Mutants of Fujinami Sarcoma Virus Which Are Temperature Sensitive for Cellular Transformation and Protein Kinase Activity

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Two temperature-sensitive mutants of Fujinami sarcoma virus were isolated and characterized. Cells infected with the mutants were temperature sensitive in focus formation, colony formation, increased sugar uptake, and synthesis of plasminogen activator. The changes between transformed and nontransformed states of cultures were completely reversible by shifting the temperature. A Fujinami sarcoma virus-specific protein of 130,000 daltons, p130, was synthesized in mutant-infected cells regardless of the temperature, but the immunoprecipitates of p130 from extracts of infected cells were active in protein kinase only when cells had been incubated at the permissive temperature. These results appear to indicate that p130 is the transforming protein of Fujinami sarcoma virus, and that its protein kinase activity plays a crucial role in cell transformation by this virus.

Mutants of RNA tumor viruses which are temperature sensitive (*ts*) in cell transformation have been extremely useful in analysis of the process of transformation by these viruses. First, the existence of mutants which are sensitive only for transformation provided the first clear indication that the product of a viral gene is directly responsible for cellular transformation (8, 10, 18, 24, 28). Second, *ts* mutants were used to establish that the product of the viral transforming gene has a particular enzyme activity such as protein kinase (3, 7, 17, 20).

Fujinami sarcoma virus (FSV) is one of the recently characterized avian sarcoma viruses (11, 16) whose transforming gene is not homologous to the *src* gene of Rous sarcoma virus (RSV). The genome of FSV consists of a relatively short (28S) RNA containing unique sequences of about 3,000 nucleotides which are flanked with 5' and 3' termini of retroviral sequences including a part of the *gag* gene (11, 16). As a product of the fused gene with *gag*, p140, a protein of 140 kilodaltons (140K), has been identified in FSV-transformed cells by immunoprecipitation with antisera against *gag* proteins. This p140 is likely to be involved directly in the process of cell transformation in FSV infection, because (i) p140 is detectable only in FSV-transformed cells and not in helper-infected cells, (ii) p140 is the only detectable product of the FSV genome, and (iii) p140 is a phosphoprotein associated with a protein kinase

activity which phosphorylates only tyrosine residues of the substrate proteins (7a), an activity similar to those of transforming proteins of RSV and Abelson leukemia virus (5, 13, 26).

In this paper, we describe the isolation of two mutants of FSV, which are both temperature sensitive in cell transformation. Like many T-class RSV *ts* mutants, these *ts* mutants of FSV produce transformed cells which can be reversibly converted to either a transformed or a non-transformed state by shifting the temperature of incubation. p140 was detectable in the *ts* mutant-infected cells at both permissive and non-permissive temperatures, but the protein kinase activity of the cells was sharply reduced at the nonpermissive temperature.

MATERIALS AND METHODS

Cells and viruses. Preparation of chicken embryo fibroblasts, FSV, Fujinami-associated virus (FAV), and Schmidt-Ruppin RSV subgroup B was described previously (11). To mutagenize the virus, chicken cells fully transformed with an FSV stock (which contains FAV) were treated with medium containing 25 μ g of 5-azacytidine (Calbiochem-Behring Corp., La Jolla, Calif.) per ml for 24 h. Virus obtained from the treated cultures was used for colony formation in soft agar. Colonies were isolated and cultured together with fresh chicken cells, and FAV was added to each culture to allow FSV production.

Sugar uptake. The rate of uptake of 2-[1,2-³H]deoxyglucose (40 Ci/mmol; New England Nuclear Corp., Boston, Mass.) was determined as de-

scribed by Kawai and Hanafusa (14), with minor modifications: phosphate-buffered saline (pH 7.4) was used instead of Hanks balanced salt solution, and the isotope solution of 2-[^3H]deoxyglucose was made at a concentration of 1 $\mu\text{Ci}/\text{ml}$ of phosphate-buffered saline.

Plasminogen activator. The level of plasminogen activator in a culture was determined by the casein overlay technique of Goldberg (9).

Labeling cultures. For labeling with [^{35}S]methionine, cultures were preincubated with methionine-free minimal essential medium (GIBCO Laboratories, Grand Island, N.Y.) containing 2% calf serum for 1 h. Then the medium was replaced with 1 ml (for 60-mm dishes) or 0.4 ml (for 35-mm dishes) of fresh medium containing 250 μCi of L-[^{35}S]methionine (1,000 to 1,200 Ci/mmol; New England Nuclear Corp.) per ml and incubated for 5 or 9 h as described in the figure legends. For $^{32}\text{P}_i$ labeling, cultures grown in 35-mm plates were incubated in 0.7 ml of phosphate-free medium 199 (GIBCO) containing 2% dialyzed calf serum. After 3 h, the medium was replaced with fresh medium containing 1.4 mCi of carrier-free $^{32}\text{P}_i$ (Amersham Corp., Arlington Heights, Ill.) per ml and incubated for 13 h, after which time the cells were harvested.

Detection of FSV protein and protein kinase assay. The procedures for preparing the cell extracts, the immunoprecipitation, the kinase assay, and the identification of the phosphoamino acids were described in detail elsewhere (7a). The antisera used in this study were raised in rabbits immunized against total viral proteins of disrupted Rous-associated virus 2 (RAV-2) virions.

Determination of ^{32}P -labeled phosphoamino acids in total cell extracts. ^{32}P -labeled cells were lysed in RIPA buffer (50 mM Tris-hydrochloride, 150 mM NaCl, 25 mM EDTA, 1% Trasylol, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [pH 7.4]), and the extraction of phosphoproteins and the analysis of phosphoamino acids was done by the method of Hunter and Sefton (13), except that the hydrolysis of proteins was for 2.5 h in vacuo.

RESULTS

Isolation of *ts* mutants. The stock of FSV used as the parental wild type in this study was obtained from a single colony derived from the original stock of FSV (11). This wild-type virus was selected because it produced round, distinctive transformed cells at 41 to 42°C. This virus was somewhat temperature dependent, in that morphological transformation proceeded faster at a temperature above 39°C, whereas the focus-forming ability was not different at 37 or 41°C. We also found that the molecular mass of the unique protein of this wild-type FSV clone was 130K rather than 140K for the original FSV (see Fig. 2). This wild-type clone was indistinguishable from the original virus stock in colony formation, in tumor formation, in extent of hybridization of viral RNA with DNA complementary to the unique sequences of FSV (22a), and by

fingerprint pattern of RNase T₁-resistant oligonucleotides.

Cultures infected with the wild-type FSV were treated with 5-azacytidine for 24 h. This treatment resulted in the reduction of the level of virus production to 1/100 of untreated control cultures. Virus obtained from these treated cultures was used for colony formation, and individual colonies were examined for the temperature sensitivity of their morphological alteration of infected cells. Of 116 colonies examined, 11 colonies appeared to be *ts*. The virus obtained from two of them, *ts*NY225 and *ts*NY240 (these will be called NY225 and NY240, respectively, in this paper), were extensively characterized.

Nature of *ts* mutants. Transformed cells derived from colonies of NY225 and NY240 were morphologically different at 37 and 42°C. The comparison of morphology of wild type- and NY240-infected cells at 37 and 41°C is shown in Fig. 1. Of the two, NY225 appeared to have a lower temperature threshold for morphological changes than had NY240. When infected cultures were placed at various temperatures, NY225 became flat at 39 to 40°C, but NY240 did not revert until 41 to 42°C. For both mutants the morphological states could be reversed by shifting the temperature up or down several times. The disappearance of the morphological transformation by shifting to a higher temperature often required more than 24 h, whereas transformation by shifting to a lower temperature was usually complete within 24 h.

The abilities of these mutants to produce foci and colonies at permissive and nonpermissive temperatures are shown in Tables 1 and 2. Formation of foci or colonies was profoundly suppressed at the nonpermissive temperatures.

Since FSV is defective in replication function, cells singly infected with this virus produce no infectious progeny (11). A stock of FSV is generally obtained from massively infected cultures which are also infected with a helper virus, FAV. Therefore, most, if not all, cells in cultures infected with the high titers of FSV are doubly infected with FSV and FAV and thus are virus producers. When such double infections with FSV and FAV were performed with NY225 or NY240 and infected cultures were held at the nonpermissive temperatures, cultures were morphologically normal, but produced normal amounts of FSV which made foci only at the permissive temperature (Table 3). The results indicate that the capacity of the *ts* mutants of FSV to be packaged as a pseudotype was not impaired at the nonpermissive temperature.

FSV-transformed chicken cells showed an increased rate of glucose uptake compared with uninfected cells, as demonstrated with RSV-

FIG. 1. Photomicrographs of uninfected, FSV wild type-infected, and *ts* mutant-infected cells. Chicken embryo cells were infected with FSV wild type or NY240, and after the cultures were fully transformed, these cultures and uninfected cultures were subcultured and incubated at 37 and 42°C for 2 days. Uninfected cells at 37°C (A) and 42°C (D); NY240-infected cells at 37°C (B) and 42°C (E); and wild type-infected cells at 37°C (C) and 42°C (F).

TABLE 1. Focus formation by *ts* mutants of FSV

Virus	Dilution	No. of foci at:			
		37°C		42°C	
Wild type	10 ⁻²	TMTC, ^a	TMTC	TMTC,	TMTC
	10 ⁻³	121,	134	166,	180
	10 ⁻⁴	11,	13	15,	12
NY225	10 ⁰	TMTC,	TMTC	0,	0
	10 ⁻¹	TMTC,	TMTC	0,	0
	10 ⁻²	305,	386	0,	0
	10 ⁻³	31,	36	0,	0
NY240	10 ⁰	TMTC,	TMTC	2,	1
	10 ⁻¹	TMTC,	TMTC	1,	0
	10 ⁻²	510,	652	0,	0
	10 ⁻³	30,	45	0,	0

^a TMTC, Too many to count.

TABLE 2. Colony formation in soft agar culture by *ts* mutants of FSV

Virus	Dilution	No. of colonies at:			
		37°C		42°C	
Wild type	10 ⁻¹	100,	120	122,	152
	10 ⁻²	50,	52	25,	43
	10 ⁻³	3,	8	3,	2
NY225	10 ⁻¹	TMTC, ^a	TMTC	3,	0
	10 ⁻²	TMTC,	TMTC	0,	0
	10 ⁻³	55,	90	0,	0
NY240	10 ⁻¹	TMTC,	TMTC	8,	10
	10 ⁻²	225,	250	1,	0
	10 ⁻³	20,	28	0,	0

^a TMTC, Too many to count.

transformed cells (10, 12). As shown in Table 4, the enhanced sugar uptake was also temperature sensitive with *ts* mutant-infected cells. This property of FSV *ts* mutant-infected cells was

also repeatedly reversible by temperature shift (data not shown).

As another parameter, the level of plasminogen activator of *ts* mutant-infected cells was examined by the casein overlay technique (9).

TABLE 3. *Virus production at permissive and nonpermissive temperatures*^a

Virus	Titer of virus produced at:	
	37°C	41°C
Wild type	5.5×10^6	6.0×10^6
NY225	3.4×10^6	2.8×10^6
NY240	2.5×10^6	2.7×10^6

^a Cells were infected with wild type, NY225, or NY240 of FSV at a multiplicity of infection of 1, and cultures were kept at 37 and 41°C for 4 days. Culture fluids were assayed for focus-forming virus at 37°C.

TABLE 4. *Sugar uptake by cells infected with ts mutants*^a

Cells infected with:	Rate of uptake of 2-deoxyglucose at:			
	37°C		41°C	
	cmp/μg of cell protein	Ratio to control	cmp/μg of cell protein	Ratio to control
Uninfected (control)	97	1.0	124	1.0
FSV wild type	482	5.0	795	6.4
FSV NY225	514	5.3	145	1.2
FSV NY240	570	5.9	158	1.3
Schmidt-Ruppin RSV subgroup B	750	7.7	960	7.7

^a Cells infected and transformed with various viruses were subcultured and incubated at 37 and 41°C for 2 days. These infected cultures and uninfected control cultures were labeled with 2-[³H]deoxyglucose for 10 min. The radioactivity associated with cells was measured as described in the text.

FSV-transformed cells formed clear plaques, as did RSV-transformed cells, and this property was *ts* for both NY225 and NY240 (data not shown).

Transforming protein of *ts* mutants. Figure 2 shows the gel electrophoretic pattern of proteins obtained from [³⁵S]methionine-labeled, FSV-transformed cells. The wild-type virus clone was found to produce a 130K protein (p130) rather than the 140K protein of the original stock of FSV. To determine the amount of p130 in *ts* mutant-infected cultures, *ts* mutant-infected cultures were kept at 37 and 42°C for at least 24 h. Cultures which were confirmed for their transformed or nontransformed state were labeled for 9 h with [³⁵S]methionine at 37 or 42°C, respectively. As shown in Fig. 3, the amount of p130 was identical at the two temperatures.

Sefton et al. (21) showed that the level of pp60^{src} protein kinase activity in extracts of some

RSV *ts* mutant-infected cells is strongly affected by the extraction buffer. Virtually no enzyme activity was demonstrated in the extracts of LA90-infected cells when the extracts were made with RIPA buffer. The extracts from the same cultures by a buffer containing only 1% Nonidet P-40 (NP40) as a detergent (10 mM sodium phosphate, 150 mM NaCl, 25 mM EDTA, 10% glycerol, 2 mM phenylmethylsulfonyl fluoride, 1% Trasylol, 1% NP40 [pH 7.4]) showed significant enzyme activity. The higher level of the enzyme activity was also recovered from BK5- and NY68-infected cells kept at nonpermissive temperatures when the NP40 buffer rather than the RIPA buffer was used. Thus, the ratio of enzyme activity in the extracts of NY68-infected cells at 41 and 37°C was as high as 0.44 with the NP40 buffer, whereas it was 0.15 with the RIPA buffer.

We compared the extraction of p130 with various buffers. Cells transformed with NY240 at 37°C were labeled with [³⁵S]methionine, and labeled p130 was extracted with various buffers and immunoprecipitated. As seen in Fig. 4B, the buffers containing NP40 (1%) or deoxycholate (0.5%) alone were less effective in extraction of

FIG. 2. Comparison of the size of FSV proteins. Cells transformed with FSV (FAV) stock (a) and a clonal FSV-FAV used as a wild type in this study (b) were labeled with [³⁵S]methionine for 5 h. Cells were lysed with RIPA buffer, and FSV proteins were immunoprecipitated with antiserum against RAV-2 proteins. The immunoprecipitates were analyzed by electrophoresis in a 5 to 15% gradient sodium dodecyl sulfate-polyacrylamide gel followed by fluorography. In addition to FSV p140 or p130, gag-pol products of helper virus (Pr180, Pr76, p27, p19, p15, and p12) were precipitated by the anti-RAV-2 serum.

FIG. 3. *In vivo* labeling of wild type and *ts* mutant p130 by [^{35}S]methionine. Wild type- and *ts* mutant-infected chicken cells were labeled with [^{35}S]methionine for 9 h. The labeled p130's were extracted with RIPA buffer, immunoprecipitated, and analyzed by 8.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by fluorography. Uninfected cells at 37°C (a); cells infected with NY240 at 41°C (b), with NY240 at 37°C (c), with NY225 at 41°C (d), with NY225 at 37°C (e), with wild type at 41°C (f), and with wild type at 37°C (g).

p130, compared with the "modified NP40 buffer" (10 mM sodium phosphate, 150 mM NaCl, 25 mM EDTA, 10% glycerol, 2 mM phenylmethylsulfonyl fluoride, 1% Trasylol, 0.5% NP40, 0.1% sodium deoxycholate [pH 7.4]) containing 0.5% NP40 and 0.1% deoxycholate or with the RIPA buffer. These four different buffers were used to extract p130 from unlabeled wild type- or NY240-transformed cells kept at 37°C, and the protein kinase activities were compared (Fig. 4A). The levels of enzyme activity appear to be proportionate to the efficiencies of extraction shown with [^{35}S]methionine-labeled p130, and the results with wild type- and NY240-infected cells were essentially the same. From these results, we concluded that FSV p130 can be extracted efficiently with either the RIPA buffer or the modified NP40 buffer.

In a previous study, we have demonstrated that FSV p140 immunoprecipitated by antiserum against RAV-2 virion proteins has an enzyme activity which transfers P_i from γ -ATP to the p140 molecule (7a). Furthermore, when casein was added to the immune complex of p140, casein was phosphorylated in addition to p140. The enzyme activity of cell extracts obtained from *ts* mutant-infected cultures was measured

by mixing the immunoprecipitates with [γ - ^{32}P]ATP in the presence or absence of casein. The protein kinase activity was significantly reduced when cultures of *ts* mutant-infected cells were incubated at the nonpermissive temperature (Fig. 5). The reduced phosphorylation of casein as well as p130 suggests a decrease in the enzyme activity rather than the reduction of the phosphate-accepting capacity of p130 as a result of a configurational alteration.

A comparison was also made for the enzyme activity extracted with the RIPA and modified NP40 buffers (Fig. 5). The extraction with the modified NP40 buffer gave slightly higher enzyme activity for both NY225- and NY240-infected cells. However, the ratio of the activity at two temperatures remained basically unchanged.

Phosphorylation of *ts* mutant p130 in vivo. We have shown that FSV is a phosphoprotein in vivo, phosphorylated at both the serine and tyrosine residues (7a). It was also shown

FIG. 4. Effects of various buffers on the extraction and kinase activity of p130. (A) Samples of lysates from cultures infected with wild-type FSV (lanes a, c, e, and g) or NY240 (lanes b, d, f, and h) at 37°C were immunoprecipitated with anti-RAV-2 antiserum, and the immunoprecipitates were tested for kinase activity. Lanes a and b, RIPA buffer; lanes c and d, NP40 buffer; lanes e and f, DOC buffer (10 mM sodium phosphate, 150 mM NaCl, 25 mM EDTA, 10% glycerol, 2 mM phenylmethylsulfonyl fluoride, 1% Trasylol, 0.5% sodium deoxycholate [pH 7.6]); and lanes g and h, modified NP40 buffer. (B) Parallel NY240-infected cultures were labeled with [^{35}S]methionine for 5 h as described in the text. Labeled cultures were extracted with the buffers indicated below. Immunoprecipitates were displayed on an 8.5% sodium dodecyl sulfate-polyacrylamide gel and visualized by fluorography. Lane a, RIPA buffer; lane b, NP40 buffer; lane c, DOC buffer; and lane d, modified NP40 buffer. Only the relevant portions of the gels are shown.

FIG. 5. Protein kinase activity of p130 obtained from cultures infected with wild-type FSV, NY225, or NY240. Immunoprecipitates from infected cells kept at 37°C (lanes a, c, and e) or 42°C (lanes b, d, and f) were tested for protein kinase activity by the *in vitro* kinase assay. The reaction mixture contained 0.5 mg of α -casein per ml as a substrate. $^{32}\text{P}_i$ was transferred from γ -ATP to both p130 and α -casein molecules. (A) Cell lysates were prepared by using RIPA buffer. Lanes a and b, wild type-infected cells; lanes c and d, NY225-infected cells; lanes e and f, NY240-infected cells. (B) Cell lysates were prepared by using modified NP40 buffer. Lanes c and d, NY225-infected cells; lanes e and f, NY240-infected cells.

that FSV p140-protein kinase phosphorylates only tyrosine residues of substrate proteins *in vitro*, and that the same tyrosine residue on FSV p140 is phosphorylated *in vivo* and *in vitro* (7a). The latter finding suggests that the *in vivo* p140 phosphorylation at tyrosine may be the result of autophosphorylation.

To examine the nature of *in vivo* phosphorylation of *ts* mutant p130, wild type- and mutant-infected cells were labeled with $^{32}\text{P}_i$ at the permissive and nonpermissive temperatures for 13 h. ^{32}P -labeled p130 was extracted, immunoprecipitated, and separated on a sodium dodecyl sulfate-polyacrylamide gel. The p130 band was cut out from the gel, and ^{32}P -labeled phosphoamino acid was analyzed after acid hydrolysis of the eluted p130. Representative data are shown in Fig. 6. The acid hydrolysate of ^{32}P -labeled p130 of FSV NY240-infected cultures kept at 37°C showed both phosphoserine and phosphotyrosine, whereas parallel *ts* mutant-infected cells incubated at 42°C showed only phosphoserine. The same results were obtained with cultures infected with NY225. On the other hand, p130 obtained from wild-type FSV-infected cells kept at both 37 and 42°C were labeled at both serine and tyrosine residues (data not shown).

By analysis of phosphoamino acid in p130 phosphorylated *in vitro*, we confirmed that NY240 p130 (kept at 37°C) maintains the same strict specificity of tyrosine phosphorylation *in vitro* as does the wild-type protein (7a) (data not shown).

Phosphorylation of tyrosine residues of cellular proteins *in vivo*. Hunter and Sefton (13, 22) showed that phosphorylation of tyrosine residues of cellular proteins is limited in normal cells and that transformation by RSV substantially increases the level of phosphotyrosine. To confirm that the protein kinase activity in FSV *ts* mutant-infected cells is temperature dependent *in vivo*, we measured the level of phosphotyrosine in NY225-infected cells (Table 5). The amounts of phosphotyrosine in NY225-infected cells expressed as the percentage of total radioactivities recovered in phosphoamino acids were temperature dependent as expected: the levels at permissive and nonpermissive temperatures were equivalent to those of wild type-infected and uninfected cells, respectively.

DISCUSSION

The two *ts* mutants of FSV described here are very similar to T class *ts* mutants of RSV in a number of ways. Cells infected with the FSV mutants were converted to either a transformed or a nontransformed state simply by shifting the incubation temperature. The reversible changes were observed with all of the parameters of cell transformation that we examined. As a minor difference from RSV mutants, the time period required for morphological alteration by shifting temperature of FSV mutant-infected cells seems to be longer than that required for cells infected with representative RSV mutants (1, 2, 14, 18, 23, 29). The kinetics of conversion, however, should be studied further by using parameters other than cell morphology, since morphological changes in FSV-infected cells are less distinct than those induced by RSV. The temperature sensitivity of the two FSV mutants was strikingly profound; there was no sign of significant leaky expression. The virus production in mutant-infected cells at nonpermissive temperatures was not significantly lower than that at permissive temperatures. This property cannot be directly compared, however, with similar results with T class RSV mutants (2, 14, 18, 29), because the virus replicative functions of FSV are defective, whereas those of RSV are nondefective. The production of infectious FSV is a result of the formation of pseudotypes, the packaging of the FSV genome in virions with all other virion proteins encoded by a helper virus.

Cells infected with FSV mutants produced

FIG. 6. Analysis of the phosphoamino acid composition of NY240 p130 phosphorylated *in vivo*. Partial acid hydrolysates of the NY240 p130 labeled *in vivo* with ^{32}P were separated in two dimensions: electrophoresis at pH 1.9 was carried out from right to left, and electrophoresis at pH 3.5 was done from bottom to top. The origin is marked $\times$. Standard phosphoamino acids: 1, phosphoserine; 2, phosphothreonine; and 3, phosphotyrosine were electrophoresed on a separate plate (C), and their position was identified by staining with ninhydrin. The ^{32}P -labeled spots in the lower right are probably partially hydrolyzed phosphopeptides. A, NY240 p130 from cultures kept at 37°C; B, NY240 p130 from cultures kept at 42°C.

TABLE 5. Level of phosphoamino acids in cells^a

Cells	Temp (°C)	% of total cpm		
		Phosphoserine	Phosphothreonine	Phosphotyrosine
Uninfected	37	92.6	7.3	0.06
FSV wild type	42	92.1	7.6	0.30
FSV NY225	37	91.4	8.3	0.26
FSV NY225	42	93.7	6.2	0.08

^a Cells were labeled with ^{32}P , for 18 h. The protein was extracted and hydrolyzed with 6 N HCl. Acid hydrolysates containing about 2×10^5 cpm were mixed with 10 μg each of phosphoserine, phosphothreonine, and phosphotyrosine. The mixture was subjected to two-dimensional electrophoresis at pH 1.9 in the first dimension and pH 3.5 in the second dimension (13). The thin-layer plates were autoradiographed, and the radioactive spots that comigrated with phosphoamino acid markers, which were identified by ninhydrin, were scraped, and the radioactivity was measured (7a).

p130 at both permissive and nonpermissive temperatures. The protein appeared to be active as a protein kinase, however, only when extracted from cultures incubated at the permissive temperature. Sefton et al. (21) showed that pp60^{src} of cells transformed by some RSV *ts* mutants is enzymatically more active when it is extracted with NP40 buffer rather than RIPA buffer. Comparison of cell extracts made by two buffers which were different in the detergent components showed that the enzymatic activity of p130 of FSV mutants was not particularly labile in the presence of ionic detergents, and that the recovery of p130 was lower with extraction

buffers containing no ionic detergents. Therefore, we consider the overall results to indicate that the level of the enzyme activity of p130 extracted with the two buffers from *ts* mutant-infected cells correlates with the level of p130 protein kinase activity within the cells. This conclusion was further supported by the fact that the level of phosphotyrosine in total cell protein was increased only when NY225-infected cells were incubated at the permissive temperature (Table 5). Thus, in addition to p60 of RSV (7, 22) and p120 of Abelson murine leukemia virus (27), the analysis of the FSV mutants seems to indicate that p130 is the transforming protein and its enzyme activity plays an essential role in FSV-induced cell transformation.

The analysis of phosphoamino acids of *in vivo* ^{32}P -labeled *ts* mutant p130 showed that at the nonpermissive temperature the mutant p130 was not phosphorylated at tyrosine residues. A similar observation was made with pp60^{src} of RSV NY68-infected cells maintained at the nonpermissive temperature (4). The lack of tyrosine phosphorylation *in vivo* can be explained by the loss of enzyme activity of mutant p130 as determined by the *in vitro* assay. However, one can also consider that the conformational changes in p130 molecules of *ts* mutants at the nonpermissive temperature may also contribute to the lack of phosphorylation of its own tyrosine site(s). In a preliminary experiment, an attempt was made to see whether NY240 p130 made at 42°C can be phosphorylated by wild-type FSV p140. FSV proteins were immunoprecipitated from a mixture of the cell extracts, NY240 p130 from cells kept at 42°C and wild-type p140, and the extent of phosphorylation of p130 in an *in vitro* protein

kinase reaction was examined. There was no significant increase in the phosphorylation of p130. The results suggest that the mutant p130 made at 42°C fails to serve as a substrate for the phosphorylation. However, this conclusion is valid if the mechanism of autophosphorylation is a *trans* reaction and if steric factors do not prevent p140 from acting on p130. These assumptions should be substantiated in further studies.

The above-described results of the mixing experiment exclude a remote possibility that the decreased activity of kinase at the nonpermissive temperature in *ts* mutant-infected cells is due to the presence of an inhibitor for the kinase.

Cellular localization of FSV p130 has not been established. The fact that the extraction of p130 from cells is more efficient with the buffers containing ionic detergents seems to suggest that p130 is also bound to cellular membrane structures, in a way similar to pp60^{src} of RSV (6, 15, 25).

The wild-type FSV used in this study was selected on the basis that it produced more distinct foci at 41°C than other clones. The change in the molecular weight of the virus protein from 140K to 130K appears to be fortuitous. The molecular weight of FSV protein of subclones of the wild type, including the *ts* mutants described here, was uniformly 130K. We have not yet studied whether this is due to a deletion in the viral genome. Similar changes in molecular weight were observed with Abelson leukemia virus transforming protein (19).

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