tyrosine phosphorylation associated with SM P120 alone were similar to those observed in immunocomplexes containing both SM-FeSV gene products.

Properties of the protein kinase activity associated with SM-FeSV gene products. Immunoprecipitates obtained by incubating SM-FeSV-transformed Mv1Lu cells with anti-SM-FeSV autologous antiserum were assayed for protein kinase activity under different experimental conditions. The in vitro protein kinase activity associated with the SM-FeSV gene products is cAMP independent, uses ATP, but not GTP, as a substrate, and is inhibited by ADP and dATP. In addition, it exhibits rapid kinetics ($V_i < 1$ min) at 30°C as well as at 0°C, requires the cation Mn^{2+} in preference to Mg^{2+}, and, as shown above, phosphorylates tyrosine residues. These properties closely resemble those of kinases associated with other transforming retroviruses including ST- and GA-FeSV's, Abelson murine leukemia virus, and the Rous, Fujinami, PRC II, and Y73 strains of avian sarcoma viruses (1, 8, 12, 19, 21, 24, 27, 28, 41, 42).

Intracellular location of the SM-FeSV gene products and their associated protein kinase activity. Identification of the subcellular structures with which the SM-FeSV gene products interact may provide valuable information regarding the mechanisms by which SM-FeSV induces cellular transformation. Clonal SM-FeSV-transformed Mv1Lu mink cells were metabolically labeled with [^{35}S]methionine, disrupted in hypotonic media, and fractionated into nuclei, cytosol, and membrane fractions by differential centrifugation. The presence of SM-FeSV gene products in each of these subcellular fractions was determined by immunoprecipitation with anti-FeLV or anti-SM-FeSV autologous antisera. Most of the SM-FeSV gene products were localized to the membrane fraction (Fig. 4), although some SM P180 and SM P120 were also detected in the nuclei. Viral proteins copurifying with the nuclei preparation could be solubilized by nonionic detergents after, but not before, nuclei disruption, suggesting that they may be associated with the nuclear membrane rather than with contaminating membranous structures. In contrast, no SM-FeSV translational products could be detected in the soluble cytoplasmic fraction.

TABLE 1. *Tyrosine phosphorylating activity in immunoprecipitates containing SM-FeSV gene products*

Sample	Immunoprecipitated SM-FeSV protein[a]	$^{32}PO_4$ incorporated[b] (total cpm × 10^4) into:		Relative abundance[c] of phosphotyrosine in:	
		SM P180	SM P120	SM P180	SM P120
A	SM P180 + SM P120	1.5	4.9	32	64
B	SM P180	0.7	NA[d]	12	NA
C	SM P120	NA	4.2	NA	82
D	None	0.1	0.1	<1	<1

[a] Around 10^8 exponentially growing SM-FeSV-transformed Mv1Lu mink cells were immunoprecipitated with anti-SM-FeSV autologous serum (sample A), anti-FeLV p30 serum (sample B), anti-SM-FeSV autologous serum after removal of SM P180 with anti-FeLV serum (sample C), and normal goat serum (sample D).

[b] Immunoprecipitates were assayed for protein kinase activity, and the resulting ^{32}P-labeled SM P180 and SM P120 were purified by preparative electrophoresis. A small sample was saved for determination of radioactivity, and the rest of the samples were submitted to phosphoamino acid analysis.

[c] Percent radioactivity eluted from spots containing a phosphotyrosine marker relative to total radioactivity coelectrophoresed at pH 3.5 with phosphoserine, phosphothreonine, and phosphotyrosine markers.

[d] NA, Not applicable.

We next examined whether the protein kinase activity detected in immunoprecipitates containing SM-FeSV gene products was also associated with the subcellular membrane fraction. [^{35}S]-methionine-labeled and unlabeled 116A cells were fractionated into nuclei, cytosol, and membrane fractions in parallel experiments. The presence of SM-FeSV gene products in each subcellular fraction was monitored by immunoprecipitation of the labeled cell extracts as described above (Fig. 4A). Protein kinase activity was assayed by immunoprecipitating the unlabeled subcellular fractions with anti-FeLV, anti-SM-FeSV autologous, or normal goat serum, followed by incubation of the resulting immunocomplexes with [γ-^{32}P]ATP in the presence of manganese ions. ^{32}P-labeled proteins were analyzed by SDS-PAGE, and the results are depicted in Fig. 4B. Only the membrane, and to a lesser extent the nuclei fraction, exhibited a protein kinase capable of phosphorylating tyrosine residues of both SM P180 and SM P120. Low levels of protein kinase activity were also found in the soluble cytoplasmic fraction (Fig. 4B). However, in this case, only serine and threonine residues became phosphorylated (data not shown). These results provide evidence suggesting that the SM-FeSV gene products may interact in vivo with cellular membrane structures.

In vivo phosphorylation of SM-FeSV gene products. Mink cells nonproductively transformed by SM-FeSV (clone 116A) were metabolically labeled with $^{32}P_i$ and immunoprecipitated with caprine antisera directed against either FeLV- or SM-FeSV-specific antigenic de-

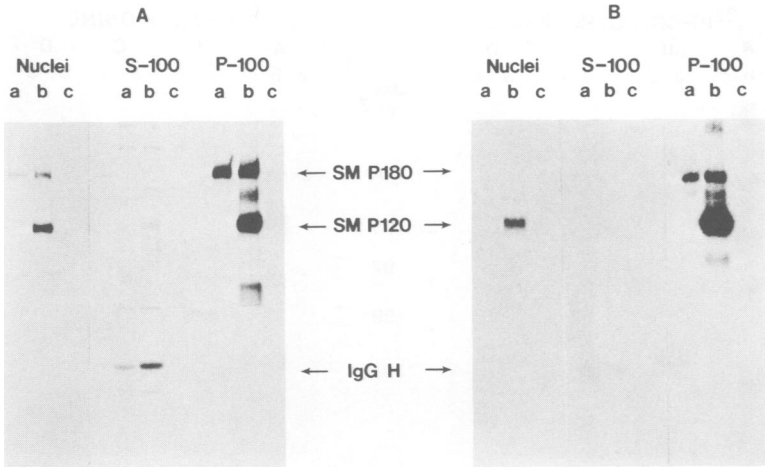

FIG. 4. *Subcellular location of the SM-FeSV gene products and their in vitro-associated protein kinase activity. (A) Exponentially growing SM-FeSV-transformed Mv1Lu cells were metabolically labeled with [^{35}S]methionine for 3 h and fractionated into subcellular fractions containing either nuclei, soluble cytoplasmic proteins (S-100), or membranes (P-100), as described in the text. Each fraction was disrupted, clarified, and immunoprecipitated with (a) anti-FeLV, (b) anti-SM-FeSV autologous, or (c) normal goat serum. Immunocomplexes collected with the aid of S. aureus protein A bound to Sepharose 4B beads were disrupted in the presence of 1% SDS and 2% 2-mercaptoethanol and analyzed by electrophoresis in 6 to 12% linear gradient SDS-polyacrylamide gels. Gels were fluorographed as described (6) and exposed to Kodak XR-5 film at −70°C for 2 days. In a parallel experiment (B), postmicrosomal cell extracts obtained from unlabeled, exponentially growing SM-FeSV-transformed Mv1Lu cells were fractionated into the nuclei, soluble cytoplasmic protein (S-100), and membrane (P-100) fractions as described in the text. Each fraction was disrupted, clarified, and immunoprecipitated as described above. Immunocomplexes were assayed for protein kinase activity as described in the legend to Fig. 1. ^{32}P-labeled proteins were analyzed by electrophoresis as described above, and the dried gels were exposed to Kodak XR-5 film for 2 days at −70°C with the aid of intensifier screens.*

terminants. In agreement with previous observations (40), both SM P180 and SM P120 incorporated low levels of $^{32}P_i$, indicating that they are poorly phosphorylated molecules (Fig. 5). Under similar conditions, the FeLV *gag* gene precursor protein Pr65 was around 20 times more efficiently labeled than SM P180 in spite of the fact that SM P180 contains a large fraction of the FeLV Pr65 molecule. Similarly, the transforming proteins of the ST and GA strains of FeSV are much more heavily phosphorylated than either SM P180 or SM P120 proteins (Fig. 5).

We next examined the phosphoamino acid content of the SM-FeSV gene products. In vivo ^{32}P-labeled SM P180 and SM P120 proteins were purified from metabolically labeled SM-FeSV-transformed cells by preparative electrophoresis and submitted to partial acid hydrolysis. The resulting phosphoamino acids were then analyzed by two-dimensional electrophoresis at pH 1.9 and 3.5 as described in Materials and Methods. Only phosphoserine and phosphothreonine, but not phosphotyrosine, could be identified as integral components of either SM P180 or SM P120 molecules (Fig. 6).

Levels of phosphorylated tyrosine residues in proteins of SM-FeSV transformants. Proteins from cells transformed by retroviruses whose transforming proteins possess tyrosine-specific protein kinases have been shown to exhibit increased levels of phosphotyrosine, an unusual phosphoamino acid (1, 5, 19, 28, 37). Because the protein kinase activity associated with SM-FeSV gene products is also capable of phosphorylating tyrosine residues in vitro, we considered it of interest to determine whether SM-FeSV transformants exhibited a similar phenotype. SM-FeSV-transformed and uninfected Mv1Lu mink cells were labeled with $^{32}P_i$, and their protein fraction was submitted to phosphoamino acid analysis. No detectable [^{32}P]-phosphotyrosine was present in hydrolysates of proteins from SM-FeSV transformants (Fig. 7). When the area of the electrophoresed cellulose plate corresponding to the cold phosphotyrosine marker was eluted from the plate, its radioactivity represented no more than 0.03% of that coelectrophoresed with phosphoserine and phosphothreonine. Similar values were obtained when the levels of [^{32}P]phosphotyrosine were determined in uninfected Mv1Lu cells (data not

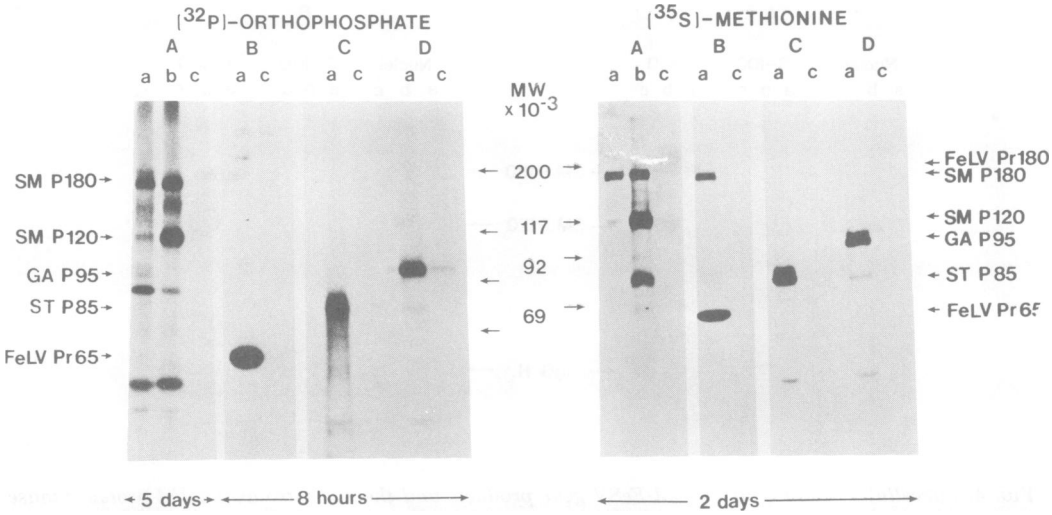

FIG. 5. *Phosphorylation levels of SM-FeSV gene products. Exponentially growing (A) SM-FeSV-transformed, (B) FeLV-infected, (C) ST-FeSV-transformed, and (D) GA-FeSV-transformed Mv1Lu cells were metabolically labeled with either $^{32}P_i$ (left) or [^{35}S]methionine (right) for 3 h, and their corresponding cell extracts were immunoprecipitated with (a) anti-FeLV p30, (b) anti-SM-FeSV autologous, and (c) normal goat sera. Immunocomplexes, collected with the aid of S. aureus protein A bound to Sepharose 4B beads, were disrupted in the presence of 1% SDS and 2% 2-mercaptoethanol and analyzed by electrophoresis in 6 to 12% linear gradient SDS-polyacrylamide gels. The migration of FeLV gag-pol Pr180, SM P180, SM P120, GA P95, ST P85 and FeLV gag Pr65 proteins is indicated by arrows. Molecular weight (MW) markers were those described in the legend to Fig. 1. Gels containing ^{32}P-labeled proteins were dried and exposed to Kodak XR-5 film at −70°C with the aid of intensifier screens. Gels containing ^{35}S-labeled proteins were fluorographed as previously described (6) and exposed to Kodak XR-5 film at −70°C. Exposure times for each of the samples are indicated at the bottom of the figure.*

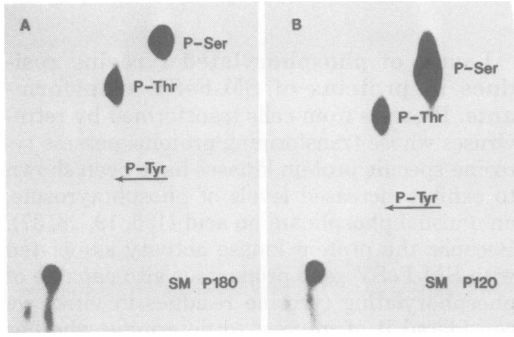

FIG. 6. *Phosphoamino acid analysis of SM-FeSV gene products. In vivo ^{32}P-labeled (A) SM P180 and (B) SM P120 proteins were purified from metabolically labeled SM-FeSV-transformed Mv1Lu cells by immunoprecipitation with anti-SM-FeSV autologous serum followed by preparative SDS-PAGE. Proteins eluted from the gel were precipitated with 20% trichloracetic acid, washed with ethanol, dried, and submitted to partial acid hydrolysis in the presence of 6 N HCl. The hydrolysate was lyophilized, suspended in 0.01 ml of a solution containing phosphoserine (P-Ser), phosphothreonine (P-Thr), and phosphotyrosine (P-Tyr) markers, and analyzed by two-dimensional electrophoresis at pH 3.5 (bottom to top) and pH 1.9 (left to right) in 20-by-20-cm cellulose plates. Electrophoresed plates were stained with ninhydrin and exposed to Kodak XR-5 film at −70°C in the presence of intensifier screens for 5 days.*

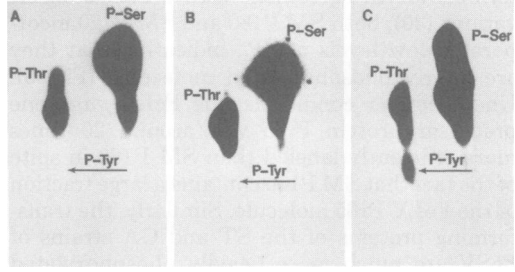

FIG. 7. *Phosphoamino acid analysis of SM-FeSV transformants. SM-FeSV-transformed Mv1Lu cells were metabolically labeled with $^{32}P_i$ for 18 h, and either (A) whole cells or (B) the membrane subcellular fraction (P-100) were extracted with phenol and subjected to partial acid hydrolysis. As a positive control, the total protein fraction from ST-FeSV-transformed Mv1Lu cells was also submitted to phosphoamino acid analysis in a parallel experiment (C). Hydrolysates were suspended in a solution containing phosphoserine (P-Ser), phosphothreonine (P-Thr), and phosphotyrosine (P-Tyr) markers, and samples (2 × 10^6 cpm) were analyzed by two-dimensional electrophoresis at pH 3.5 (bottom to top) and pH 1.9 (left to right) in 20-by-20-cm cellulose plates. Electrophoresed plates were stained with ninhydrin and exposed to Kodak XR-5 film at −70°C in the presence of intensifier screens for 6 days.*

shown). In contrast, ST-FeSV-transformed mink cells processed in the same experiment as positive controls (Fig. 7C) revealed [^{32}P]phosphotyrosine levels comparable to those published elsewhere (1).

Considering the intracellular location of the SM-FeSV gene products and their associated protein kinase, we isolated the membrane fraction from ^{32}P-labeled SM-FeSV-transformed mink cells and determined the phosphoamino acid content of their protein fraction. We did not detect any relative increase in the levels of phosphotyrosine residues in the membrane proteins of SM-FeSV transformants (Fig. 7B). Thus, if SM-FeSV-induced transformation were to proceed via phosphorylation of tyrosine residues, the viral gene products must interact with a very limited number of cellular target proteins.

DISCUSSION

Characterization of the structural and functional properties of the SM-FeSV gene products should help to elucidate the mechanisms by which this virus induces malignant transformation. The structure of the SM-FeSV transforming gene, the 5′ portion of the helper virus *gag* gene fused to sequences of cellular origin, conforms to a pattern found in a variety of acute transforming retroviruses (10). As a consequence, the SM-FeSV primary translational product, SM P180, contains the amino-terminus of the FeLV *gag* gene product covalently linked to a sarcoma virus-specific polypeptide (3, 29, 34, 40). In addition, SM-FeSV transformants exhibit the unusual property of expressing high levels of another virus-coded molecule, SM P120, which is highly related to the sarcoma virus-specific domain of SM P180 (3).

The results of the present study indicate that both SM-FeSV gene products are intimately associated with the membrane fraction of SM-FeSV transformants. Low levels of SM P180 and SM P120 were also found in structures comprising the nuclear envelope. However, no detectable SM-FeSV proteins (less than 1% of the total) were detected in the soluble cytoplasmic fraction. Other retroviral transformation proteins, such as RSV pp60^{src} and Abelson P120, have been found to be affiliated with the plasma membrane (for a review, see reference 20). Yet, unlike SM P180 and SM P120, these proteins can also be detected in a soluble form in the cytoplasm (7, 22; M. Barbacid, unpublished data). Although the biological significance of these differences may be trivial, it is possible that the more specific subcellular location of the SM-FeSV gene products may have implications with respect to the nature and number of different cellular targets with which these viral proteins interact.

Immunoprecipitates containing SM-FeSV gene products possess a protein kinase activity whose enzymatic properties, and in particular its ability to phosphorylate tyrosine residues, closely resembled those associated with the transforming proteins of a variety of avian (Rous, Fujinami, PRC II, and Y73 strains of avian sarcoma virus) and mammalian (Abelson murine leukemia virus and ST and GA strains of FeSV) retroviruses (1, 8, 12, 19, 21, 24, 27, 28, 41, 42). The specificity of these findings was demonstrated by a series of control experiments in which tyrosine phosphorylation was limited to those immunoprecipitates containing SM-FeSV gene products. Moreover, such enzymatic reactivity was exclusively observed in those subcellular fractions known to harbor the SM P180 and SM P120 molecules. Recent reports (31, 40) have failed to demonstrate protein kinase activity associated with the SM-FeSV gene products. A possible explanation for this discrepancy is that these studies failed to detect SM P120, the viral gene product which exhibits most of the in vitro SM-FeSV-associated protein kinase. Alternatively, it could be argued that the use of magnesium, instead of the preferred cation manganese, is responsible for the lack of detection of the virus-associated enzyme in these studies (31, 40).

The presence of an enzymatic activity capable of phosphorylating tyrosine residues in immunoprecipitates containing SM P180 and SM P120 suggests superficially that SM-FeSV may transform cells by a mechanism similar to that utilized by the above-mentioned retroviruses. However, the structural properties of the SM-FeSV gene products do not support this hypothesis. Retroviral transforming proteins such as Rous sarcoma virus pp60^{src} (7, 24), Fujinami sarcoma virus P140 (16, 23), PRC II P105 (26), Y73 P90 (21), Abelson P120 (44), ST P85 (3), and GA P95 (3, 41) are highly phosphorylated and contain phosphotyrosine residues (1, 4, 12, 19, 21, 27, 28, 41, 42). These structural properties are not present in the corresponding gene products coded for by transformation-defective mutants, suggesting that they may be important for the biological activity of these retroviral molecules (2, 8, 15, 24, 28, 30, 32, 36, 43). As shown here, wild-type SM P180 and SM P120 are neither highly phosphorylated nor contain phosphotyrosine. More importantly, we and others (31) have not been able to detect elevated levels of phosphorylated tyrosine residues in proteins of SM-FeSV transformants, a phenotype readily detected in cells transformed by all of the other known "protein kinase-containing" retroviruses (1, 5, 19, 28, 37). Our results closely resemble those obtained with cells transformed by polyoma, a DNA tumor virus. Immunoprecipitates

containing wild-type, but not mutant, polyoma middle T antigen possess an associated protein kinase capable of phosphorylating the tyrosine residues of the viral gene product (11, 38), yet levels of phosphotyrosine in the protein fraction of polyoma-transformed cells are comparable to those found in control uninfected cells (36).

One interpretation of our present findings obtained with SM-FeSV as well as those previously reported with polyoma virus (11, 36, 38) is that the in vitro phosphorylation of tyrosine residues does not have physiological significance. Thus, if SM-FeSV (or polyoma)-induced transformation were to proceed by pathways that do not involve phosphorylation of tyrosine residues, these results could raise a note of caution in the interpretation of similar in vitro findings with other transforming viruses. An alternative explanation would be to postulate that the SM-FeSV gene products may act in vivo as, or in close association with, a protein kinase that phosphorylates a small subset of cellular proteins, which do not represent a significant fraction of the total cellular protein content. In either case, our findings support the concept that SM-FeSV induces malignant transformation by a mechanism different from those of other transforming retroviruses, including ST- and GA-FeSV, whose gene products exhibit an associated tyrosine-specific protein kinase activity. Thus, the cat cellular genome possesses at least two subsets of transducible genetic information with different oncogenic properties.

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LITERATURE CITED

1. **Barbacid, M., K. Beemon, and S. G. Devare.** 1980. Origin and functional properties of the major gene product of the Snyder-Theilen strain of feline sarcoma virus. Proc. Natl. Acad. Sci. U.S.A. **77**:5158–5163.
2. **Barbacid, M., L. Donner, S. K. Ruscetti, and C. J. Scherr.** 1981. Transformation-defective mutants of Snyder-Theilen feline sarcoma virus lack tyrosine-specific protein kinase activity. J. Virol. **39**:246–254.
3. **Barbacid, M., A. V. Lauver, and S. G. Devare.** 1980. Biochemical and immunological characterization of polyproteins coded for by the McDonough, Gardner-Arnstein, and Snyder-Theilen strains of feline sarcoma virus. J. Virol. **33**:196–207.
4. **Barbacid, M., A. V. Lauver, L. Long, and S. Devare.** 1980. The major gene products of the Snyder-Theilen and Gardner-Arnstein strains of feline sarcoma viruses have a common cellular origin and similar functional properties, p. 309–320. *In* W. D. Hardy, M. Essex, and A. J. McClelland (ed.), Feline leukemia virus. Elsevier/North Holland Press, New York.
5. **Beemon, K.** 1981. Transforming proteins of some feline and avian sarcoma viruses are related structurally and functionally. Cell **24**:145–153.
6. **Bonner, W. M., and R. Laskey.** 1974. A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. **46**:83–88.
7. **Burr, J. G., G. Dreyfuss, S. Penman, and J. M. Buchanan.** 1980. Association of the *src* gene product of Rous sarcoma virus with cytoskeletal structures of chicken embryo fibroblasts. Proc. Natl. Acad. Sci. U.S.A. **77**:3484–3488.
8. **Collett, M. S., and R. L. Erikson.** 1978. Protein kinase activity associated with the avian sarcoma virus *src* gene product. Proc. Natl. Acad. Sci. U.S.A. **75**:2020–2024.
9. **Courtneidge, S. A., A. D. Levinson, and J. M. Bishop.** 1980. The protein encoded by the transforming gene of avian sarcoma virus ($pp60^{src}$) and a homologous protein in normal cells ($pp60^{proto\text{-}src}$) are associated with the plasma membrane. Proc. Natl. Acad. Sci. U.S.A. **77**:3783–3787.
10. **Duesberg, P. H.** 1980. Transforming genes of retroviruses. Cold Spring Harbor Symp. Quant. Biol. **44**:13–29.
11. **Eckhart, W., M. A. Hutchison, and T. Hunter.** 1979. An activity phosphorylating tyrosine in polyoma T antigen immunoprecipitates. Cell **18**:925–933.
11a. **Fedele, L. A., J. Even, C. F. Garon, L. Donner, and C. J. Sherr.** 1981. Recombinant bacteriophages containing the integrated transforming provirus of Gardner-Arnstein feline sarcoma virus. Proc. Natl. Acad. Sci, U.S.A. **78**:4036–4040.
12. **Feldman, R. A., T. Hanafusa, and H. Hanafusa.** 1980. Characterization of protein kinase activity associated with the transforming gene product of Fujinami sarcoma virus. Cell **22**:757–765.
13. **Frankel, A. E., J. H. Gilbert, K. J. Porzig, E. M. Scolnick, and S. A. Aaronson.** 1979. Nature and distribution of feline sarcoma virus nucleotide sequences. J. Virol. **30**:821–827.
14. **Gardner, M. B., R. W. Rongey, P. Arnstein, J. D. Estes, P. Sarma, R. J. Huebner, and C. G. Richard.** 1970. Experimental transmission of feline fibrosarcoma to cats and dogs. Nature (London) **226**:807–809.
15. **Hanafusa, T., B. Mathey-Prevot, R. A. Feldman, and H. Hanafusa.** 1981. Mutants of Fujinami sarcoma virus which are temperature sensitive for cellular transformation and protein kinase activity. J. Virol. **38**:347–355.
16. **Hanafusa, T., L. H. Wang, S. M. Anderson, R. E. Karess, W. S. Hayward, and H. Hanafusa.** 1980. Characterization of the transforming gene of Fujinami sarcoma virus. Proc. Natl. Acad. Sci. U.S.A. **77**:3009–3013.
17. **Hay, A. J.** 1974. Studies on the formation of the influenza virus envelope. Virology **60**:398–418.
18. **Henderson, I. C., M. M. Lieber, and G. J. Todaro.** 1974. Mink cell line Mv1Lu (CCL64). Focus formation and the generation of "nonproducer" transformed cell lines with murine and feline sarcoma viruses. Virology **60**:282–287.
19. **Hunter, T., and B. M. Sefton.** 1980. The transforming gene product of Rous sarcoma virus phosphorylates tyrosine. Proc. Natl. Acad. Sci. U.S.A. **77**:1311–1315.
20. **Hynes, R. O.** 1980. Cellular location of viral transforming proteins. Cell **21**:601–602.
21. **Kawai, S., M. Yoshida, K. Segawa, H. Sugiyama, R. Ishizaki, and K. Toyoshima.** 1980. Characterization of Y73, a newly isolated avian sarcoma virus: a phosphopolyprotein with protein kinase activity. Proc. Natl. Acad. Sci. U.S.A. **77**:6199–6203.
22. **Krzyzek, R. A., R. L. Mitchell, A. F. Lau, and A. J. Faras.** 1980. Association of $pp60^{src}$ and *src* protein kinase activity with the plasma membrane of nonpermissive and permissive avian sarcoma virus-infected cells. J. Virol. **36**:805–815.
23. **Lee, W. H., K. Bister, A. Pawson, T. Robins, C. Mos-**

covici, and P. H. Duesberg. 1980. Fujinami sarcoma virus: an avian RNA tumor virus with a unique transforming gene. Proc. Natl. Acad. Sci. U.S.A. 77:2018–2022.

24. Levinson, A. D., H. Oppermann, L. Levintow, H. E. Varmus, and J. M. Bishop. 1978. Evidence that the transforming gene of avian sarcoma virus encodes a protein kinase associated with a phosphoprotein. Cell 15:561–572.
25. McDonough, S. K., S. Larsen, R. S. Brodey, N. D. Stock, and W. D. Hardy, Jr. 1971. A transmissible feline fibrosarcoma of viral origin. Cancer Res. 31:953–956.
26. Neil, J. C., M. L. Breitman, and P. K. Vogt. 1981. Characterization of a 105,000 molecular weight *gag*-related phosphoprotein from cells transformed by the defective avian sarcoma virus PRC II. Virology 108:98–110.
27. Neil, J. C., J. Ghysdael, and P. K. Vogt. 1981. Tyrosine-specific protein kinase activity associated with p105 of avian sarcoma virus PRC II. Virology 109:223–228.
28. Pawson, T., J. Guyden, T. Kung, K. Radke, T. Gilmore, and G. S. Martin. 1980. A strain of Fujinami sarcoma virus which is temperature-sensitive in protein phosphorylation and cellular transformation. Cell 22: 767–775.
29. Porzig, K. J., M. Barbacid, and S. A. Aaronson. 1979. Biological properties and translational products of three independent isolates of feline sarcoma virus. Virology 92:91–107.
30. Reynolds, F. H., W. J. M. Van de Ven, J. Blomberg, and J. R. Stephenson. 1981. Involvement of a high-molecular-weight polyprotein translational product of Snyder-Theilen feline sarcoma virus in malignant transformation. J. Virol. 37:643–653.
31. Reynolds, F. H., W. J. M. Van de Ven, J. Blomberg, and J. R. Stephenson. 1981. Differences in mechanisms of transformation by independent feline sarcoma virus isolates. J. Virol. 38:1084–1089.
32. Reynolds, F. H., W. J. M. Van de Ven, and J. R. Stephenson. 1980. Abelson murine leukemia virus transformation-defective mutants with impaired P120-associated protein kinase activity. J. Virol. 26:374–386.
33. Robbins, K. C., M. Barbacid, K. J. Porzig, and S. A. Aaronson. 1979. Involvement of different exogenous FeLV subgroups in the generation of independent feline sarcoma virus isolates. Virology 97:1–11.
34. Ruscetti, S. K., L. P. Turek, and C. J. Sherr. 1980. Three independent isolates of feline sarcoma virus code for three distinct *gag*-X polyproteins. J. Virol. 35:259–264.
35. Sarma, P. S., and T. Log. 1977. Defectiveness of a feline sarcoma virus demonstrable by the acquisition of a new envelope antigenic type by phenotype mixing. Virology 78:336–339.
36. Sefton, B. M., T. Hunter, K. Beemon, and W. Eckhart. 1980. Evidence that the phosphorylation of tyrosine is essential for cellular transformation by Rous sarcoma virus. Cell 20:807–816.
37. Sefton, B. M., T. Hunter, and W. C. Raschke. 1981. Evidence that the Abelson virus protein functions *in vivo* as a protein kinase that phosphorylates tyrosine. Proc. Natl. Acad. Sci. U.S.A. 78:1552–1556.
38. Smith, A. E., R. Smith, B. Griffin, and M. Fried. 1979. Protein kinase activity associated with polyoma virus middle T antigen in vitro. Cell 18:915–924.
39. Snyder, S. P., and G. H. Theilen. 1969. Transmissible feline fibrosarcoma. Nature (London) 221:1074–1075.
40. Van de Ven, W. J. M., F. H. Reynolds, Jr., R. P. Nalewaik, and J. R. Stephenson. 1980. Characterization of a 170,000-dalton polyprotein encoded by the McDonough strain of feline sarcoma virus. J. Virol. 35: 165–175.
41. Van de Ven, W. J. M., F. H. Reynolds, and J. R. Stephenson. 1980. The nonstructural components of polyproteins encoded by replication-defective mammalian transforming retroviruses are phosphorylated and have associated protein kinase activity. Virology 101: 185–197.
42. Witte, O. M., A. Dasgupta, and D. Baltimore. 1980. Abelson murine leukemia virus protein is phosphorylated *in vitro* to form phosphotyrosine. Nature (London) 283:826–831.
43. Witte, O. N., S. Goff, N. Rosenberg, and D. A. Baltimore. 1980. A transformation-defective mutant of Abelson murine leukemia virus lacks protein kinase activity. Proc. Natl. Acad. Sci. U.S.A. 77:4993–4997.
44. Witte, O. N., N. Rosenberg, M. Paskind, A. Shields, and D. A. Baltimore. 1978. Identification of an Abelson murine leukemia virus-encoded protein present in transformed fibroblast and lymphoid cells. Proc. Natl. Acad. Sci. U.S.A. 75:2488–2492.