Snyder-Theilen Feline Sarcoma Virus P85 Contains a Single Phosphotyrosine Acceptor Site Recognized by Its Associated Protein Kinase

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Received 2 December 1980/Accepted 19 February 1981

Cells nonproductively transformed by a variant of the Snyder-Theilen strain of feline sarcoma virus (FeSV) expressed an 85,000-dalton polyprotein (P85) with associated tyrosine-specific protein kinase activity. We identified within this polyprotein a single tyrosine acceptor site for its enzyme activity. This acceptor site, as well as two serine phosphorylation sites localized within the p12 structural component of Snyder-Theilen FeSV P85, was phosphorylated in cells nonproductively transformed by Snyder-Theilen FeSV. In contrast, infection by Snyder-Theilen FeSV transformation-defective mutants resulted in phosphorylation only of the two serine acceptor sites, indicating phosphorylation of the tyrosine acceptor site to be transformation specific. In addition, we describe *in vitro* labeling conditions, using unfractionated cell extracts, which resulted in preferential phosphorylation of the single Snyder-Theilen FeSV tyrosine-specific acceptor site.

Several isolates of replication-defective transforming viruses of chicken (3, 7, 9, 10, 21, 24), mouse (15, 17, 22, 32), and cat (2, 12, 23, 26, 29) origin encode as their major translational products high-molecular-weight polyproteins. These generally consist of amino-terminal *gag* gene structural proteins covalently linked to acquired sequence-encoded nonstructural components. Of the mammalian type C virus-encoded polyproteins studied to date, several have been shown to be highly phosphorylated (15, 23, 27, 30, 33) and to possess intrinsic or closely associated protein kinase activities (1, 20, 30, 31). In each case, these protein kinases exhibit the somewhat novel property of phosphorylating tyrosine residues (1, 16, 19, 30, 31). Evidence for the involvement of this enzymatic activity in transformation has been obtained through analysis of transformation-defective (td) mutants of both Abelson murine leukemia virus (4, 18, 32) and the Snyder-Theilen strain of feline sarcoma virus (FeSV) (16). Moreover, the well-characterized avian sarcoma virus transforming protein pp60^{src} (5, 6, 11, 14) and the polyoma virus middle T antigen (8) have been shown to possess tyrosine-specific protein kinase activity.

Polyproteins of around 115,000 molecular weight (*M_r*) encoded by the Gardner and Snyder-Theilen strains of FeSV (27, 30) as well as

the 120,000-*M_r* Abelson leukemia virus-encoded polyprotein (P120) (18, 30, 31) are known to represent efficient substrates for their associated protein kinase. Cells transformed by an independent variant of Snyder-Theilen FeSV express an 85,000-*M_r* polyprotein (P85) which also exhibits both enzymatic and substrate activity (1, 16). In addition, a 150,000-*M_r* cellular substrate for the Gardner and Snyder-Theilen FeSV polyprotein-associated enzymatic activities has been described (19, 20). Tryptic peptide analysis of Abelson leukemia virus P120 labeled in immunoprecipitate form by its associated protein kinase has revealed a single major tyrosine acceptor site (18). Similarly, under appropriate labeling conditions, avian sarcoma virus pp60^{src} is subject to apparent autophosphorylation involving a single tyrosine-containing peptide (6, 11).

The identification and characterization of an acceptor site(s) for viral polyprotein-associated protein kinases is important in elucidating mechanisms of malignant transformation. The present study was thus undertaken in an effort to identify the tyrosine phosphorylation acceptor site(s) within the Snyder-Theilen FeSV polyprotein translational product. For this purpose, a variant of Snyder-Theilen FeSV encoding an 85,000- rather than a 115,000-*M_r* product was

used. This variant was chosen because of the availability of a number of well-characterized td mutants encoding kinase-defective polyproteins (16). By this means, we have identified a single tyrosine phosphorylation acceptor site in Snyder-Theilen FeSV P85 and established *in vivo* phosphorylation of this residue to be transformation specific.

MATERIALS AND METHODS

Cells and viruses. Cells were grown in Dulbecco's modification of Eagle medium supplemented with 10% calf serum (Colorado Serum Co., Denver) in petri dishes (60 by 15 mm; Falcon Plastics, Los Angeles, Calif.). Cell lines used have been described previously (16) and include subclones of the CCL64 mink cell line nonproductively infected with either of two wild-type (wt) Snyder-Theilen FeSV isolates (ST-FeSV 64-Cl 56 and ST-FeSV 64-Cl 103) or four td variants (ST-FeSV 64-Cl 82, ST-FeSV 64-Cl 13, ST-FeSV 64-Cl 54, and ST-FeSV 64-Cl 79) of Snyder-Theilen FeSV.

Metabolic labeling, immunoprecipitation and SDS-PAGE. Tissue culture cells (10^7) were radioactively labeled by incubation for 2 h in medium containing $^{32}\text{P}_i$ (100 $\mu\text{Ci}/\text{ml}$, carrier-free; New England Nuclear Corp., Boston, Mass.) (29). 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% sodium dodecyl sulfate [SDS]). Normal goat serum (25 μl) 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 a further 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 μl of specific hyperimmune goat antiserum and 100 μl 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 multiple times in PBSTDS, suspended in 20 μl of 0.65 M Tris-hydrochloride (pH 6.7), 1% SDS, 10% glycerol, 2.5% 2-mercaptoethanol, and 0.1% bromophenol blue (electrophoresis sample buffer), and heated for 2 min at 90°C . Samples were analyzed by SDS-polyacrylamide gel electrophoresis (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 (13). Radioactivity was visualized by autoradiography, using Kodak X-Omat S film (28).

Two-dimensional tryptic peptide analysis. Proteins were digested by incubation of SDS-PAGE slices in tolylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin (Worthington Biochemicals Corp., Freehold, N.J.; 50 $\mu\text{g}/\text{ml}$ in 0.05 M ammonium bicarbonate, pH 8.0) for 6 h at 37°C . Supernatant fluids were replaced with a fresh TPCK-trypsin solution for additional digestion at room temperature for 16 h; fractions were pooled, filtered, lyophilized, washed by

suspension in water, and rehydrophilized. Labeled digests were incubated for 2 h at 4°C in 0.1 ml of chilled performic acid (30% H_2O_2 and 90% formic acid [1:9] preincubated for 2 h at room temperature), diluted with 2 ml of water, lyophilized, suspended in electrophoresis buffer (acetic acid-formic acid-water, 15:5:80) (pH 1.9), and spotted on cellulose-coated thin-layer chromatography (TLC) glass plates (10 by 10 cm; EM Laboratories, Inc., Elmsford, N.Y.). Electrophoresis was performed for 30 min at 500 V, and chromatography was conducted in the second dimension in buffer containing butanol, pyridine, acetic acid, and water (32:25:5:20). Radiolabeled tryptic peptides were visualized by autoradiography.

Protein kinase assay. Tissue culture cells (10^7) were disrupted by repeated aspiration through a 25-gauge needle in 5 ml of 10 mM phosphate buffer, pH 7.2, containing 100 mM NaCl, 1.0% Triton X-100, and 5 mM MgCl_2 (PBSTM). Lysates were preabsorbed by addition of normal goat serum (25 μl) 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 μl of specific antiserum, lysates (1 ml) were incubated for 18 h at 4°C , 50 μl 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 resuspension of immunoprecipitates in PBSTM containing 10 μCi of [$\gamma\text{-}^{32}\text{P}$]ATP (2,000 Ci/mmol; New England Nuclear) and incubation for 10 min at 30°C . Reactions were terminated by addition of 3 ml of ice-cold PBSTDS, and non-protein-bound ^{32}P was removed by repeated centrifugation in excess PBSTDS. After the final wash, immunoprecipitates were analyzed by SDS-PAGE as described above.

In other experiments, cells (10^6) were lysed by incubation in 50 μl of PBSTM for 1.0 min at 4°C , 50 μCi of [$\gamma\text{-}^{32}\text{P}$]ATP was added, and incubation was continued for a further 1 min. Reactions were terminated by addition of 50 μl of electrophoresis sample buffer. Alternatively, for samples to be subjected to immunoprecipitation analysis, 5 ml of ice-cold PBSTDS was used to stop reactions.

Phosphoamino acid analysis. ^{32}P -labeled phosphoproteins were digested from polyacrylamide gel slices with TPCK-trypsin, eluted, and lyophilized as described above. Hydrolysis was performed by a 1-h incubation at 100°C in 0.2 ml of 6 N HCl under nitrogen. In other experiments, peptides were scraped from TLC plates, eluted in 0.2 ml of 6 N HCl, and centrifuged at $1,000 \times g$ before hydrolysis. HCl was removed by lyophilization, and phosphoamino acids were dissolved in 10 μl of acetic acid, formic acid, and water (15:5:80) before spotting on TLC plates. Two-dimensional electrophoretic and chromatographic analysis was performed as described previously (19).

RESULTS

Phosphorylation of Snyder-Theilen FeSV P80 in immunoprecipitates. Both we and others have previously reported efficient phos-

phorylation of Snyder-Theilen FeSV P85 in immunoprecipitate form and have shown this activity to primarily involve tyrosine residues (1, 16). In the present study, in order to identify the acceptor site(s) involved in this *in vitro* phosphorylation reaction, Snyder-Theilen FeSV P80 was immunoprecipitated from the previously described (16) nonproductively transformed mink cell line ST-FeSV 64-Cl 56 by anti-FeLV and subjected to *in vitro* phosphorylation in the presence of [γ - 32 P]ATP. In addition to FeSV P85, both the previously described cellular substrate, P150, and immunoglobulin G were phosphorylated (Fig. 1, lane A). In contrast, using either normal goat serum for precipitation (Fig. 1, lane B) or an extract of cells nonproductively infected by a td variant of Snyder-Theilen FeSV cells

(Fig. 1, lane C), no significant 32 P incorporation was observed. After *in vitro* [γ - 32 P]ATP labeling and purification by SDS-PAGE, FeSV P85 was subjected to two-dimensional tryptic peptide analysis as described in Materials and Methods. A single major site of phosphorylation was observed (Fig. 2). Although a number of additional weakly phosphorylated peptides were also present, their number and distribution were somewhat variable from one experiment to the next.

To further characterize the major phosphorylated peptide described above, and to determine its significance with respect to transformation, both *in vitro* [γ - 32 P]ATP-labeled FeSV P85 and the major phosphorylated peptide were hydrolyzed and subjected to two-dimensional phosphoamino acid analysis. Authentic unlabeled phosphoserine, phosphothreonine, and phosphotyrosine standards were included as internal controls. The results obtained establish that although the predominant FeSV P85 phosphorylated amino acid labeled in immunoprecipitates is phosphotyrosine, there are, in addition, minor amounts of phosphoserine and phosphothreonine (Fig. 3A). In contrast, analysis of the major phosphorylated tryptic peptide revealed significant levels only of phosphotyrosine (Fig. 3B). These findings argue that FeSV P85 contains, within a single tryptic peptide, one or more tyrosine acceptor residues for its associated protein kinase activity. Background phosphory-

FIG. 1. SDS-PAGE analysis of protein kinase activity associated with Snyder-Theilen FeSV P85. Cells were disrupted, immunoprecipitated by either goat antiserum against FeLV (A, C) or normal goat serum (B), assayed for [γ - 32 P]ATP phosphotransferase activity, and analyzed by SDS-PAGE as described in the text. Cells tested included the wt Snyder-Theilen FeSV clone ST-FeSV 64 Cl-56 (lanes A and B) and a td mutant clone designated ST-FeSV 64 Cl-82 (lane C). 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). IgG, Immunoglobulin G.

FIG. 2. Two-dimensional tryptic peptide analysis of Snyder-Theilen FeSV P85 immunoprecipitated by anti-FeLV from the ST-FeSV 64 Cl-56 nonproductively transformed cell line, phosphorylated in the presence of [γ - 32 P]ATP, and purified by SDS-PAGE. Tryptic digests (8,000 cpm) were suspended in electrophoresis buffer and spotted on TLC glass plates (0) as described in the text. Separation was by electrophoresis in the first dimension (left to right) and by ascending chromatography in the second dimension.

FIG. 3. Phosphoamino acid analysis of Snyder-Theilen FeSV P85 labeled in immunoprecipitates in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Samples analyzed included: (A) total ^{32}P -labeled Snyder-Theilen P85 prepared by immunoprecipitation, *in vitro* phosphorylation, and SDS-PAGE as described in Fig. 1; (B) the major Snyder-Theilen FeSV *in vitro*-phosphorylated tryptic peptide isolated as described in Fig. 2. Phosphoamino acid determinations were performed as described in the text; the positions of unlabeled phosphoamino acid standards including phosphothreonine (p-thr), phosphotyrosine (p-tyr), and phosphoserine (p-ser) are indicated by dotted circles.

lation of serine and threonine residues was presumably due to low levels of contaminating cellular protein kinases in immunoprecipitates.

Snyder-Theilen FeSV P85 tyrosine acceptor site is phosphorylated *in vivo*. In view of the above findings, it was important to determine whether the major FeSV P85 phosphorylation site was of physiological significance or simply represented an artifact of the *in vitro* labeling conditions used. Snyder-Theilen FeSV P85 was thus labeled by incubation of the ST-FeSV 64-Cl 56 cell line in $^{32}\text{P}_i$ -containing medium for 2 h. FeSV P85 was recovered by immunoprecipitation using a high-titered antiserum against FeLV, purified by SDS-PAGE, and subjected to two-dimensional tryptic peptide analysis. A total of three well-resolved peptides were observed (Fig. 4A). Phosphoamino acid analysis indicated that two of these, designated "a" and "b" (Fig. 5A and B), were found to

FIG. 4. Tryptic peptide analysis of *in vivo* ^{32}P -labeled Snyder-Theilen FeSV P85. Mink cells nonproductively infected by either (A) wt (ST FeSV 64 Cl 56) or (B) td (ST-FeSV 64 Cl 82) FeSV were pulse-labeled by overnight incubation in $^{32}\text{P}_i$ -containing medium. ^{32}P -labeled P85 was immunoprecipitated by antiserum to FeLV and purified by SDS-PAGE. Tryptic peptide analysis was performed as described in the legend to Fig. 2.

contain phosphoserine, whereas the third, "c" (Fig. 5C), contained tyrosine as its predominant phosphorylated species. The migration position of the *in vivo*-labeled phosphotyrosine-containing peptide appeared analogous to that of the tryptic peptide containing the tyrosine acceptor site labeled in immunoprecipitates. This was confirmed by a mixing experiment (data not shown).

To determine whether phosphorylation of this major tyrosine acceptor site, ^{32}P labeled both in immunoprecipitates and *in vivo*, is relevant to transformation, P85 was isolated by immunoprecipitation from mink cells nonproductively in-

FIG. 5. Phosphoamino acid analysis of individual *in vivo* $^{32}\text{P}_i$ -labeled Snyder-Theilen FeSV P85 tryptic peptides purified by immunoprecipitation and SDS-PAGE as described in Fig. 4A. Phosphoamino acid analysis was performed as described in the legend to Fig. 3. Individual peptides are designated (A) "a," (B) "b," and (C) "c" on the basis of their migration positions indicated in Fig. 4A. Analysis was performed as described in the legend to Fig. 3.

fected by a representative td variant of Snyder-Theilen FeSV and subjected to tryptic peptide mapping analysis. Two peptides were observed migrating at positions corresponding to peptides "a" and "b" in the wt Snyder-Theilen FeSV-transformed clone (Fig. 4B). Upon further analysis, both peptides were found to contain phosphoserine as their predominant phosphorylated

species (data not shown). In contrast, the single Snyder-Theilen FeSV P85 phosphotyrosine-containing tryptic peptide was not detectably labeled. Similarly, in a more extensive analysis involving an additional wt and three additional td Snyder-Theilen FeSV nonproductively infected clones, expression of transformation was invariably associated with phosphorylation of the single tyrosine acceptor site (Table 1). These findings establish that phosphorylation of the major P85 tyrosine phosphate acceptor site is transformation specific and are consistent with our previous results indicating the FeSV P85-associated protein kinase to be defective in cell clones nonproductively infected by td variants of Snyder-Theilen FeSV (16).

Localization of phosphoserine residues within the p12 structural component of Snyder-Theilen FeSV P85. In a previous study, we described an isolate of Snyder-Theilen FeSV encoding, as its major translational product, a 115,000-*M_r* polyprotein (28). This virus-encoded protein was shown to contain several major phosphorylated tryptic peptides, two of which were localized within its p12 structural component (28, 30). We therefore reasoned that since P85 also contains p12, the two above-described phosphoserine-containing peptides may be p12 specific. To directly test this possibility, we compared the tryptic peptide maps of FeSV P85 and FeLV p12 after $^{32}\text{P}_i$ labeling. The results

TABLE 1. Phosphorylation of P85 in wt and td Snyder-Theilen nonproductive infected cell clones

Cell clone	Acceptor sites phosphorylated ^a		
	Serine (a)	Serine (b)	Tyrosine (c)
wt FeSV-infected			
ST-FeSV 64			
Cl-56	+	+	+
Cl-103	+	+	+
td FeSV-infected			
ST-FeSV 64			
Cl-82	+	+	—
Cl-13	+	+	—
Cl-54	+	+	—
Cl-79	+	+	—
Uninfected control, CCL64	—	—	—

^a After labeling of cells overnight in $^{32}\text{P}_i$ containing medium, FeSV P85 was purified by immunoprecipitation and SDS-PAGE and subjected to two-dimensional tryptic peptide analysis as described in the legend to Fig. 4. Results are expressed as either the presence (+) or absence (—) of phosphorylation of individual phosphopeptides designated as "a," "b," or "c" corresponding to their TLC migration positions in Fig. 4A.

obtained indicated correspondence in migration position between the two FeLV p12 phosphopeptides (Fig. 6A) and two P85 phosphoserine-containing tryptic peptides (Fig. 4). Moreover, phosphoamino acid analysis of the FeLV p12 peptides established both to consist predominantly of phosphoserine (data not shown). Finally, in a reconstruction experiment involving a mixture of the two *in vivo*-labeled p12 phosphoserine peptides (Fig. 6A) and the single *in vitro*-labeled phosphotyrosine peptide (Fig. 6B), a tryptic map was obtained which was indistinguishable from that derived by direct analysis of *in vivo* ^{32}P -labeled wt FeSV P85 (Fig. 6C). Although these findings do not establish the location of the single phosphotyrosine acceptor site within FeSV P85, it is clear that both phosphoserine acceptor sites are localized in the p12 structural component.

Phosphorylation of P85 in extracts of Snyder-Theilen FeSV-transformed cells. In an effort to develop an *in vitro* system for phosphorylation of the FeSV P85 tyrosine acceptor

in the absence of immunoglobulin, labeling in total unfractionated cell extracts was examined. For this purpose, phosphotransferase reactions were carried out for 1 min at 4°C, conditions that were found to result in preferential phosphorylation of tryosine acceptor sites. Phosphorylation of a single major protein of around 85,000 *M*, as well as, to a lesser extent, numerous minor proteins distributed over a relatively wide molecular weight range was obtained (Fig. 7, lane A). The major 85,000-*M*, phosphorylated protein was precipitated by anti-FeLV (Fig. 7, lane B) but not by normal goat serum (Fig. 7, lane C), identifying it as Snyder-Theilen FeSV P85. That the phosphorylation observed was attributable to the P85-associated protein kinase was indicated by the absence of P85 labeling in extracts of Snyder-Theilen FeSV td mutant-infected cells (Fig. 7, lane d).

To determine whether the above preferential labeling of P85 in extracts of Snyder-Theilen FeSV-transformed cells did, in fact, correspond to phosphorylation of tyrosine residues, the la-

FIG. 6. Comparison by two-dimensional tryptic peptide analysis of (A) the two individual phosphopeptides localized within the p12 component of FeLV and (B) the single Snyder-Theilen FeSV P85 peptide containing the tyrosine acceptor site for P85-associated protein kinase activity. ^{32}P -labeled FeLV p12 was prepared as previously described (30); the major P85 phosphotyrosine-containing peptide was ^{32}P labeled and purified as described in Fig. 2. Panel C shows the results obtained from analysis of a 1:1 mixture of samples tested in panels A and B. Tryptic peptide analysis was performed as described in the legend to Fig. 2.

beled protein was purified by immunoprecipitation and SDS-PAGE and subjected to phosphoamino acid analysis. Under these labeling conditions, the major ^{32}P -labeled FeSV P85 amino acid was tyrosine (Fig. 8A). In fact, even in the total unfractionated cell extract, tyrosine was the most intensely labeled amino acid (Fig. 8B). That ^{32}P labeling of FeSV P85 involves exclusively a single site indistinguishable from that phosphorylated *in vivo* in wt FeSV-transformed cells was established by tryptic peptide analysis (Fig. 9). Finally, the identity of this single ^{32}P -labeled peptide as phosphotyrosine was confirmed by phosphoamino acid analysis (data not shown).

DISCUSSION

In this study, we have identified within the major 85,000- M_r translational product of a var-

iant of the Snyder-Theilen strain of FeSV a single tyrosine acceptor site for its associated protein kinase activity. This peptide was phosphorylated in immunoprecipitates and in unfrac-

FIG. 7. $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ -mediated phosphorylation of Snyder-Theilen FeSV P85 in unfractionated total cell extracts. Cells (10^6) including the wt Snyder-Theilen FeSV clone ST-FeSV 64 Cl-56 (lanes A-C) and a *td* mutant, ST-FeSV 64 Cl-82 (lane D), were lysed by incubation in 50 μl of PBSTM for 1 min at 4°C. $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (50 μCi) was added, and incubation was continued for a further 1 min. Reactions were terminated by addition of 50 μl of electrophoresis sample buffer, and samples were subjected to direct SDS-PAGE analysis as described in the text (lane A). Alternatively, 5 ml of cold PBSTDs was used to stop reactions, and samples were immunoprecipitated by either anti-FeLV (lanes B and D) or normal goat (lane C) serum before SDS-PAGE analysis. Molecular weight standards are as described in the legend to Fig. 1.

FIG. 8. Phosphoamino acid analysis of Snyder-Theilen FeSV P85, ^{32}P labeled as described in the legend to Fig. 7, and purified by either (A) SDS-PAGE after immunoprecipitation by anti-FeLV or (B) SDS-PAGE only. Phosphoamino acid analysis was performed as described in the legend to Fig. 3.

FIG. 9. Tryptic peptide analysis of Snyder-Theilen FeSV P80, ^{32}P labeled and purified by immunoprecipitation and SDS-PAGE as described in the legend to Fig. 7. Tryptic peptide analysis was performed as described in the legend to Fig. 2.

tionated cell extracts, using [γ - 32 P]ATP as phosphate donor, and also after 32 P_i labeling of cells in vivo. Lower levels of phosphorylation of additional peptides in immunoprecipitates, primarily involving serine and threonine residues, appear to be nonspecific and may represent the presence of a protein kinase associated with previously described 150,000-*M*, cellular protein with binding affinity for the Snyder-Theilen FeSV P85 enzyme (19). That phosphorylation of the major P85 tyrosine acceptor in vivo is attributable to the Snyder-Theilen FeSV-associated protein kinase is established by the absence of labeling in Snyder-Theilen FeSV td mutant-infected cells. In addition to the major tyrosine phosphorylation acceptor site, two in vivo-labeled serine acceptor sites are identified. Although we have localized the two serine phosphorylation acceptor sites within the p12 structural component of P85, the location of the tyrosine acceptor site remains to be established.

Of interest, by carrying out phosphorylation reactions with total unfractionated cell extracts at 4°C for very short times (1 min), preferential labeling of a single major substrate was observed. This protein was identified as Snyder-Theilen FeSV P85 based on molecular weight and immunological properties. Moreover, by tryptic peptide analysis all of the phosphorylation observed was localized within the single P85 tyrosine acceptor site. The preferential phosphorylation of FeSV P85 by this means was striking, thus favoring the possibility that this protein kinase activity represents an intrinsic property of the polyprotein itself, rather than an associated cellular enzyme. Available genetic evidence in which the 85,000-*M*, polyprotein translational products of each of a number of Snyder-Theilen FeSV td mutants were shown to lack detectable protein kinase activity is also consistent with the possibility that Snyder-Theilen FeSV is subject to autophosphorylation (16).

An as yet unresolved question is whether phosphorylation of the above-described P85 tyrosine acceptor site is a necessary event for Snyder-Theilen FeSV-induced transformation. Alternatively, the presence of a tyrosine acceptor site within FeSV P85 could be fortuitous and serve only as a convenient phenotypic marker for the P85-associated protein kinase activity. One line of evidence arguing that phosphorylation of a tyrosine acceptor site(s) within virus-encoded transforming proteins is of functional significance is the fact that, of the viral proteins studied to date, all those with protein kinase activity have also proven to represent active substrates (1, 27, 30, 31). Similarly, the 21,000-*M*, translational product of the Harvey strain of murine sarcoma virus has been shown to repre-

sent a natural substrate for an associated threonine-specific GTP phosphotransferase activity (25). It is possible, however, that although phosphorylation of virus-encoded transforming proteins may be necessary for transformation, other primary acceptor sites may also be involved. In this respect, a recently described 150,000-*M*, cellular phosphoprotein which both binds to and contains one or more tyrosine acceptor sites phosphorylated by the Gardner and Snyder-Theilen FeSV protein kinases is of potential importance (19, 20).

The identification of a single tryptic peptide containing one or more phosphorylation acceptor sites for the FeSV P85-associated protein kinase may provide a means for determining the significance of tyrosine phosphorylation in transformation. For instance, it should be possible to purify and subject this peptide to amino acid sequence analysis. Such information should be of value for comparing the primary structure of phosphorylation acceptor sites for tyrosine-specific protein kinases within translational products of independent transforming virus isolates of avian and mammalian origin. In addition, the synthesis of competitive inhibitors for the tyrosine acceptor sites might be feasible, thus providing a direct test of their involvement in transformation.

ACKNOWLEDGMENTS

This work was supported under Public Health Service contract N01-CO-75380 from the National Cancer Institute and by a fellowship to J.B. from the Nordic Cancer Union.

The excellent secretarial assistance provided by Barbara Eader is gratefully acknowledged.

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**:5158-5162.
2. 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.
3. Bister, J., M. J. Hayman, and P. K. Vogt. 1977. Defectiveness of avian myelocytomatosis virus MC29: isolation of long-term nonproducer cultures and analysis of virus-specific polypeptide synthesis. *Virology* **82**:431-448.
4. Blomberg, J., F. H. Reynolds, Jr., W. J. M. Van de Ven, and J. R. Stephenson. 1980. Abelson murine leukaemia virus transformation involves loss of epidermal growth factor binding sites. *Nature (London)* **286**:504-507.
5. 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**:2021-2024.
6. Collett, M. S., A. F. Purchio, and R. L. Erikson. 1980. Avian sarcoma virus-transforming protein pp60^{src} shows kinase activity specific for tyrosine. *Nature (London)* **285**:167-169.
7. Duesberg, P. H., and P. K. Vogt. 1979. Avian acute

- leukemia viruses MC29 and MH2 share specific RNA sequences: evidence for a second class of transforming genes. *Proc. Natl. Acad. Sci. U.S.A.* **76**:1633-1637.
8. Eckhart, W., M. A. Hutchinson, and T. Hunter. 1979. An activity phosphorylating tyrosine in polyoma T antigen immunoprecipitates. *Cell* **18**:925-933.
 9. 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.
 10. Hayman, M. J., B. Royer-Pokora, and T. Graf. 1979. Defectiveness of avian erythroblastosis virus: synthesis of a 75K gag-related protein. *Virology* **92**:31-45.
 11. 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.
 12. 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.
 13. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680-685.
 14. 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.
 15. Reynolds, F. H., Jr., T. L. Sacks, D. N. Deobagkar, and J. R. Stephenson. 1978. Cells nonproductively transformed by Abelson murine leukemia virus express a high molecular weight polypeptide containing structural and nonstructural components. *Proc. Natl. Acad. Sci. U.S.A.* **75**:3974-3978.
 16. Reynolds, F. H., Jr., W. J. M. Van de Ven, J. Blomberg, and J. R. Stephenson. 1981. Involvement of a high molecular weight polypeptide translational product of Snyder-Theilen FeSV in malignant transformation. *J. Virol.* **37**:643-653.
 17. Reynolds, R. K., W. J. M. Van de Ven, and J. R. Stephenson. 1978. Translation of type C viral RNAs in *Xenopus laevis* oocytes: evidence that the 120,000-molecular-weight polypeptide expressed in Abelson virus-transformed cells is virus coded. *J. Virol.* **28**:665-670.
 18. 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.
 19. 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.
 20. 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**:409-412.
 21. Roussel, M., S. Saule, C. Lagrou, C. Rommens, H. Beug, T. Graf, and D. Stehelin. 1979. Three new types of viral oncogenes of cellular origin specific for haematopoietic cell transformation. *Nature (London)* **281**:452-455.
 22. Sacks, T. L., F. H. Reynolds, Jr., D. N. Deobagkar, and J. R. Stephenson. 1978. Murine leukemia virus (T-8) transformed cells: identification of a precursor polypeptide containing *gag* gene-coded proteins (p15 and p12) and a nonstructural component. *J. Virol.* **27**:809-814.
 23. 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.
 24. 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. Shih, T. Y., A. G. Papageorge, P. E. Stokes, M. O. Weeks, and E. M. Scolnick. 1980. Guanine nucleotide-binding and autophosphorylating activities associated with the p21^{src} protein of Harvey murine sarcoma virus. *Nature (London)* **287**:686-691.
 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, F. H. Reynolds, Jr. 1980. Mammalian RNA type-C transforming viruses: characterization of viral-coded polypeptides containing phosphorylated nonstructural components with possible transforming function. *Proc. Cold Spring Harbor Symp.* **44**:865-874.
 28. 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.
 29. Van de Ven, W. J. M., F. H. Reynolds, Jr., R. P. Nalewaik, and J. R. Stephenson. 1980. Characterization of a 170,000-dalton polypeptide encoded by the McDonough strain of feline sarcoma virus. *J. Virol.* **35**:165-175.
 30. Van de Ven, W. J. M., F. H. Reynolds, Jr., and J. R. Stephenson. 1980. The nonstructural components of polypeptides encoded by replication-defective mammalian transforming retroviruses are phosphorylated and have associated protein kinase activity. *Virology* **101**:185-197.
 31. 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.
 32. Witte, O. N., S. Goff, N. Rosenberg, and D. 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.
 33. Witte, O. N., N. Rosenberg, M. Paskind, A. Shields, and D. Baltimore. 1978. Identification of an Abelson murine leukemia virus-encoded protein present in transformed fibroblasts and lymphoid cells. *Proc. Natl. Acad. Sci. U.S.A.* **75**:2488-2492.