Temperature-Sensitive Mutants of Fujinami Sarcoma Virus: Tumorigenicity and Reversible Phosphorylation of the Transforming p140 Protein

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Several clones of Fujinami sarcoma virus (FSV) isolated from a laboratory stock or from mutagenized virus were temperature sensitive (*ts*) in transformation of cells in culture. When shifted from the permissive (37°C) to the nonpermissive (41.5°C) temperature, the cellular phenotype reverted to normal within 2 h, but it required about 48 h at 37°C to revert back to the transformed morphology. A temperature-resistant (*tr*) FSV clone was isolated from a tumor of an animal. All *ts* mutants were tumorigenic in animals but induced tumors only after latent periods of 12 to 25 days, compared to 5 to 6 days with *tr* virus. The *ts* lesions of the FSV mutants affected 90% of the phosphorylation of the nonstructural, *gag*-related 140,000-kilodalton phosphoprotein coded by FSV (p140), but did not affect virus replication or the synthesis of p140. Upon shifting from the permissive to the nonpermissive temperature, p140 was 90% dephosphorylated with an approximate ³²P half-life of 20 min. When shifted back to the permissive temperature, the preexisting p140 was rephosphorylated in the absence of protein synthesis within a 90-min test period. Likewise, most of the phosphate of fully phosphorylated p140 was exchanged at the permissive temperature within 30 to 90 min even when protein synthesis was inhibited. However, the protein structure of p140 had a half-life of 5 h at both temperatures. These results prove p140 to be a substrate of reversible phosphorylation. Superinfection and transformation of *ts* FSV-infected cells maintained at the nonpermissive temperature with acute leukemia virus MC29 failed to phosphorylate p140. It would follow that in vivo phosphorylation of *ts* p140 is controlled by an FSV-specific mechanism and is a prerequisite, not a consequence, of transformation. p140 of *ts* FSV recovered from cells maintained at 41.5°C with anti-*gag* serum was over 10 times less phosphorylated by associated kinase than the same protein recovered from cells at 37°C if assayed in vitro at 20°C. This kinase activity associated with or dissociated from p140 with a half-life of less than 30 min during temperature shifts of *ts* FSV-infected cells. However, p140 recovered from *ts* FSV-infected cells maintained at 37°C was phosphorylated by associated kinase in vitro not only at 20°C but also, and essentially at the same level, at 41.5°C. This suggests that the kinase associated with the immunocomplex of p140 of *ts* FSV is not temperature sensitive. p140 translated in vitro from *ts* and *tr* FSV RNA lacked kinase activity. We conclude that a fully phosphorylated p140 is necessary for the maintenance of transformation by FSV. This is consistent with the notion that other highly oncogenic viruses also code for nonstructural phosphoproteins with probable transforming function. A model which postulates that p140 is a substrate of reversible phosphorylation and that the lesion of the *ts* FSV clones described herein affects association of p140 with a cellular kinase rather than a hypothetical intrinsic kinase activity of the protein is most compatible with our data.

Fujinami sarcoma virus (FSV) is a defective avian sarcoma virus with a 4.5-kilobase RNA genome which requires a helper virus, such as Fujinami-associated virus (FAV), for replication (10). The RNA contains a *gag*-related (1-kilo-

base) sequence and an adjacent specific (3-kilobase) sequence which together code for a nonstructural phosphoprotein of 140 kilodaltons termed p140 (3, 4, 10). This protein is thought to have transforming function because it repre-

sents approximately all of the coding capacity of the viral RNA and because of its structural analogy with the transforming phosphoproteins of avian acute leukemia viruses (2-4, 10). The p140 FSV protein is also phosphorylated in vitro by an associated kinase activity which is removed from the protein or inactivated by prior incubation with preimmune serum, followed by centrifugation at $100,000 \times g$ (3). However, there is as yet no definitive genetic evidence to show that the p140 FSV protein is a transforming protein and to indicate whether it functions as a kinase.

To determine whether synthesis and phosphorylation of the p140 FSV protein are necessary for the maintenance of cellular transformation, we have isolated several spontaneous and chemically induced mutants temperature sensitive (*ts*) in transformation and have compared these mutants with each other and with a temperature-resistant (*tr*) FSV clone at the permissive and nonpermissive temperatures. Using one of our spontaneous *ts* FSV mutants, Pawson et al. (11) have shown a positive correlation between cellular transformation and phosphorylation of p140 in vivo and in vitro. This mutant was the same clonal isolate that we had selected, initially oblivious of its temperature-sensitive properties, for our previous analyses of FSV because this clone contained an excess of FSV over the associated virus, FAV (3, 10). Here we have shown that phosphorylation of the p140 proteins of all *ts* mutants isolated by us is temperature sensitive in vivo. Furthermore, we have found that at the nonpermissive temperature the p140 FSV protein not only remains unphosphorylated but is rapidly dephosphorylated while it continues to be synthesized. At the permissive temperature, the protein is rephosphorylated even in the presence of cycloheximide. Likewise, the phosphate of fully phosphorylated p140 was exchanged at a rapid rate both in the presence and in the absence of protein synthesis. In vitro, p140 of *ts* FSV isolated from cells maintained at 37°C was phosphorylated at 41.5°C essentially at the same level as if assayed at 20°C. We submit, as the simplest explanation, that p140 of our *ts* FSVs is a substrate of reversible phosphorylation that associates with a presumably cellular kinase only at the permissive temperature. Once isolated by immunoprecipitation, the resulting complex phosphorylates p140 in vitro at 20 and 41.5°C.

MATERIALS AND METHODS

Cells and viruses. Quail embryo fibroblasts were prepared from eggs that were obtained from Life Sciences, Inc., St. Petersburg, Fla., through the auspices of the National Cancer Institute. Chickens were

hatched from SPAFAS eggs (Norwich, Conn.). The origin of our FSV strain and the associated virus (FAV) has been described (10). Avian erythroblastosis virus chicken cell nonproducer clone C23 was a gift from G. S. Martin. The avian myelocytomatosis virus (MC29)-ring-neck pheasant helper virus (RPV) complex, quail nonproducer cells transformed by the Bryan strain of Rous sarcoma virus [RSV(-)], transformation-defective Schmidt-Ruppin strain RSV, subgroup A (tdSR-A), and avian myeloblastosis virus-associated helper virus (MAV-1), subgroup A, were as described (2, 7).

Reagents. 5-Azacytidine and cycloheximide were from Sigma Chemical Co.

Focus assay of FSV. The focus assay of FSV followed published procedures (16). Typically, 10^6 C/O chicken embryo fibroblasts were seeded per 60-mm petri dish and infected with virus in 4 ml of medium containing 2% tryptose phosphate broth, 2% calf serum, 1% chick serum, and polybrene at 2 μ g/ml. After 6 to 12 h at 37°C, the medium was removed, and the culture was overlaid with 5 ml of the same medium containing 0.75% agar. Subsequently, cultures were incubated at 37 or at 41.5°C with additional agar overlays of 3 ml added at 3-day intervals. Foci of FSV were recorded after 5 to 10 days. The foci consisted of fusiform cells, which were easily distinguishable from foci induced by other avian retroviruses such as RSV and MC29.

Agar colony assay. The method for agar colony assay (4, 8) employed a bottom layer of 0.9% agar and a top layer containing 0.36% agar in which infected cells were suspended. The compositions of the agar media were as follows. Top agar contained in 50 ml: 10 ml of double-strength F10 medium; 3 ml of fetal calf serum; 1 ml of chicken serum; 5 ml of tryptose phosphate broth, 5 ml of 100 $\times$ vitamin of Eagle minimal medium (GIBCO); 20 ml of conditioned growth medium (2% tryptose phosphate broth, 2% calf serum, 1% chicken serum) from primary cultures of Japanese quail fibroblast; 10 ml of 1.8% agar; 0.3 ml of 100 $\times$ penicillin, streptomycin, and amphotericin B (GIBCO). Bottom agar had the same composition except that conditioned medium was omitted. About 4 ml of bottom agar was pipetted into 60-mm dishes and allowed to harden at room temperature. Thereafter the bottom layer was overlaid with different numbers of FSV-infected quail cells suspended in 3 ml of top agar. These were prepared from trypsin-dissociated quail primary cells which were seeded at 6×10^6 cells per 60-mm dish and then incubated for 3 h with 0.05 focus-forming unit (FFU) of virus per cell. Samples of dilutions of the infected cells were added to 3 ml of top agar in tubes at 45°C. The agar mix was then immediately poured onto the bottom agar. Upon incubation at 37°C, FSV-transformed cells grew into large colonies after 15 to 20 days. These colonies were picked and grown into mass cultures in medium containing F10, 2% calf serum, 2% tryptose phosphate broth, and 1% chicken serum.

Immunoprecipitation of radiolabeled cell lysates. To label cellular proteins with ^{32}P or ^{35}S , around 1.5×10^6 cells in 60-mm petri dishes were starved by incubation at 37°C for 30 min in phosphate-free or in methionine-free medium and then incubated for 1 to

2 h in 2 ml of phosphate-free medium supplemented with $^{32}\text{PO}_4^{3-}$ (1 mCi/ml) or of methionine-free medium supplemented with [^{35}S]methionine (50 $\mu\text{Ci}/\text{ml}$) (3). All subsequent operations were at 4°C . Cell extracts were prepared in lysis buffer containing 25 mM Tris-hydrochloride (pH 7.4), 50 mM NaCl, 0.2% Nonidet P-40, 0.5% deoxycholate, and 200 units of Kallikrein inactivator (Calbiochem) per ml. The lysate was clarified at 4°C at $20,000 \times g$ for 20 min. Immunoprecipitation was carried out with serum prepared against p27 of avian myeloblastosis virus which was obtained from the Division of Cancer Cause and Prevention, National Cancer Institute, then adsorbed to Formalin-fixed *Staphylococcus aureus* (the Enzyme Center) and subsequently washed with (i) lysis buffer, (ii) 1 M NaCl, 10 mM Tris-hydrochloride (pH 7.4), and 0.1% Nonidet P-40; (iii) 0.15 M NaCl, 10 mM Tris-hydrochloride (pH 7.4), 0.1% Nonidet P-40; and (iv) lysis buffer. The immunoprecipitated proteins were prepared for electrophoresis as described previously (3, 10).

Immunocomplex-kinase assay. Proteins immunoprecipitated from cells as described above were incubated in 20 to 50 μl of buffer containing 10 mM MgCl_2 , 20 mM Tris-hydrochloride (pH 7.5), 0.15 M NaCl, and 10 to 100 μCi of [$\gamma\text{-}^{32}\text{P}$]ATP (ICN; 4,500 Ci/mmol) at temperatures ranging between 20 and 41.5°C for 10 to 15 min. The reaction was terminated by adding an equal volume of 0.5 M EDTA. After the precipitate was washed twice with lysis buffer, the immunocomplex was dissolved by incubation for 2 min at 100°C in sodium dodecyl sulfate-gel sample buffer. The bacteria were removed by centrifugation at $10,000 \times g$ (3).

Cell-free translation. Isolation of polyadenylic acid-containing, heat-denatured 50 to 70S viral RNA and its translation in the messenger-dependent rabbit reticulocyte lysate were as described (10, 12).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Discontinuous slab gels were prepared and run as described (3). Gradient gels of 6 to 18% polyacrylamide were used. Gels of ^{35}S -labeled and ^{32}P -labeled proteins were fluorographed at -70°C after impregnation with dimethyl sulfoxide-based 2,5-diphenyloxazole (6). To quantitate the radioactivity in a band, it was excised and counted in a liquid scintillation counter in the presence of scintillation fluid.

RESULTS

Isolation of *ts* and *tr* clones of FSV. In an initial experiment, a cloned stock of our complex of FSV and FAV [FSV(FAV)] was observed to be temperature sensitive for focus formation in tissue culture, with a titer of 10^5 to 10^6 FFU at 37°C and 10^2 to 10^3 FFU at 41.5°C (Table 1). This spontaneous *ts* clone of FSV, termed here L-5, was derived by focus cloning from a stock received by H. M. Temin (10).

Additional and less leaky *ts* FSV mutants were obtained by chemical mutagenesis of the L-5 clone. For this purpose, FSV L-5 was propagated in the presence of 5-azacytidine at 100 $\mu\text{g}/\text{ml}$ of

TABLE 1. Focus assay of *ts* and *tr* FSV

Virus	Titer (FFU/ml) at:	
	37°C	41.5°C
FSV L-5 (FAV)	10^5 – 10^6	10^2 – 10^3
FSV A-31 (FAV)	10^4 – 10^5	<1
FSV A-53 (FAV)	10^4 – 10^5	<1
FSV A-45 (FAV)	10^2 – 10^3	<1
FSV L-15 ^a	0	0
FSV L-15 (td SR-A) ^b	10^4 – 10^5	<10
FSV L-15 (MAV-1)	10^4 – 10^5	<10
FSV A-31-1 (FAV)	10^4 – 10^5	<1
FSV A-31-2 (FAV)	10^4 – 10^5	<1
<i>tr</i> FSV (FAV)	10^5 – 10^6	10^5 – 10^6

^a A nonproducer quail fibroblast clone.

^b td SR-A, Transformation-defective Schmidt-Ruppin RSV, subgroup A.

cell culture medium. Quail embryo fibroblasts, infected with the mutagenized stock at a multiplicity of less than 0.05 FFU/cell, were then allowed to grow at 37°C into colonies in soft agar (see above). Several colonies were selected and allowed to grow into mass cultures of transformed cells at 37°C . The supernatant media were then assayed for viral FFU at two temperatures. Virus produced by three colonies termed A-31, A-53, and A-45 produced no foci at 41.5°C (Table 1). In addition, a nonproducer colony was obtained which contained a *ts* FSV termed L-15. The culture was transformed at 37°C and had a flat morphology at 41.5°C (Fig. 1). *ts* FSV L-15 was rescuable from this culture with transformation-defective Schmidt-Ruppin RSV or avian myeloblastosis-associated virus MAV-1 (Table 1). The morphology of the culture remained *ts* upon superinfection with the transformation-defective Schmidt-Ruppin RSV (Fig. 1). Rescue of a *ts* FSV from nonproducer cells directly proved that the *ts* lesion of L-15 is associated with the defective FSV rather than with helper virus or the cell. The cell morphology and focus titer of a *tr* FSV isolated from a chicken tumor (see below) were the same at 37 and 41.5°C (Fig. 1, Table 1).

Given an apparent *ts* lesion in the transforming function of our *ts* FSV clones, we examined the kinetics of cellular transformation and reversion to the normal phenotype after temperature shifts. Quail cells transformed by *ts* or *tr* FSV and adherent to the plastic petri dish showed a fusiform morphology with an upper layer of rounded cells (Fig. 1A, B, C, and E). Upon shifting to the nonpermissive temperature (41.5°C), the cells assumed a flat morphology and formed clusters of parallel cells within 2 h (Fig. 1D and F). By contrast, it took about 48 h for the cells to return to the transformed phe-

notype when shifted from 41.5 to 37°C. It would appear that the kinetics of transformation are significantly slower than those of phenotype reversion to normal.

To determine whether the *ts* lesion of our mutants affected RNA replication in addition to transforming function of the defective FSV, FSV production at the permissive (37°C) and nonpermissive (41.5°C) temperature was compared. In one type of experiment, culture media harvested at two different temperatures were assayed for focus-forming virus at the permissive temperature. It was found that the focus titer of all *ts* virus strains propagated at 41.5°C was only two to three times lower than that of virus propagated at 37°C (data not shown). Likewise, the amounts of virus particles assayed by virus-associated reverse transcriptase were only reduced five- to eightfold at 41.5°C as compared to the values obtained at 37°C (not shown). The somewhat reduced yield of virus at 41.5°C compared to that of 37°C is thought to be the result of the transformed (37°C) versus the normal (41.5°C) phenotype of the host cell. Transformed cells generally produce more virus than untransformed counterparts [unpublished observations with the production of avian leukosis viruses, in particular those of subgroup A propagated by untransformed cells versus production by cells superinfected and transformed by RSV(-)]. We conclude that the *ts* lesion of our mutants affects transforming function without significantly affecting replicative functions of FSV.

Tumorigenicity of *tr* and *ts* FSV. We have described previously that the *ts* FSV clone L-5 caused tumors within 10 to 15 days upon inoculation of 10⁴ FFU into a chicken (Table 2) (10). We have since observed that virus recovered from these tumors induced tumors in subsequent experiments within 5 to 6 days (Table 2). This virus was *tr* if assayed under all conditions described in this paper (see above) and was, therefore, termed *tr* FSV (Table 2). Tumor formation was also observed with *ts* FSV A-31 in all of four animals inoculated, after a latent period of 14 to 25 days. The longer latent period of A-31 compared to L-5 is consistent with the higher degree of temperature sensitivity of A-31 (Table 2). Virus (FSV A-31-1, A-31-2) recovered from these tumors was still *ts* in terms of phosphorylation of p140 (see below) and focus formation if assayed in our conditions in cell culture (Table 1).

It appears that in one case tumor formation in the animal has selected for a *tr* clone derived from the relatively leaky *ts* mutant FSV L-5 (Table 1). However, *tr* clones were not detected in the virus stocks obtained from the tumors

induced by the tight *ts* mutants FSV A-31-1 and A-31-2 (Table 1). This suggests that mutants *ts* in the parameters measured here in cell culture can cause tumors in the animal, albeit after longer latent periods than *tr* FSV.

Synthesis and phosphorylation in vivo of p140 of *ts* FSV at the permissive and nonpermissive temperature. Next, we examined the synthesis and phosphorylation of the p140 proteins of the *ts* FSV mutants at the permissive and nonpermissive temperatures. By measuring incorporation of [³⁵S]methionine we found that mutants L-15, A-45 (see Fig. 3), A-31, and A-53 (not shown) synthesized p140 at both the permissive and nonpermissive temperature, as shown elsewhere (11) with one of our mutants, FSV L-5. By contrast, the p140 of these mutants was only 10% phosphorylated at the nonpermissive temperature compared to the value of p140 isolated from cells kept at the permissive temperature. No difference in synthesis and phosphorylation of p140 of *tr* FSV was observed at 37 or 41.5°C (Fig. 2). Therefore, it is concluded that the *ts* lesion of these FSV mutants affects phosphorylation of the p140 protein but does not affect its synthesis.

It is noted that our anti-*gag* serum precipitates other viral and nonviral proteins from FSV-infected cells. The nonviral proteins were identified as such because they were also precipitated by preimmune serum (data not shown). For example, a nonviral ³⁵S-protein of >200,000 daltons was detected (Fig. 2) in all FSV-infected cells in addition to known viral primary gene products pr180, pr76 and its processing product p27, and other processing intermediates migrating between p140 and p27. Moreover, viral [³²P]pr76 and probably nonviral ³²P-labeled proteins are detectable in Fig. 2. It deserves particular notice that phosphorylation of these nonviral proteins was temperature sensitive in FSV-infected cells (Fig. 2A, cf. lanes 3 and 4 with lanes 9 and 10). By contrast, phosphorylation of the presumably cellular proteins is not temperature sensitive in cells transformed by *tr* FSV (Fig. 2C, lanes 15 and 16) or by *ts* FSV and MC29 (see Fig. 6, lanes c and d). This suggests that phosphorylation of these proteins may be a (necessary) consequence rather than the primary cause of transformation by FSV.

Phosphorylation of *ts* mutant p140 is reversible in vivo. It may be argued that the failure to detect normal levels of phosphorylation in p140 of *ts* FSV at 41.5°C in vivo or to detect phosphorylation in vitro of p140 isolated from cells kept at 41.5°C reflects instability of the mutant proteins at the nonpermissive temperature. To test for this possibility, the half-life

FIG. 1. Cell morphology of *ts* and *tr* FSV-infected quail embryo fibroblasts at permissive and nonpermissive temperatures. Phase-contrast photomicrographs ($\times 125$) are shown of quail embryo fibroblasts infected with *tr* FSV (FAV) and maintained at (A) 37°C and (B) 41.5°C ; of nonproducer clone *ts* FSV L-15, maintained at (C) 37°C and (D) 41.5°C ; and of quail embryo fibroblasts infected with *ts* FSV L-15 (td SR-A), maintained at (E) 37°C and (F) 41.5°C .

of ^{35}S -labeled and ^{32}P -labeled p140 protein was determined *in vivo*. It was found that the half-lives of p140's of *ts* FSV A-45 pulse-labeled with [^{35}S]methionine and chased at the permissive and nonpermissive temperatures were about the same, i.e., 300 min (Fig. 3). The half-life of p140 was significantly longer than that of the pr76 precursor of the virion *gag* proteins, which was 30 to 40 min (not shown).

By contrast, the half-life of most of the ^{32}P label associated with p140 of *ts* FSV A-45 in cells labeled and chased at 37°C was 20 to 30 min. The half-life of the relatively low level of ^{32}P associated with p140 of *ts* FSV at the nonpermissive temperature (10% of level at permissive temperature; Fig. 2) was around 300 min (Fig. 3). Thus the half-life of this background level of

^{32}P associated with p140 was the same as that of [^{35}S]p140, suggesting phosphorylation of nonreversible sites.

It would appear that p140 of *ts* FSV is dephosphorylated at 41.5°C while it continues to be synthesized. To test this directly, dephosphorylation of *ts* FSV A-45 p140, ^{32}P -labeled for 90 min in cells maintained at 37°C and then shifted to 41.5°C still in the presence of $^{32}\text{PO}_4^{3-}$ -containing medium, was examined. It can be seen (Fig. 4) that under these conditions p140 became

TABLE 2. Tumorigenicity of *ts* and *tr* FSV^a

Virus	No. of chickens with tumors/no. of chickens injected	Day of tumor appearance
<i>tr</i> FSV (FAV)	4/4	5-6
FSV L-5 (FAV)	4/4	12-14
FSV A-31 (FAV)	4/4	14-25

^a One-week-old chicks hatched from the same batch of SPAFAS eggs were injected in the wing web with $\sim 10^4$ FFU of viruses of each stock.

FIG. 3. The half-lives of ^{32}P - and ^{35}S -labeled p140 of *ts* FSV A-45 in cells maintained at 37 and 41.5°C . p140 protein was labeled, isolated, and electrophoretically analyzed as described for Fig. 2.

FIG. 2. Synthesis and phosphorylation *in vivo* at 37 and 41.5°C and phosphorylation *in vitro* at 20°C of the nonstructural p140 protein of *ts* (A, B) and *tr* FSV (C). Radioactive proteins were analyzed by electrophoresis in 6 to 18% polyacrylamide gels and detected by autoradiography. p140 was isolated by immunoprecipitation with anti-*gag* serum from lysates of quail embryo fibroblasts infected by (A) *ts* FSV L-15 (FAV), (B) *ts* FSV L-15 in the absence of helper virus (nonproducer), and (C) *tr* FSV (FAV), under the following conditions: Labeling of cells for 2 h with [^{35}S]methionine at 37°C (lanes 1, 7, and 13) and 41.5°C (lanes 2, 8, and 14); labeling of cells for 2 h with $^{32}\text{PO}_4^{3-}$ at 37°C (lanes 3, 9, and 15) and 41.5°C (lanes 4, 10, and 16). *In vitro* phosphorylation with [γ - ^{32}P]ATP at 20°C of immunoprecipitates prepared from cells maintained at 37°C (lanes 5, 11, and 17) and at 41.5°C (lanes 6, 12, and 18).

FIG. 4. Dephosphorylation of p140 of *ts* FSV A-45 (FAV) at the nonpermissive temperature (41.5°C). Parallel cultures of *ts* FSV A-45(FAV)-infected quail cells (1.5×10^6) were labeled with $^{32}\text{PO}_4^{3-}$ -containing medium for 90 min at 37°C (see the text). Lane 1, p140 prepared from cells kept at 37°C. Lanes 2 to 7, p140 prepared from cells labeled at 37°C for 90 min and then shifted to 41.5°C for 10, 20, 30, 60, 90, and 120 min, still in the presence of $^{32}\text{PO}_4^{3-}$ -containing medium.

about 90% dephosphorylated within 20 min and was not further dephosphorylated during 2 h under the same conditions. By contrast, the ^{32}P associated with pr76 continued to increase over the 2-h observation period.

To test whether unphosphorylated p140 can be phosphorylated and whether phosphorylated p140 can be reversibly de- and rephosphorylated when de novo protein synthesis is inhibited, the following experiments were done. Cells infected with *ts* FSV A-45 and kept at 41.5°C were treated with cycloheximide (15 $\mu\text{g}/\text{ml}$) to inhibit synthesis of p140 and then incubated at 37°C in the presence of $^{32}\text{PO}_4^{3-}$ to allow phosphorylation to occur. It was found that within 1.5 h at 37°C p140 was phosphorylated (Fig. 5b), whereas p140 from cells kept at 41.5°C was not phosphorylated (Fig. 5a) over the background that is temperature resistant. A control experiment indicated that cycloheximide at 15 $\mu\text{g}/\text{ml}$ completely inhibited de novo synthesis of p140 (not shown). Furthermore, p140 in cells which had been maintained at 37°C and were then ^{32}P -labeled for 1.5 h at 37°C in the presence of cycloheximide was phosphorylated (Fig. 5c).

These results indicate that unphosphorylated p140 can be phosphorylated in the absence of protein synthesis. Moreover, since even p140 in

FIG. 5. Reversible phosphorylation of p140 of *ts* FSV in the presence of cycloheximide. (a) One of three parallel quail cell cultures, infected with *ts* FSV A-45(FAV), had been maintained at 41.5°C. It was then labeled with ^{32}P for 90 min at 41.5°C in the presence of cycloheximide (15 $\mu\text{g}/\text{ml}$). p140 and other viral proteins were prepared and analyzed by polyacrylamide electrophoresis as described for Fig. 2. (b) A second culture was maintained at 41.5°C, then shifted to 37°C and labeled with ^{32}P for 90 min at 37°C in the presence of cycloheximide. p140 was isolated and electrophoretically analyzed as described for Fig. 2. (c) A third culture was maintained at 37°C and otherwise treated as described for (b).

cells that had been maintained at 37°C was phosphorylated, although it must have been fully phosphorylated at the time when cycloheximide and ^{32}P were added, it follows that preexisting phosphate must have been exchanged. Thus p140 is subject to reversible phosphorylation and dephosphorylation at a rate of between 30 and 90 min based on the tests shown in Fig. 3 to 5.

Phosphorylation of p140 in cells infected by *ts* FSV and superinfected with avian acute leukemia virus MC29. To determine whether phosphorylation of p140 was mediated by its own specific mechanism or can be phosphorylated upon infection by other oncogenic viruses, *ts* FSV A-45(FAV)-transformed cells maintained at 41.5°C were superinfected and transformed with MC29 virus. It was found that upon transformation with MC29(RPV) at the permissive temperature both the p140 FSV and p110 MC29 proteins (3), as well as copurifying

cellular proteins, were phosphorylated (Fig. 6, lane c). However, at the nonpermissive temperature, the p140 FSV protein was only slightly phosphorylated (10% of the value at 37°C), whereas the p110 MC29 and the cellular proteins were phosphorylated to the same degree as, or even higher than, at 37°C (Fig. 6, lane d). A parallel experiment using [³⁵S]methionine showed that the synthesis of all proteins was essentially the same at both temperatures (Fig. 6, lanes a and b).

When avian erythroblastosis virus or RSV(−) nonproducer cells were superinfected with *ts* FSV A-31(FAV), essentially the same results were obtained as those just described with MC29; in each case, phosphorylation of the p140 FSV protein was temperature sensitive (data not shown).

It follows from the above experiments that phosphorylation of p140 FSV protein is controlled by an FSV-specific mechanism and that it is not a consequence of but a prerequisite for transformation by FSV. Furthermore, it appears that phosphorylation of those cellular proteins that are precipitated with our anti-*gag* serum

FIG. 6. Synthesis and phosphorylation of p140 at 37 and 41.5°C in cells infected with *ts* FSV A-45(FAV) and superinfected with MC29(RPV). Proteins precipitated by anti-*gag* serum from lysates of cells labeled with [³⁵S]methionine for 2 h at 37°C (a) and at 41.5°C (b). Proteins from parallel cultures labeled with ³²PO₄^{3−} for 2 h at 37°C (c) and at 41.5°C (d). Proteins were electrophoresed as described for Fig. 2.

(compare Fig. 2, lanes 3 and 4 and 9 and 10, with Fig. 2, lanes 15 and 16, and with Fig. 6, lanes c and d) is controlled by the transformed state of the cell, rather than by FSV or MC29, since phosphorylation of these proteins was temperature sensitive in cells transformed by *ts* FSV but was not temperature sensitive in cells transformed by either *tr* FSV or both MC29 and *ts* FSV.

p140 obtained by in vitro translation of viral RNA lacks kinase activity. In vitro translation of the RNA of RSV generates a nonstructural protein that is indistinguishable from the viral p60 *src* protein found in transformed cells (13). This protein also shares an associated kinase activity with its counterpart isolated from transformed cells.

In an effort to determine whether the p140 of FSV is itself a kinase or is associated with a cellular kinase, we asked whether in vitro-synthesized p140 (10) is associated with kinase activity. For this purpose RNA of *tr* FSV(FAV) was translated in vitro at 41.5°C in a rabbit reticulocyte lysate in the presence of [³⁵S]methionine. After immunoprecipitation with anti-*gag* serum, [³²P]ATP was added to one of two samples, and the proteins, either labeled with [³⁵S]methionine or double-labeled with ³⁵S and ³²P, were analyzed by polyacrylamide gel electrophoresis. It can be seen in Fig. 7a and b that p140 was synthesized but that no ³²P label was associated with p140 (over the background of the ³⁵S label associated with p140) (Fig. 7b). In a third translation reaction, *tr* FSV(FAV) RNA was translated in the absence of [³⁵S]methionine, and the immunoprecipitated reaction products were incubated with [³²P]ATP under the conditions that allowed phosphorylation of p140 in immunoprecipitates from cellular lysates (Fig. 7c). Again, no phosphorylation of p140 was observed.

Additional translations of *ts* FSV(FAV) RNA at 41.5°C and of *tr* and *ts* FSV(FAV) RNA at 41.5°C and of *tr* and *ts* FSV(FAV) RNAs at 30 and 37°C gave the same results. At each temperature, p140 protein was made in quantities comparable to those reported previously for p60 translated in vitro from RSV RNA (13), but phosphorylation of p140 was not detected.

One interpretation of these experiments is that p140 is not intrinsically a kinase; alternatively, the in vitro-made protein may lack undetected elements present in p140 from transformed cells.

Association of kinase with p140 of *ts* FSV is temperature sensitive. The p140 proteins of *ts* FSV clones L-15 and A-45, prepared from cells kept at the nonpermissive temperature,

FIG. 7. p140 translated *in vitro* from *tr* FSV(FAV) RNA is not phosphorylated. (a, b) About 1 μ g of 50S *tr* FSV(FAV) RNA was heated at 100°C for 30 s to dissociate the RNA complex and was translated in a rabbit reticulocyte lysate in the presence of [35 S]methionine. The proteins of the *in vitro* reaction were immunoprecipitated with anti-gag serum. Electrophoresis of the immunoprecipitates was as described for Fig. 2. (b) The immunoprecipitate was incubated with 50 μ Ci of [γ - 32 P]ATP in 20 μ l of reaction buffer for 20 min at 20°C as described in the text. (c) A third translation reaction was carried out in the absence of [35 S]methionine, but the immunoprecipitate was incubated with [γ - 32 P]ATP as described for (b), under the same conditions that allow phosphorylation of immunoprecipitated p140 from cellular lysates (Fig. 2), before electrophoretic analysis.

lacked kinase activity if assayed at 20°C, whereas immunoprecipitates prepared from *ts* FSV-infected cells kept at 37°C were associated with kinase (Fig. 2, lanes 5 and 6, 11 and 12, and 17 and 18). By contrast, p140 from *tr* FSV-infected cells kept at either 37 or 41.5°C was associated with kinase (Fig. 2, lanes 17 and 18). A kinetic analysis showed that most of the kinase activity associated with p140 was lost within 45 min upon shifting *ts* FSV A-45-infected cultures from 37 to 41.5°C before isolating the protein. Conversely, the kinase was recovered with similar kinetics upon shifting infected cells

from 41.5 to 37°C (Fig. 8). The dissociation kinetics of kinase associated with p140 of *ts* FSV roughly paralleled those of the *in vivo* phosphorylation of the p140 protein in infected cells (Fig. 3). Moreover, these *in vitro* results conform with those described recently by Pawson et al. (11). The p140's of all other mutants isolated by us behaved identically in this regard (not shown).

These *in vitro* results could be explained in terms of two models: one postulates that p140 of *ts* FSV is a temperature-sensitive autokinase; the other suggests that association with, and subsequent phosphorylation of, p140 by a presumably cellular kinase is temperature sensitive. To distinguish between these alternatives, p140 was prepared by immunoprecipitation with anti-gag serum from *tr* and *ts* FSV A-45(FAV)-infected cells kept at 37°C. *In vitro* phosphorylation was determined with aliquots during 10-min reaction periods at 20°C, at 41.5°C, and at 41.5 and then at 20°C for another 10 min. Within a factor of about two, phosphorylation of p140 of *tr* (Fig. 9A, lanes 1 to 3) and *ts* FSV (Fig. 9A, lanes 4 to 6) was the same under all three conditions. It follows that the kinase activity associated with *ts* p140 recovered from cells kept at 37°C is active at 41.5°C. Other investigators

FIG. 8. Kinetic analysis of the dissociation and association of kinase activity and p140 after isolation from cells shifted from the permissive to the nonpermissive and from the nonpermissive to the permissive temperature. p140 from quail cells infected with *ts* FSV A-45 (FAV) was isolated, subjected to *in vitro* kinase tests, and subsequently analyzed by gel electrophoresis as described for Fig. 2. Lanes 1 to 4: Phosphorylation of p140 from infected quail cells kept at 37°C and then at 41.5°C for 45, 90, and 180 min. Lanes 5 to 8: Phosphorylation of p140 from quail cells kept at 41.5°C and then at 37°C for 45, 90, and 180 min.

FIG. 9. *In vitro* phosphorylation at 41.5°C (A) and thermostability (B) of p140 of *tr* and *ts* FSV prepared from cells maintained at 37°C. (A) Immunoprecipitates of p140 from cells maintained at 37°C and infected with *tr* FSV (FAV) (lanes 1 to 3) and *ts* FSV A-45 (FAV) (lanes 4 to 6) were divided into three aliquots: one portion was incubated with [γ - 32 P]ATP for 10 min at 20°C (lanes 1 and 4); another aliquot was incubated with [γ - 32 P]ATP for 10 min at 41.5°C (lanes 2 and 5); and the third sample was incubated with [γ - 32 P]ATP for 10 min at 41.5°C and then for 10 min at 20°C (lanes 3 and 6). Electrophoresis of immunoprecipitated protein was as described for Fig. 2. (B) Immunoprecipitates of p140 from cells maintained at 37°C and infected with *tr* FSV (FAV) (lanes 1 to 6) and *ts* FSV A-45 (FAV) (lanes 7 to 12) were incubated at 41.5°C for 0, 1, 2, 4, 8, and 16 min (lanes 1 and 7, 2 and 8, 3 and 9, 4 and 10, 5 and 11, and 6 and 12, respectively). Subsequently the reactions were chilled on ice for 3 min, and [γ - 32 P]ATP was added to allow phosphorylation of p140 during 20-min reaction periods at 20°C. The proteins were analyzed and autoradiographed as described for Fig. 2.

have shown that the kinase activity associated with the p60 protein of RSV can be assayed *in vitro* at 41.5°C (14).

However, it may be argued that the kinase associated with p140 of *ts* FSV is heat inactivated more slowly than would be detected during our 10-min assay period. To test this, the immunoprecipitates of p140 from *ts* and *tr* FSV, prepared as described above, were incubated for various periods of time at 41.5°C, and phosphorylation was subsequently assayed at 20°C. It can be seen in Fig. 9B that the kinase activities associated with p140 of *tr* (Fig. 9B, lanes 1 to 6) and *ts* FSV (Fig. 9B, lanes 7 to 12) were inactivated with approximately identical kinetics within a period of about 16 min. It appears that the kinase activities associated with *tr* and *ts* p140 are thermolabile, but that the activity associated with p140 of *ts* FSV is not temperature sensitive as compared to that of *tr* FSV.

We submit that p140 of *ts* FSV associates with a presumably cellular kinase at the permissive but not at the nonpermissive temperature. Once associated, the immunoprecipitated complex can phosphorylate p140 at the permissive and nonpermissive temperatures.

DISCUSSION

Phosphorylated p140 is necessary for transformation. The isolation of clones from our FSV strain which are temperature sensitive and temperature resistant in cell transformation allows the definition of viral components necessary for the maintenance of transformation. Comparison of viral components identified phos-

phorylation of the nonstructural p140 protein as the only detectable *ts* viral marker correlating with cellular transformation by these *ts* FSV clones. In addition, our experiments show that p140 functions as a specific substrate of reversible phosphorylation with a half-life of about 30 min, compared to the 5-h half-life of the protein structure of p140. Moreover, our observation that p140 of *ts* FSV is not phosphorylated at the nonpermissive temperature in cells transformed by MC29 indicates that phosphorylation of p140 is not a consequence of the transformed state of the cell but a prerequisite of transformation by FSV. Thus, our experiments prove that phosphorylation of p140 is necessary for the maintenance of transformation which extends previous evidence for a transforming function of this protein (3, 4, 10, 11). It remains to be shown that tumor induction by *ts* FSV, which occurs after longer latent periods than that by *tr* FSV, involves phosphorylation of p140.

Since p140 of FSV and the nonstructural, *gag*-related proteins of other highly oncogenic viruses, including the p110 of MC29 (3), the p100 of MH2 (5), the p90 of CMII (3), the p200 of OK10 (5), the p75 of avian erythroblastosis virus (3), the p60 protein of RSV of the avian tumor virus group (1, 9, 14), the p120 of murine Abelson virus (17), and the nonstructural proteins of the feline sarcoma viruses (15), all have in common the fact that they are phosphorylated, it is plausible that phosphorylation is necessary for transforming function of all of these proteins.

Preliminary evidence (see Fig. 2 and 6) suggests that phosphorylation of some cellular pro-

teins is also temperature sensitive in cells infected by *ts* FSV. This implies that phosphorylation of certain cellular proteins is a consequence of transformation by FSV and probably also by MC29.

Is the FSV p140 a component of a kinase complex or a kinase? The nonstructural, probable transforming proteins of a number of genetically unrelated RNA tumor viruses have been shown to be associated with kinases and, therefore, are believed to have intrinsic kinase activity (9, 11, 15, 17). If correct and if also true for FSV, this would mean that genetically unrelated transforming genes of viruses of the same taxonomic group, e.g., FSV and RSV, and of viruses of different groups, e.g., RSV, Abelson virus, and feline sarcoma viruses, all may transform by the same mechanism.

We have tried to fit the results described here and elsewhere for p140 of *ts* FSV into two models: (i) model I, which assumes that p140 is a kinase capable of autophosphorylation; and (ii) model II, which assumes that p140 is not a kinase but a specific substrate of phosphorylation that associates directly or via a third substance with a cellular kinase and phosphatase.

Model I could most readily explain temperature-sensitive phosphorylation of p140 in vivo. However, it does not shed any light on the ready dephosphorylation of *ts* p140 at the nonpermissive temperature. In fact, if one considers phosphorylation as an essential intermediate of a hypothetical kinase function of the protein, dephosphorylation may be expected not to occur at the nonpermissive temperature in at least some of the four *ts* FSV mutants we analyzed.

The observation that p140 is only phosphorylated in vivo at the permissive temperature of *ts* FSV has been considered, among other possibilities, as consistent with the view that p140 may by itself be a kinase (11). However, our results that p140 not only remains unphosphorylated, but is rapidly dephosphorylated at the nonpermissive temperature while it continues to be synthesized, are consistent with the view that p140 may instead be merely a temperature-sensitive substrate of reversible phosphorylation (see discussion of model II). Moreover, since p140 isolated from FSV-transformed cells should be fully phosphorylated, it is surprising that it is a good substrate for in vitro phosphorylation, unless in vivo different sites are phosphorylated than in vitro. Indeed, Pawson et al. (11) have shown that only a subset of the in vivo sites are phosphorylated in vitro, which may suggest that in vitro phosphorylation would not necessarily measure a physiological viral function.

Furthermore, model I fails to explain why *ts*

p140 isolated at the nonpermissive temperature is not phosphorylated when assayed in vitro at 20°C, although the protein is rephosphorylated in the cell when shifted from the nonpermissive to the permissive temperature in the presence of cycloheximide. Likewise, the model fails to explain why p140 of *ts* FSV isolated at the permissive temperature was rephosphorylated if assayed at the nonpermissive temperature in vitro (Fig. 9). The assumption that anti-*gag* serum bound to p140 of *ts* FSV renders the hypothetical kinase activity of the protein temperature resistant cannot be excluded. However, it appears unlikely, because the antibody does not seem to bind to the active center of the associated kinase, since the kinase is active in the immune complex and because it is not plausible that the *gag*-related portion of the protein is the active site of a hypothetical kinase function.

Model I also does not explain why p140 synthesized in vitro lacks kinase activity and why p140 prepared by immunoaffinity chromatography has nondetectable kinase activity (unpublished data).

Model II could explain temperature-sensitive phosphorylation of p140 in vivo if one assumes that association of p140 of *ts* FSV with a cellular kinase is temperature sensitive. The lack of associated kinase activity in *ts* p140 isolated from cells at 41.5°C and assayed at 20°C in vitro could then be explained by assuming that the p140 protein is not associated with kinase in the non-transformed cell at the nonpermissive temperature. If allowed to renature in vivo, even in the presence of cycloheximide, the protein would reassociate with a kinase while the cell starts regaining the transformed morphology. This model could also explain why p140 of *ts* FSV isolated from cells kept at 37°C, and hence associated with cellular kinase, is phosphorylated in vitro at 41.5°C. Furthermore, model II best explains our previous observation that p140 can be recovered from cells by immunoprecipitation without associated kinase (3). Finally, the second model would explain the functional analogies of p140 FSV, the p110 of MC29, the p75 of avian erythroblastosis virus, and the p60 of RSV—all of which have in common that they are phosphorylated, but only p60 of RSV, p140 of FSV, and possibly p110 of MC29 are associated with kinases (3)—and, in particular, the analogy that the p140 of *ts* FSV and p60 of *ts* RSV (1) are only fully phosphorylated at the permissive temperature.

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