Involvement of a High-Molecular-Weight Polyprotein Translational Product of Snyder-Theilen Feline Sarcoma Virus in Malignant Transformation

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The previously described high-molecular-weight polyprotein major translational product of the Snyder-Theilen strain of feline sarcoma virus (FeSV) was shown to possess protein kinase activity with specificity for tyrosine acceptor sites. Cells transformed by Snyder-Theilen FeSV exhibited constitutively elevated levels of phosphotyrosine and a concomitant reduction in epidermal growth factor (EGF) binding sites. By endpoint cloning in microtiter plates, a number of transformation-defective (td) mutants of the Snyder-Theilen strain of FeSV were isolated. Mink cells nonproductively infected by such mutants were morphologically nontransformed, failed to grow in soft agar, bound EGF as efficiently as control mink cells, and lacked rescuable transforming virus. Although the level of expression of the major viral polyprotein translational product in td mutant-infected clones was comparable to that of wild-type (wt) transformants, the polyprotein in mutant clones lacked detectable protein kinase activity and total cellular phosphotyrosine levels were not elevated significantly above control values. Of a large number of wt Snyder-Theilen FeSV-transformed mink cell clones isolated, the majority were found to revert to a nontransformed morphology upon continuous passage in cell culture. Such nontransformed variants, as well as a Gardner FeSV-transformed mink cell revertant, lacked detectable polyprotein expression and exhibited levels of phosphotyrosine and EGF binding similar to those of control mink cells. These findings provide strong evidence favoring the involvement of the Snyder-Theilen FeSV-encoded high-molecular-weight polyprotein and its associated tyrosine-specific protein kinase activity in transformation.

Several independent isolates of feline sarcoma virus (FeSV) have been described (7, 13, 21, 25). Each, in the presence of an appropriate type C helper virus, transforms embryo fibroblasts in cell culture and induces fibrosarcomas in vivo. The two isolates studied in greatest detail, Gardner and Snyder-Theilen, encode as their major translational products polyproteins of around 115,000 molecular weight (M_r) (10, 26, 30). In addition, cells transformed by these isolates exhibit possible cleavage products of 72,000 and 80,000 M_r , respectively (30). An independently derived clone of Snyder-Theilen FeSV has been shown to encode a somewhat lower 85,000- M_r polyprotein (1, 14, 24). A third FeSV isolate, originally described by McDonough et al. (13), encodes a 170,000- M_r polyprotein as well as a 55,000- M_r probable cleavage product (1, 31). On the basis of immunological and tryptic peptide analysis, the major polyproteins expressed in FeSV-transformed cell lines have been shown to

contain structural components encoded by the 5'-terminal region of the type C viral *gag* gene covalently linked to nonstructural components encoded by acquired cellular sequences represented within the viral genome. Translation is apparently initiated at the 5' terminus of the viral *gag* gene and continues in phase into adjacent acquired cellular sequences (1, 4, 9, 10, 23, 24, 26).

We have recently shown the nonstructural components of the 115,000-polyproteins (P115) encoded by Gardner and Snyder-Theilen FeSV to contain multiple sites of phosphorylation (32). In addition, we have described a protein kinase activity intrinsic to, or associated with, Gardner FeSV P115 which, in contrast to most eucaryotic protein kinases, exhibits specificity for tyrosine acceptor sites (17a, 32). In this respect, Gardner FeSV P115 resembles polyoma virus middle-T antigen (5) and type C virus-encoded proteins with documented transforming function, such as

avian sarcoma virus (ASV) pp60^{src} (3, 8) and Abelson murine leukemia virus (MuLV) P120 (2, 32, 33).

In the present study, a genetic approach was undertaken in an effort to directly demonstrate the involvement of the Gardner and Snyder-Theilen FeSV-encoded polypeptides in transformation. This has involved the isolation and characterization of transformation-defective (td) mutants and morphological revertants of FeSV-transformed cell lines. In addition, we have extended our studies of type C virus-associated protein kinases to show that the polypeptide translational products of the Snyder-Theilen strain of FeSV possess such activity and have attempted to relate viral protein kinase activities to the decrease in epidermal growth factor (EGF) binding sites characteristic of FeSV-transformed cells (29). The results establish the involvement of protein kinase activities associated with the Snyder-Theilen and Gardner FeSV-encoded polypeptides, and thus the polypeptides themselves, in transformation.

MATERIALS AND METHODS

Cells and virus. Cells were grown in Dulbecco's modification of Eagle medium supplemented with 10% calf serum (Colorado Serum Co., Denver, Colo.) in petri dishes (60 by 15 mm; Falcon Plastics, Los Angeles, Calif.). Cell lines used have been previously described and include a highly contact inhibited mink lung cell line, CCL64 (16), and a Fisher rat embryo line designated FRE 3A (16). A Snyder-Theilen FeSV(FeLV B)-transformed mink cell line, MSTF, was generously provided by M. Essex, and a rat cell line nonproductively transformed by the Snyder-Theilen strain of FeSV, designated ST-NRK, was a gift of E. M. Scolnick. Other nonproductively transformed cell lines included Gardner FeSV-transformed mink, 64F3 (24); McDonough FeSV-transformed rat, McD-FeSV FRE CL3 (30); and McDonough FeSV-transformed mink, McD-FeSV C115-1. Type C viruses including feline leukemia virus (FeLV) subgroup B (30) and a wild mouse amphotropic virus isolate designated 4070-A (21) have been previously described.

RNA-dependent DNA polymerase assay. Culture fluids (4 ml) were harvested from petri dishes (60 by 15 mm) and clarified by centrifugation for 30 min at 10,000 rpm. Virus was collected by centrifugation for 60 min at 30,000 rpm and assayed for virion-associated reverse transcriptase activity as previously described (22).

Competition immunoassays. FeLV structural proteins including p12, p30, and gp70 were quantitated by competition immunoassay according to previously described methods (10).

Metabolic labeling, immunoprecipitation, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Tissue culture cells (10^7) were radioactively labeled by incubation for 2 h in medium containing [³H]lysine (100 μ Ci/ml, 80.5 Ci/mmol) or ³²P_i (100 μ Ci/ml, carrier-free) (New England Nuclear Corp., Boston, Mass.) (16). Labeled cell monolayers

were washed twice in serum-free medium and disrupted at 4°C by repeated aspiration through a 25-gauge needle in 5 ml of PBSTDS (10 mM sodium phosphate, pH 7.2, 0.9% NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS). Normal goat serum (25 μ l) and 1 ml of a 10% (vol/vol) suspension of protein A-Sepharose (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) were added, and extracts were incubated at 4°C for 18 h. Extracts were clarified by centrifugation at 100,000 $\times g$ for 1 h. Specific immunoprecipitates were prepared by incubating 1 ml of labeled extract with 10 μ l of specific hyperimmune goat sera and 100 μ l of a 10% (vol/vol) suspension of protein A-Sepharose at 4°C for 18 h. Immunoprecipitates were collected by centrifugation at 2,000 $\times g$ for 10 min, washed six times in PBSTDS, suspended in 20 μ l of 0.65 M Tris-hydrochloride, pH 6.7, 1% SDS, 10% glycerol, 2.5% 2-mercaptoethanol, and 0.1% bromophenol blue, and heated for 2 min at 90°C. Samples were analyzed by SDS-PAGE, using a 5% acrylamide-0.133% bisacrylamide stacking gel and a 9-cm 5 to 20% linear polyacrylamide separation gel (30:0.8 acrylamide-bisacrylamide) in a Tris-glycine-SDS buffer system at 20 to 30 mA as described by Laemmli (11). Radioactivity was visualized by autoradiography, using Kodak X-Omat S film (30).

Protein kinase assay. Tissue culture cells (10^7) were disrupted by repeated aspiration through a 25-gauge needle in 5 ml of 10 mM Tris-hydrochloride buffer, pH 7.2, containing 100 mM NaCl, 1 mM EDTA, and 1.0% Triton X-100. Lysates were preabsorbed by addition of normal goat serum (25 μ l) and 1.0 ml of a 10% suspension of protein A-Sepharose, incubated overnight at 4°C, and clarified by centrifugation at 100,000 $\times g$ for 1 h. After addition of 5 μ l of specific antiserum, 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 PBSTDS. Protein kinase activity was assayed by suspension of immunoprecipitates in 20 mM Tris-hydrochloride, pH 7.2-5 mM MgCl₂ buffer containing 1 μ Ci of [γ -³²P]ATP (2,000 Ci/mmol; New England Nuclear Corp.) and incubation for 10 min at 30°C. Reactions were terminated by addition of 3 ml of ice-cold PBSTDS, and non-protein-bound ³²P was removed by repeated centrifugation in excess PBSTDS. After the final wash, immunoprecipitates were analyzed by SDS-PAGE as described above.

Phosphoamino acid analysis. Total cellular phosphoproteins were labeled by incubation of 10^7 cells in medium containing 1 mCi of ³²P_i for 12 h at 37°C. Cell monolayers were washed twice with serum-free medium and disrupted in 1 ml of PBSTDS. Phosphoproteins were prepared by twice extracting cell lysates with an equal volume of TNE buffer (50 mM Tris-hydrochloride, pH 7.5, 0.1 M NaCl, and 5 mM EDTA)-equilibrated phenol. The combined phenol phases were twice back-extracted with an equal volume of TNE buffer and diluted to 40 ml with water, and the phosphoproteins were precipitated by the addition of trichloroacetic acid to a final concentration of 15% (vol/vol). Precipitates were collected by centrifugation at 2,000 $\times g$ for 30 min, extracted with a mixture of chloroform and methanol (2:1), and dried

under vacuum; then phosphoamino acid analysis was performed as described elsewhere (17a).

EGF and insulin binding. Cells were trypsinized and distributed to 24-well cluster plates (Costar, Cambridge, Mass.) at around 2×10^5 cells per well. After 24 h of incubation, individual wells were washed once in culture medium containing 10% calf serum and then incubated for 3 h at 37°C in serum-free medium and for an additional 4 h at 4°C in 0.1 ml of serum-free medium containing 0.1% ovalbumin and 2×10^5 cpm of either ^{125}I -labeled mouse EGF (134 $\mu\text{Ci}/\mu\text{g}$; Collaborative Research, Inc., Waltham, Mass.) or ^{125}I -labeled porcine insulin (≈ 100 $\mu\text{Ci}/\mu\text{g}$; New England Nuclear Corp.). Cells were subsequently washed three times in serum-free medium and dissolved in 1% SDS (1 ml), and radioactivity was measured in a gamma scintillation counter.

RESULTS

Isolation of td variants of the Snyder-Theilen strain of FeSV. In an effort to obtain mutants of Snyder-Theilen FeSV defective in expression of transformation, we followed a procedure similar to that previously used for the isolation of mutants of the Abelson strain of MuLV (16, 20). For this purpose, we selected as virus source a culture of Snyder-Theilen FeSV-transformed mink cells producing approximately equal amounts of replication-defective transforming virus and infectious FeLV subgroup B helper virus. Exponentially growing CCL64 mink cell cultures, pretreated for 24 h with medium containing 2 μg of polybrene per ml, were infected with Snyder-Theilen FeSV pseudotype virus at a multiplicity of around 1. Infected cultures were incubated at 37°C for 12 h, trypsinized, and transferred to 96-well Microtest II plates (Falcon Plastics) at a concentration of 0.5 cells per well. Cultures were media changed after 7 days of incubation, and single-cell clones were removed from individual wells by trypsinization and transferred to petri plates (15 by 60 mm) on day 16. After maintenance at 37°C for about 1 week, plates were scored for morphological transformation. Of 280 clones analyzed, 63 were morphologically transformed whereas the remaining 217 were indistinguishable from control CCL64 mink cells.

The above-described clones were subsequently assayed for virus production by analysis of culture fluids for virion-associated RNA-dependent DNA polymerase activity. Of 63 transformed cultures examined, 21 lacked any evidence of detectable virus release. Although, as will be discussed below, each of these clones was morphologically transformed and grew efficiently in soft agar, they all exhibited relatively high frequencies of morphological reversion to the nontransformed phenotype. This phenomenon was observed even after multiple cycles of

recloning, excluding the possibility that the nontransformed cells represented contaminants present in the original cultures (data not shown).

Of 217 nontransformed clones examined, 163 were virus positive, as measured by the reverse transcriptase assay, and were discarded. The remaining 54 cultures were analyzed for FeLV p12 antigenic reactivity by competition immunoassay; of these, 12 were positive. Upon analysis in competition immunoassays for FeLV p30 and gp70, nine of these clones were nonreactive. The remaining three clones reacted positively in both assays and, upon immunoprecipitation with anti-FeLV and subsequent SDS-PAGE analysis, were found to represent replication-defective variants of FeLV subgroup B. These were analogous to other replication-defective type C and type D virus mutants described by our laboratory previously (19) and were not studied further.

Properties of cell clones nonproductively infected by wild-type (wt) and td variants of Snyder-Theilen FeSV. Two of the above transformed and five p12 positive nontransformed clones were selected for further analysis. Both of the morphologically transformed clones exhibited relatively high frequencies (19 to 20%) of colony formation in soft agar in contrast to the nontransformed clones, none of which grew to significant extent ($<0.01\%$ colony formation) (Table 1). By competition immunoassay analysis, levels of expression of FeLV p12 antigenic reactivity were similar in transformed and nontransformed clones, whereas none of the clones was detectably reactive in assays for FeLV p30 or gp70. These findings strongly favor the possibility that the nontransformed clones express the previously described Snyder-Theilen FeSV major high-molecular-weight polyprotein translational product and that the level of expression is comparable to that characteristic of transformed clones. To test this directly, one representative transformed clone, ST-FeSV 64 clone 56, and each of the five nontransformed clones were analyzed by immunoprecipitation and SDS-PAGE. The wt Snyder-Theilen FeSV-transformed and nontransformed clones all expressed an 85,000- M_r protein (P85) precipitable by goat anti-FeLV but not normal goat serum (Fig. 1). Additional proteins seen to varying extents in the different td, but not wt, FeSV-infected clones represented posttranslational cleavage products of P85 (data not shown). Although the major wt Snyder-Theilen FeSV-encoded protein, P85, differed in molecular weight from the 115,000- and 80,000- M_r translational products of the Snyder-Theilen FeSV isolate previously studied by our laboratory (26, 30), its molecular weight was consistent with that pre-

TABLE 1. *Biological properties of cell clones nonproductively infected with td variants of Snyder-Theilen FeSV*

Cell	Morphology	Growth in soft agar ^a	FeLV antigen expression (ng/mg of cell protein) ^b			Retransformation by FeSV (FeLV) ^c	Rescue of transformation competent FeSV ^d
			p12	p30	gp70		
wt FeSV-infected							
ST-FeSV 64							
Clone 56	Transformed	22	320	<2	<10	—	3.8
Clone 103	Transformed	19	290	<2	<10	—	4.1
td FeSV-infected							
ST-FeSV 64							
Clone 82	Nontransformed	<0.01	210	<2	<10	4.2	0.0
Clone 13	Nontransformed	<0.01	430	<2	<10	4.3	0.0
Clone 54	Nontransformed	<0.01	180	<2	<10	3.9	0.0
Clone 79	Nontransformed	<0.01	360	<2	<10	4.1	0.0
Clone 41	Nontransformed	<0.01	270	<2	<10	3.7	0.0
Uninfected control, CCL64	Nontransformed	<0.01	<8	<2	<10	4.0	0.0

^a Plating efficiency in soft agar was tested as previously described (16, 20). Results are expressed as the number of colonies observed at 14 days expressed as a percentage of the input cell number.

^b Cell extracts were prepared and analyzed at serial twofold dilutions in homologous competition immunoassays as described in the text. Results represent mean values from three separate determinations.

^c Susceptibility of individual cell clones to transformation was determined by infection with wt Snyder-Theilen FeSV(4070-A) pseudotype virus at serial 10-fold dilutions according to previously described procedures. Results are expressed as virus titer in focus-forming units per milliliter obtained by using individual clones as recipient assay cells and represent mean values from two separate determinations.

^d Individual cultures were infected with 4070-A amphotropic virus at a multiplicity of infection of around 1. Fourteen days later, culture fluids were harvested and assayed for focus-forming activity at serial 10-fold dilution on control CCL64 mink cells. Cultures were scored for number of transformed foci at 10 days after infection, and results are expressed as number of foci per milliliter of culture fluid.

viously reported for the variant of Snyder-Theilen FeSV produced by the MSTF cell clone (1, 24a).

Lack of expression of transformation in Snyder-Theilen FeSV nonproducer clones is due to a viral rather than cellular defect. In an initial effort to determine whether the transformation defectiveness of the above-described Snyder-Theilen FeSV nonproductively infected clones was due to a lesion in the viral genome itself or represented a defect in cellular function(s) required for expression of the transformed phenotype, clones were analyzed for susceptibility to retransformation by wt Snyder-Theilen FeSV(FeLV) pseudotype virus. All five td Snyder-Theilen FeSV clones examined were as susceptible to retransformation as control CCL64 mink cells. Moreover, although FeSV transforming activity was rescuable from wt Snyder-Theilen FeSV-transformed clones by superinfection with 4070-A amphotropic MuLV, none of the td mutant clones contained rescuable wt transforming virus. The fact that cells infected with FeSV rescued from td mutant clones expressed P80 at levels comparable to those exhibited by wt FeSV-infected clones established that the td FeSV genome was efficiently rescued and transmitted to recipient cultures

(data not shown). These findings argue that the defects in transformation exhibited by the Snyder-Theilen FeSV td clones isolated in the present study are virus rather than cell specific.

Snyder-Theilen FeSV P85 expressed in td clones is defective in protein kinase activity. In view of the above findings, it was of interest to determine whether the 85,000-*M*_r Snyder-Theilen FeSV-encoded polypeptide exhibited associated protein kinase activity analogous to enzymatic activities described for other RNA tumor viruses (3, 8, 16, 32, 33) and, if so, whether any of the td mutant clones exhibited lesions in this activity. Such a possibility would account for expression of levels of P85 in td mutant clones comparable to those characteristic of wt nonproductively transformed clones. P85 immunoprecipitated from a representative wt Snyder-Theilen FeSV-transformed clone, ST-FeSV 64 clone 56, was efficiently labeled in vitro in the presence of [γ -³²P]ATP. In contrast, ³²P labeling of P85 was not observed in immunoprecipitates of any of the four mutant clones examined (Table 2). In a second experiment, we used an antiserum that recognizes a previously described cellular protein kinase, P150 (17, 17a), in addition to Snyder-Theilen FeSV P85. The extent of autophosphorylation of P150 was ap-

FIG. 1. Immunoprecipitation and SDS-PAGE analysis of high-molecular-weight polyproteins expressed in CCL64 mink cells nonproductively infected with either a representative wt Snyder-Theilen FeSV clone ST-FeSV 64 clone 56 (A, B) or td variants of Snyder-Theilen FeSV including ST-FeSV 64 clone 82 (C, D); ST-FeSV 64 clone 13 (E, F); ST-FeSV 64 clone 54 (G, H); ST-FeSV clone 79 (I, J); ST-FeSV 64 clone 41 (K, L). Cell clones were pulse-labeled in [35 S]methionine-containing medium (250 μ Ci/ml) for 30 min, immunoprecipitated with either goat anti-FeLV (lanes A, C, E, G, I, and K) or control goat serum (lanes B, D, F, H, J, and L), and analyzed by SDS-PAGE as described in the text. Molecular weight standards include 14 C-labeled phosphorylase B (98,000), bovine serum albumin (69,000), ovalbumin (46,000), carbonic anhydrase (30,000), and cytochrome (12,000).

proximately the same in control mink cells as in wt and td Snyder-Theilen FeSV clones despite pronounced differences in extent of [γ - 32 P]ATP-mediated labeling of P85 (Fig. 2).

We next undertook to determine whether Snyder-Theilen FeSV P85-associated protein kinase had specificity for phosphotyrosine and, if so, whether constitutive levels of cellular phosphotyrosine were higher in Snyder-Theilen FeSV-transformed clones than in control or td mutant-infected mink clones. Snyder-Theilen FeSV P85 was immunoprecipitated from the ST-FeSV 64 clone 56 cell line, labeled *in vitro* in the presence of [γ - 32 P]ATP, and subjected to phosphoamino acid analysis as described in Materials and Methods. The results were analogous to those reported elsewhere for Gardner FeSV P115 (17a) in that tyrosine represented the major site of polyprotein phosphorylation (data not shown).

To explore the possibility of constitutively higher levels of phosphotyrosine in Snyder-Theilen FeSV-transformed as compared with

control mink cells, 32 P_i-labeled extracts were analyzed with respect to total phosphoamino acid composition. A significant concentration of phosphotyrosine was observed in Snyder-Theilen FeSV-transformed but not control mink cells (Fig. 3). Upon development of autoradiographs for longer time periods, phosphotyrosine was also observed in control mink cells, although at only around 10% of the level in Snyder-Theilen FeSV-transformed cells. Most importantly, of four td Snyder-Theilen FeSV clones subjected to similar analysis, none exhibited levels of phosphotyrosine significantly above those in control mink cells.

The above findings establish that protein kinase activities associated with the 85,000-M_r polyproteins expressed in td Snyder-Theilen FeSV mutant clones are defective or, alternatively, that the polyproteins are unaltered with respect to enzymatic activity but are inactive as substrates. To resolve these alternatives, polyproteins immunoprecipitated from wt or td Snyder-Theilen FeSV-transformed mink clones

TABLE 2. Analysis of tyrosine-specific protein kinase activity in wt and td Snyder-Theilen FeSV nonproductively infected cell clones

Cell	Protein kinase activity ^a	Phosphotyrosine in ^b :	
		Total cell extract	In vitro-labeled casein
wt FeSV-infected			
ST-FeSV 64			
Clone 56	0.6	2.8	5.6
Clone 103	0.5	3.1	4.1
td FeSV-infected			
ST-FeSV 64			
Clone 82	<0.02	<0.3	<0.3
Clone 13	<0.02	<0.3	<0.3
Clone 54	<0.02	<0.3	<0.3
Clone 79	<0.02	<0.3	<0.3
Uninfected control, CCL64	<0.02	<0.3	<0.3

^a Protein kinase activity associated with Snyder-Theilen FeSV P85 was determined as described in the text using anti-FeLV for immunoprecipitation of P85 from extracts. Incorporated radioactivity was quantitated by liquid scintillation counting, and results are expressed as picomoles of ³²P incorporated into trichloroacetic acid-insoluble precipitates corrected for nonspecific radioactivity at zero reaction time.

^b Phosphotyrosine as a percentage of total cellular phosphoamino acids was determined as described in Fig. 3 with the exception that individual ³²P-labeled phosphoamino acids were scraped from thin-layer chromatography plates and radioactivity was quantitated by liquid scintillation counting. Results are expressed as the ratio of ³²P-labeled phosphotyrosine to total ³²P in phosphotyrosine, phosphothreonine, and phosphoserine. The ratio of ³²P-labeled phosphotyrosine to total ³²P-labeled phosphoamino acids in casein phosphorylated in vitro by the Snyder-Theilen P85-associated protein kinase was similarly determined, using labeling conditions described in the legend to Fig. 4.

were analyzed for ability to phosphorylate the tyrosine acceptor site(s) of an external substrate, casein. As shown in Fig. 4 for a representative clone, and summarized for a number of wt and td clones in Table 2, dephosphorylated casein labeled in vitro in the presence of P85 immunoprecipitated from wt Snyder-Theilen FeSV-transformed clones contained phosphotyrosine representing between 4.1 and 5.6% of its total ³²P-labeled phosphoamino acids. In contrast, under similar conditions in the presence of td Snyder-Theilen FeSV P85, no appreciable phosphorylation of casein tyrosine acceptor sites was observed. These findings argue that the defect in [γ -³²P]ATP-mediated in vitro phosphorylation of the 85,000-M_r polypeptides encoded by td mutants isolated in the present study represents lesions in enzymatic rather than substrate activity.

Comparison of EGF binding by wt and td Snyder-Theilen FeSV nonproductively infected cells. Morphological transformation of cells in culture by several RNA transforming viruses, including Snyder-Theilen FeSV, has been shown to result in a pronounced decrease in available EGF binding sites (2, 29). It was thus of interest to examine EGF binding by cell clones infected with td variants of Snyder-Theilen FeSV. ¹²⁵I-labeled EGF binding by Snyder-Theilen FeSV-transformed mink cells was around 10-fold lower than by uninfected control CCL64 mink cells, although extents of ¹²⁵I insulin binding were similar (Table 3). In contrast, none of the five td Snyder-Theilen FeSV clones examined exhibited reduced levels of EGF binding. These findings thus raise the possibility of an association between Snyder-Theilen P85-associated protein kinase activity and EGF binding properties.

Morphological revertants of Snyder-Theilen and Gardner FeSV-transformed cells lack detectable P85 expression. As described above, each of the Snyder-Theilen FeSV nonproductively transformed cell lines isolated in the present study exhibited high frequencies of reversion to nontransformed phenotype. As an independent approach to establish the role of Snyder-Theilen FeSV P85 in transformation, several revertant clones of two independent wt Snyder-Theilen FeSV transformants were further examined. In addition, a previously described morphological revertant of mink cells nonproductively transformed by the Gardner strain of FeSV (27) was subjected to similar analysis. Each of the revertant clones resembled nontransformed control mink cells with respect to lack of growth in soft agar and large numbers of available EGF binding sites (Table 4). Although wt transforming virus was rescuable from each of the revertants examined, the levels of rescue were around 100- to 1,000-fold lower than from morphologically transformed clones. These findings raise the possibility that the defect in expression of transformation by revertant clones may be cellular rather than viral in nature.

Analysis of each of the Snyder-Theilen FeSV revertant clones by competition immunoassay and immunoprecipitation-SDS-PAGE analysis revealed a lack of detectable P85 expression. In contrast, all 21 of the original wt Snyder-Theilen FeSV nonproducer clones exhibited high levels of P85 expression. Finally, constitutive levels of phosphotyrosine in transformed and revertant clones were analyzed. In contrast to each wt transformed clones, none of the morphological revertants exhibited levels of phosphotyrosine significantly above those characteristic of non-

FIG. 2. Gel electrophoretic analysis of protein kinase activities associated with polyproteins expressed in *wt* or *td* Snyder-Theilen FeSV nonproductively infected CCL64 mink cells. Cell clones including CCL64 (A), a *wt* transformant, ST-FeSV 64 clone 56 (B, C), and four *td* isolates, ST-FeSV 64 clone 82 (D), ST-FeSV 64 clone 13 (E), ST-FeSV 64 clone 54 (F); and ST-FeSV clone 79 (G) were disrupted, immunoprecipitated by either a previously described goat serum with specificity for both FeSV P115 and cellular P150 (17a) (lanes A, B, D-G) or control goat serum (C), assayed for [γ^{32} P]ATP phosphotransferase activity, and subjected to SDS-PAGE as described in the text. Molecular weight standards are as described in the legend to Fig. 1.

transformed control mink cells (Table 4). Similarly, the Gardner FeSV nonproductively transformed clone 64 FeSV-14, but not its revertant subclone, 64 FeSV-14-1, expressed P115 at high levels and exhibited elevated levels of total cellular phosphotyrosine. Thus, high levels of polyprotein expression and elevated levels of cellular phosphotyrosine appear to be correlated with expression of transformation in both Snyder-Theilen and Gardner FeSV nonproductively infected clones.

DISCUSSION

The results of the present study favor the possible involvement in transformation of polyprotein translational products of two independently derived FeSV isolates. This was done through the isolation of a series of *td* mutants of the Snyder-Theilen strain of FeSV following an approach analogous to that previously used for

the successful isolation of Abelson MuLV *td* mutants (16, 20). A similar approach was also used by Donner et al. (4) for the isolation of Snyder-Theilen FeSV mutants. Although the levels of P85 expression in each of the mutant clones isolated in the present study were comparable to those characteristic of *wt* FeSV-transformed clones, polyproteins expressed in the mutant clones were subject to much more rapid posttranslational cleavage and lacked detectable associated protein kinase activity. Moreover, the increase in total levels of cellular phosphotyrosine characteristic of mink cell clones transformed by *wt* FeSV was not observed in *td* Snyder-Theilen FeSV clones. These findings, in combination with the invariable presence of P85-associated protein kinase in each of a large number of *wt* FeSV-transformed clones and the loss of P85 expression in morphological revert clones, strongly favor the possibility that P85 is

FIG. 3. Identification of major phosphoamino acids in representative wt and td Snyder-Theilen FeSV-transformed mink cell clones. Cells were labeled in $^{32}\text{P}_i$ -containing medium and subjected to two-dimensional phosphoamino acid analysis as described in the text. Phosphoamino acid standards including phosphothreonine (a), phosphotyrosine (b), and phosphoserine (c) were mixed at equimolar concentration with ^{32}P -labeled hydrolysates of cellular phosphoproteins before analysis. Cell clones analyzed included a wt isolate, ST-FeSV 64 clone 56 (A), and a representative td mutant, ST-FeSV 64 clone 82 (B).

required for Snyder-Theilen FeSV-induced transformation and that the polyprotein-associated protein kinase activity is involved in this process.

The previous demonstration of three methionine-containing tryptic peptides common to the nonstructural components of Gardner and Snyder-Theilen FeSV-encoded polyproteins in combination with the results of molecular hybridization studies (6) argues that the two independently derived isolates of FeSV contain common regions within their acquired sequences (1, 17,

30). Evidence that similarities between the two polyproteins are not only structural but also functional derives from the present demonstration that both possess associated protein kinase activities with specificity for tyrosine acceptor sites. Thus, although genetic studies summarized above were limited to analysis of Snyder-Theilen FeSV, the conclusions with respect to the role of virus-encoded polyproteins in transformation also pertain to Gardner FeSV. Of interest, the variant of Snyder-Theilen FeSV used in the present studies, chosen for its favorable ratio of transforming activity to infectious virus, encodes a polyprotein translational product of around 85,000 M_r , in contrast to the Snyder-Theilen FeSV transformant initially studied by our laboratory, which encodes a major poly-

FIG. 4. Analysis of phosphoamino acid acceptor sites in casein labeled by $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in the presence of Snyder-Theilen FeSV P85. ST-FeSV 64 clone 56 cells (10^7) were disrupted in 5 ml of 10 mM Tris-hydrochloride buffer, pH 7.2, containing 100 mM NaCl, 1 mM EDTA, and 1.0% Triton X-100 (buffer A). Snyder-Theilen FeSV P85 was immunoprecipitated by using 50 μl of goat anti-FeLV as described in the text. Partially dephosphorylated casein (5 μg ; Sigma Chemical Co., St. Louis, Mo.) and 100 μCi of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in 50 μl of buffer A were added to immunoprecipitates, which were then incubated at 25°C for 10 min. After addition of 0.5 ml of PBSTDS, reaction mixtures were subjected to centrifugation at 2,500 rpm for 10 min at 4°C. ^{32}P -labeled casein in supernatant fluids was separated from excess $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ by gel filtration through a column (1 by 20 cm) of Bio-Gel P-10, 100 to 200 mesh (Bio-Rad, Richmond, Calif.) preequilibrated with 10 mM ammonium bicarbonate, lyophilized, and subjected to SDS-PAGE as described in the text. Gel slices containing ^{32}P -labeled casein were washed in 10% methanol, lyophilized, hydrolyzed in 6 N HCl for 1 h at 100°C, mixed with unlabeled phosphoamino acid standards, and subjected to phosphoamino acid analysis as described in the legend to Fig. 3.

protein of 115,000 M_r and an 80,000- M_r probable cleavage product (30). These differences in molecular weights of polyproteins encoded by independent clonal isolates of Snyder-Theilen FeSV might be analogous to the demonstration of polyproteins ranging from 90,000 to 160,000

M_r encoded by Abelson MuLV (18).

The availability of Snyder-Theilen FeSV td mutants and of morphological revertants of Gardner and Snyder-Theilen FeSV-transformed cells provides a genetically controlled system for determining the significance of reduced EGF binding with respect to transformation. Using such clones, we have confirmed and extended previous studies indicating a reduced EGF binding by Gardner (29) and Snyder-Theilen (4) FeSV-transformed mink cells. Although both the results of the present study and our previous findings derived from analysis of cell lines infected by wt and td variants of Abelson MuLV (2) indicate a close correlation between EGF binding and tyrosine-specific protein kinase activity, the significance of this relationship is not known. Such a correlation could reflect the existence of tyrosine acceptor sites within or close to the cellular receptor for EGF, phosphorylation of which results in an impairment to EGF binding. Alternatively, cellular factors such as the recently described sarcoma growth factor (28) could be activated as a result of phosphorylation of specific cellular tyrosine acceptor sites and compete with EGF binding surface receptors.

Although the results of the present study pertain directly only to the role in transformation of polyprotein translational products of Snyder-

TABLE 3. *Polypeptide hormone binding by mink cell clones nonproductively infected by wt and td variants of Snyder-Theilen FeSV*

Cell	¹²⁵ I-labeled polypeptide binding (cpm) ^a		Ratio EGF/insulin binding
	EGF	Insulin	
wt FeSV-infected ST-FeSV 64			
Clone 56	1,650 ± 271	3,541 ± 312	0.47
Clone 103	2,032 ± 401	3,383 ± 413	0.60
td FeSV-infected ST-FeSV 64			
Clone 82	23,680 ± 903	4,852 ± 511	5.17
Clone 13	20,075 ± 212	3,094 ± 681	6.49
Clone 54	28,793 ± 712	3,307 ± 550	8.71
Clone 79	18,995 ± 874	4,201 ± 712	4.52
Clone 41	28,094 ± 720	4,408 ± 519	6.37
Uninfected control, CCL64	19,011 ± 460	2,033 ± 332	9.35

^a Binding of ¹²⁵I-labeled EGF and insulin was measured as described in the text. Results represent mean values ± 1 standard error of triplicate determinations corrected for non-specific binding (1.5 to 4.0% of total) determined in parallel by addition of a 100-fold excess of nonradioactive polypeptide to reaction mixtures.

TABLE 4. *Properties of morphological revertants of mink cells transformed by either the Snyder-Theilen or Gardner strain of FeSV*

Cell	Morphology	Growth in soft agar ^a	Rescue of transformation-competent FeSV ^b	Ratio of EGF/insulin binding ^c	Polyprotein expression (μg/mg of cell protein) ^d	Phosphotyrosine ^e
wt ST-FeSV-infected ST-FeSV 64						
Clone 56	Transformed	21	4.2	0.63	1.3	3.1
Clone 56 R-1	Nontransformed	<0.01	0.9	6.35	<0.02	<0.3
Clone 56 R-8	Nontransformed	<0.01	1.2	5.27	<0.02	<0.3
wt ST-FeSV-infected ST-FeSV 64						
Clone 103	Transformed	19	3.7	0.58	1.4	2.7
Clone 103 R-4	Nontransformed	<0.01	0.3	6.12	<0.02	<0.3
Clone 103 R-7	Nontransformed	<0.01	1.1	4.93	<0.02	<0.3
Clone 103 R-12	Nontransformed	<0.01	0.9	4.80	<0.02	<0.3
wt Gardner FeSV-infected 64 FeSV						
Clone 14	Transformed	25	1.9	0.72	1.6	3.4
Clone 14-1	Nontransformed	<0.01	0.3	6.31	<0.2	<0.3

^a See Table 1.

^b See footnote d, Table 1.

^c See footnote a, Table 3. Extents of binding of ¹²⁵I-labeled insulin were relatively constant, never differing by more than two- to threefold between individual clones.

^d Levels of Snyder-Theilen FeSV P85 and Gardner FeSV P115 expression were calculated on the basis of the results of competition immunoassay for their p12 structural components.

^e See footnote a, Table 2.

Theilen and Gardner strains of FeSV, they also have probable significance with respect to other type C transforming viruses. For instance, there are now a number of examples of type C virus isolates known to encode polypeptide translational products with associated protein kinase activity. These include the two FeSV isolates discussed in the present study, Abelson MuLV (2, 33), and the Fujinami strain of ASV (H. Hanafusa, personal communication). Genetic evidence for the involvement of Abelson MuLV P120 in transformation has been previously reported (2, 16, 18, 20), and we present evidence here that the Gardner and Snyder-Theilen FeSV-encoded polypeptides are involved in transformation. Fujinami ASV has been shown not only to be highly related to a second avian transforming virus, PRC II, on the basis of sequence homology within its acquired components, but also to cross-hybridize with the acquired sequence components of the Snyder-Theilen and Gardner strains of FeSV (24a). Thus, on the basis of both structural and functional considerations, it appears highly probable that the acquired sequence-encoded components of high-molecular-weight polypeptides encoded by the Fujinami and PRC II strains of ASV are also involved in transformation. A further argument favoring the involvement of these diverse virus-encoded polypeptides in transformation relates to their functional similarity to ASV pp60^{src} with respect to phosphorylation of tyrosine acceptor sites (3, 8).

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LITERATURE CITED

- Barbacid, M., A. V. Lauver, and S. G. Devare. 1980. Biochemical and immunological characterization of polypeptides coded for by McDonough, Gardner-Arnstein, and Snyder-Theilen strains of feline sarcoma virus. *J. Virol.* **33**:196-207.
- Blomberg, J., F. H. Reynolds, Jr., W. J. M. Van de Ven, and J. R. Stephenson. 1980. Abelson murine leukemia virus transformation involves loss of epidermal growth factor binding sites. *Nature (London)* **286**:504-507.
- Collett, M. S., A. F. Purchio, and R. L. Erikson. 1980. Avian sarcoma virus-transforming protein kinase activity specific for tyrosine. *Nature (London)* **285**:167-169.
- Donner, L., L. P. Turek, S. K. Ruscetti, L. A. Fedele, and C. J. Sherr. 1980. Transformation-defective mutants of feline sarcoma virus which express a product of the viral *src* gene. *J. Virol.* **35**:129-140.
- Eckhart, W., M. A. Hutchinson, and T. Hunter. 1979. An activity phosphorylating tyrosine in polyoma T antigen immunoprecipitates. *Cell* **18**:925-933.
- 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.
- 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 of cats and dogs. *Nature (London)* **226**:807-809.
- Hunter, T., and B. M. Sefton. 1980. Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. *Proc. Natl. Acad. Sci. U.S.A.* **77**:1311-1315.
- Khan, A. S., D. N. Deobagkar, and J. R. Stephenson. 1978. Isolation and characterization of a feline sarcoma virus-coded precursor polypeptide. *J. Biol. Chem.* **253**:8894-8901.
- Khan, A. S., and J. R. Stephenson. 1977. Feline leukemia virus: biochemical and immunological characterization of *gag* gene-coded structural proteins. *J. Virol.* **23**:599-607.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T₄. *Nature (London)* **227**:680-685.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **192**:262-275.
- 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.
- 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.
- Porzig, K. J., K. C. Robbins, and S. A. Aaronson. 1979. Cellular regulation of mammalian sarcoma virus expression: a gene regulation model for oncogenesis. *Cell* **16**:875-884.
- Reynolds, F. H., Jr., 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.* **36**:374-386.
- Reynolds, F. H., Jr., W. J. M. Van de Ven, and J. R. Stephenson. 1980. Feline sarcoma virus polypeptide P115 binds a host phosphoprotein in transformed cells. *Nature (London)* **286**:504-507.
- Reynolds, F. H., Jr., W. J. M. Van de Ven, and J. R. Stephenson. 1980. Feline sarcoma virus P115-associated protein kinase phosphorylates tyrosine: identification of a cellular substrate conserved during evolution. *J. Biol. Chem.* **255**:11040-11047.
- Rosenberg, N., and O. N. Witte. 1980. Abelson murine leukemia virus mutants with alterations in the virus-specific P120 molecule. *J. Virol.* **33**:340-348.
- Sacks, T. L., S. G. Devare, G. T. Blennerhassett, and J. R. Stephenson. 1978. Nonconditional replication mutants of type C and type D retroviruses defective in *gag* gene coded polypeptide post-translational processing. *Virology* **91**:352-363.
- Sacks, T. L., E. J. Hershey, and J. R. Stephenson. 1979. Abelson murine leukemia virus infected cell lines defective in transformation. *Virology* **97**:231-240.
- 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.
- Scolnick, E. M., E. Rands, S. A. Aaronson, and G. J. Todaro. 1970. RNA-dependent DNA polymerase activity in five viruses: divalent cation requirements. *Proc. Natl. Acad. Sci. U.S.A.* **67**:1789-1796.
- Sherr, C. J., L. A. Fedele, L. Donner, and L. P. Turek. 1979. Restriction endonuclease mapping of unintegrated proviral DNA of Snyder-Theilen sarcoma virus: localization of sarcoma-specific sequences. *J. Virol.* **32**:860-875.
- Sherr, C. J., A. Sen, G. J. Todaro, A. Sliski, and M. Essex. 1978. Pseudotypes of feline sarcoma virus contain an 85,000-dalton protein with feline oncornavirus-associated cell membrane antigen (FOCMA) activity.

- Proc. Natl. Acad. Sci. U.S.A. 75:1505-1509.
- 24a. Shibuya, M., T. Hanafusa, H. Hanafusa, and J. R. Stephenson. 1980. Homology exists among the transforming sequences of avian and feline sarcoma viruses. Proc. Natl. Acad. Sci. U.S.A. 77:6536-6540.
25. Snyder, S. P., and G. H. Theilen. 1969. Transmissible feline fibrosarcoma. Nature (London) 221:1074-1075.
26. Stephenson, J. R., A. S. Khan, A. H. Sliski, and M. Essex. 1977. Feline oncornavirus-associated cell membrane antigen: evidence for an immunologically cross-reactive feline sarcoma virus-coded protein. Proc. Natl. Acad. Sci. U.S.A. 74:5608-5612.
27. Stephenson, J. R., W. J. M. Van de Ven, A. S. Khan, and F. H. Reynolds, Jr. 1980. Characterization of viral-coded polyproteins containing phosphorylated nonstructural components with possible transforming function. Proc. Cold Spring Harbor Symp. 44:865-874.
28. Todaro, G. J., and J. E. DeLarco. 1978. Growth factors produced by sarcoma virus-transformed cells. Cancer Res. 38:4147-4154.
29. Todaro, G. J., J. E. DeLarco, and S. Cohen. 1978. Transformation by murine and feline sarcoma viruses specifically blocks binding of epidermal growth factor to cells. Nature (London) 264:26-30.
30. Van de Ven, W. J. M., A. S. Khan, F. H. Reynolds, Jr., K. T. Mason, and J. R. Stephenson. 1980. Translational products encoded by newly acquired sequences of independently derived feline sarcoma virus isolates are structurally related. J. Virol. 33:1034-1045.
31. 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.
32. Van de Ven, W. J. M., F. H. Reynolds, Jr., 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.
33. Witte, O. N., A. Dasgupta, and D. Baltimore. 1980. Abelson murine leukaemia virus protein is phosphorylated *in vitro* to form phosphotyrosine. Nature (London) 283:826-831.