Differences in the Ribosomal Protein Gel Profile after Infection of L Cells with Wild Type or Temperature-Sensitive Mutants of Vesicular Stomatitis Virus†

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ABSTRACT: The possible modification of host ribosomal proteins after infection of mouse L cells by vesicular stomatitis virus was examined. L cells were infected for 4.5 h with wild type virus or with different temperature sensitive mutants and simultaneously incubated in the presence of [^{32}P]orthophosphate. The ribosomal proteins were extracted from high salt washed ribosomes or subunits and analyzed by two-dimensional gel electrophoresis. Two differences were observed in the basic ribosomal protein pattern derived from infected cells. A new protein (or a modification of an existing ribosomal protein), SLX, was identified on the amido black stained pattern of 80S ribosomal proteins. The same protein was also found on the stained pattern of the ribosomal proteins from the 40S and 60S subunits. Also, several phosphorylated components (Pi 1-5) comigrated with the 80S ribosomal proteins, or more specifically, with the 40S subunit proteins. The capacity of temperature sensitive mutants to induce the appearance of these components was investigated. The results are discussed in terms of their capacity to kill the cell or to inhibit host protein synthesis. At low multiplicities of infection and at restrictive temperatures, only mutant W10 (IV), and not G114 (I), G22 (II), or G41 (IV), was able to promote the appearance of these components. The non-viral-induced inhibition of protein synthesis by high salt concentration did not promote the appearance of the components. We conclude that the differences observed in the ribosomal protein pattern from uninfected and infected cells are a consequence of virus infection and the establishment of a cytopathic state and are probably not responsible for the inhibition of host protein synthesis. The nature of the phosphorylated components is not known.

After infection of vertebrate cells with vesicular stomatitis virus, there is a rapid inhibition of host protein synthesis (Yamazaki and Wagner, 1970; Wertz and Youngner, 1970, 1972) and the development of a cytopathic effect expressed as cell killing (Marcus and Sekellick, 1974, 1975, 1976). We have attempted to define the viral function(s) responsible for these effects by comparing the capacity of wild type VS[1] virus and different temperature-sensitive mutants to inhibit cellular protein synthesis and to kill cells (Marvaldi et al., 1977).

Temperature-sensitive mutants of VS virus fall into five complementation groups (Wagner, 1975), representing the five known VS proteins, namely, L, G, M, N, and NS. The group I mutants carry a mutation in the cistron coding for the viral RNA transcriptase (L) (Szilagyi and Pringle, 1972; Hunt and Wagner, 1974); groups III and V mutants are localized in the cistrons for the envelope proteins M and G, respectively (Lafay, 1974); and mutants of groups II and IV are present in the cistrons designating proteins NS and N, respectively (Wagner, 1975), both of which are found in the nucleocapsid of the virion.

Our results (Marvaldi et al., 1977) indicate that minimally functional viral protein L and newly synthesized viral proteins N and NS are required for the expression of both cellular protein synthesis inhibition and cell killing. The role that these proteins play in these processes is not understood. Since cellular protein synthesis inhibition is generally accompanied by a decrease in the number of cellular polysomes (Martin and Kerr, 1968), the inhibition is thought to occur at the level of polypeptide chain initiation. Because of the important role that ribosomes play in this step, we focused our attention in this report on possible viral modifications of cellular ribosomes after infection, particularly ribosomal protein phosphorylation.

Phosphorylation of ribosomal proteins occurs in vivo in several eukaryotic systems (Krystosek et al., 1974; Zinker and Warner, 1976). Using two-dimensional polyacrylamide gel electrophoresis to separate the ribosomal proteins, Gressner and Wool (1974) showed that only a single protein, S6 (nomenclature of Sherton and Wool, 1972), is phosphorylated. The phosphorylation of S6 is enhanced by a number of stimuli such as cAMP or glucagon (Gressner and Wool, 1976a). In diabetic rats the phosphorylation of S6 is also enhanced, but is decreased toward the normal level in the presence of insulin (Gressner and Wool, 1976b).

Certain eukaryotic ribosomal proteins can be phosphorylated in vitro by several different kinases either present in the ribosome free supernatant fraction (Traugh and Porter, 1976) or bound to ribosomes (Eil and Wool, 1973a). The number of ribosomal proteins phosphorylated in vitro is considerably larger than the number found in vivo. The biological role of the phosphorylation of ribosomal proteins is still unresolved. No significant differences in cell-free protein synthetic activity have been demonstrated with phosphorylated or dephosphorylated ribosomes (Eil and Wool, 1973b).

Bacterial ribosomal proteins are not normally phosphorylated in vivo (Gordon, 1971); but after infection of *E. coli* by bacteriophage T7, some ribosomal proteins become phosphorylated (Rahmsdorf et al., 1973). The consequence of this modification on cellular functions is not understood. Apparently, such a modification occurs also in eukaryotic systems. While our work was in progress, Kaerlein and Horak (1976) reported the phosphorylation of ribosomal proteins of HeLa cells infected with vaccinia virus.

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[1] Abbreviations used: VS virus, vesicular stomatitis virus; cAMP, cyclic adenosine 3′,5′-monophosphate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; MEM, Eagle minimum medium.

In this report we have compared the two-dimensional gel electrophoretic pattern of the basic ribosomal proteins from uninfected and VS virus infected L cells. In addition to the proteins and phosphorylated components found in uninfected cells, a new protein and five phosphorylated components are detected in the gel pattern from infected cells. We have utilized temperature-sensitive mutants of VS virus that do not inhibit host protein synthesis at the nonpermissive temperature in an attempt to determine if any correlation exists between the appearance of the components and protein synthesis inhibition. Our results suggest that the differences seen in the ribosomal protein profile from infected cells are a consequence of viral infection rather than a cause of protein synthesis inhibition. The nature of the phosphorylated components and new protein is not known.

Methods

Cells: Growth Conditions. Monolayers of mouse L cells were cultivated in Eagle minimum medium (MEM) supplemented with 9% fetal calf serum. Confluent monolayers on 100-mm (Falcon plastic) dishes contained approximately 2×10^7 cells.

Viruses: Stock Preparation. The following wild type and mutants of VS virus were used: wild type (HR-C), a heat resistant strain of Indiana serotype (Holloway et al., 1970); temperature sensitive (ts) mutants G-114 (I), G22 (II), G41 (IV) carrying the G (Glasgow) designation (Pringle, 1970); and W10 (IV) carrying the W (Winnipeg) designation (Holloway et al., 1970). Confluent monolayers of mouse L cells in 100-mm dishes were infected with ts mutants or wild type HR-C at a multiplicity of infection (m_{pfp}) of 10^{-3}. The virus stock was harvested after 36–48 h at 30 °C for the ts mutants or 18–20 h at 37 °C for the wild type, when essentially all the cells manifested a gross cytopathic effect. The cell debris was removed from the medium by centrifugation for 20 min at 1000*g*. The supernatant was centrifuged for 90 min at 30 000 rpm in a Beckman 60 Ti rotor. The pelleted virus was resuspended in a buffer containing: Tris-HCl, 10 mM (pH 7.4); NaCl, 100 mM; EDTA, 1 mM. This material constituted the standard virus stock. The yield of VS virus in the standard stock solution was about 500–1000 plaque forming particles per mouse L cell.

Incorporation of [^{32}P]Orthophosphate. Five confluent monolayers each containing 2×10^7 mouse L cells (100-mm dishes) were preincubated for 2 h in fresh MEM supplemented with 9% fetal calf serum. After the preincubation period, the cells were mock infected or infected at m_{pfp} = 3, 10, 50, 100, or 200. Virus in 0.6 mL of attachment solution (phosphate-buffered saline) was adsorbed for 35 min at 37 °C. After the adsorption period, the remaining attachment solution was removed and the cells were washed with 10 mL of MEM without phosphate but containing 9% fetal calf serum. The cells were then overlaid with 3 mL of the same, serum supplemented MEM without phosphate and containing 80 μCi/mL of [^{32}P]orthophosphate. After 4.5-h incubation at 30, 37, or 40 °C, the radioactive medium was removed and 1.5 mL of cooled 0.4% (v/v) Triton X-100 in a buffer containing Tris-HCl, 10 mM (pH 7.8), magnesium acetate, 1.5 mM, KCl, 10 mM, was added to each plate. In the "salt shift" experiments, the cells were incubated for 40 min with MEM supplemented with 100 mM NaCl in the presence of 80 μCi/mL of [^{32}P]orthophosphate.

Labeling of Viral Proteins. Confluent monolayers of mouse L cells were infected at m_{pfp} = 100 as described above except that after the adsorption period, 15 mL of MEM containing 9% fetal calf serum was added, and the incubation continued for 6 h at 37 °C. After the incubation period, the cells were washed with phosphate-buffered saline and 3 mL of prewarmed (37 °C) MEM lacking amino acids and containing 16 μCi/mL of ^{3}H-labeled amino acid mixture was added immediately. After 30-min incubation at 37 °C the radioactive medium was removed and further incorporation of radioactivity was stopped by adding 1.5 mL of the 0.4% Triton X-100 buffered solution described above.

Preparation of Ribosomes and Ribosomal Subunits. The Triton X-100 treated, [^{32}P]phosphate labeled cells (approximately 10^8 cells) were scraped from the plates and the suspension was clarified by centrifugation at 30 000*g* for 10 min at 4 °C. All subsequent operations were carried out at 4 °C. The supernatant fluid was centrifuged for 3 h at 150 000*g*. The ribosomal pellet obtained was rinsed twice with buffer A (Tris-HCl, 20 mM, pH 7.6; KCl, 500 mM; $MgCl_2$, 3 mM; β-mercaptoethanol, 20 mM) and resuspended in the same buffer. The ribosomal suspension was clarified by centrifugation at 30 000*g* for 10 min. The suspension was incubated (in the presence of 500 mM KCl) for 15 additional min and then centrifuged for 3 h at 150 000*g* in a Beckmann 50 Ti rotor. The ribosome pellet was rinsed and resuspended in buffer A, and the suspension was clarified as described above.

The clear ribosomal suspension was made 0.5 mM in puromycin and incubated for 15 min at 0 °C and 15 min at 37 °C. One milliliter of this suspension was layered on a 9-mL, 0.5 M sucrose cushion in buffer A and centrifuged for 6 h at 150 000*g*. This pellet constituted the washed 80S ribosomal fraction.

For the isolation of ribosomal subunits, 1.5 mL of the puromycin treated ribosomal suspension was layered on a linear 5–20% (w/v) sucrose gradient in buffer A. The gradient was centrifuged for 16 h at 16 000 rpm in a Beckmann SW 27 rotor. The fractions were collected and those containing the 40S or 60S subunits were pooled and pelleted by centrifugation (10 h at 150 000*g*).

Extraction and Analysis of Ribosomal Proteins. The ribosomal proteins were extracted from ribosomes or ribosomal subunits with 67% acetic acid according to the procedure of Sherton and Wool (1974). The final concentration of magnesium acetate was adjusted to 67 mM during the extraction. All the proteins thus obtained (in a volume of approximately 1 mL) were dialyzed for 24 h against 1 L of 1 N acetic acid with two changes of acid. The dialyzed proteins were lyophilized. Electrophoresis was performed as described by Sherton and Wool (1972) except that the first dimension (pH 8.6) was run for 23 h at 75 V. The second dimension (pH 4.2) was run for 12 h at 105 V on a 0.9-mm slab gel using a modified version of the apparatus described by Kaltschmidt and Wittmann (1970). Approximately 0.5–0.8 mg of 80S ribosomal proteins or 0.2–0.4 mg of the 40S or 60S ribosomal proteins was analyzed. The gels were stained for 20 min in 1% amido black in 7.5% acetic acid and destained electrophoretically. The lyophilized proteins were dissolved in the sample gel and layered on the first dimension gel. These migrated toward the cathode. Under these conditions the acidic proteins migrated out of the gel into the upper buffer chamber.

When viral markers were analyzed, the sample gel containing the proteins was polymerized in the middle of the first dimension gel. Under these conditions the acidic proteins migrate toward the anode in the upper separation gel and the basic proteins migrate toward the cathode in the lower separation gel.

Preparation of Viral Markers and Polyacrylamide Gel Electrophoresis in Sodium Dodecyl Sulfate. The Triton X-100 treated cells were scraped from the dishes and the suspension

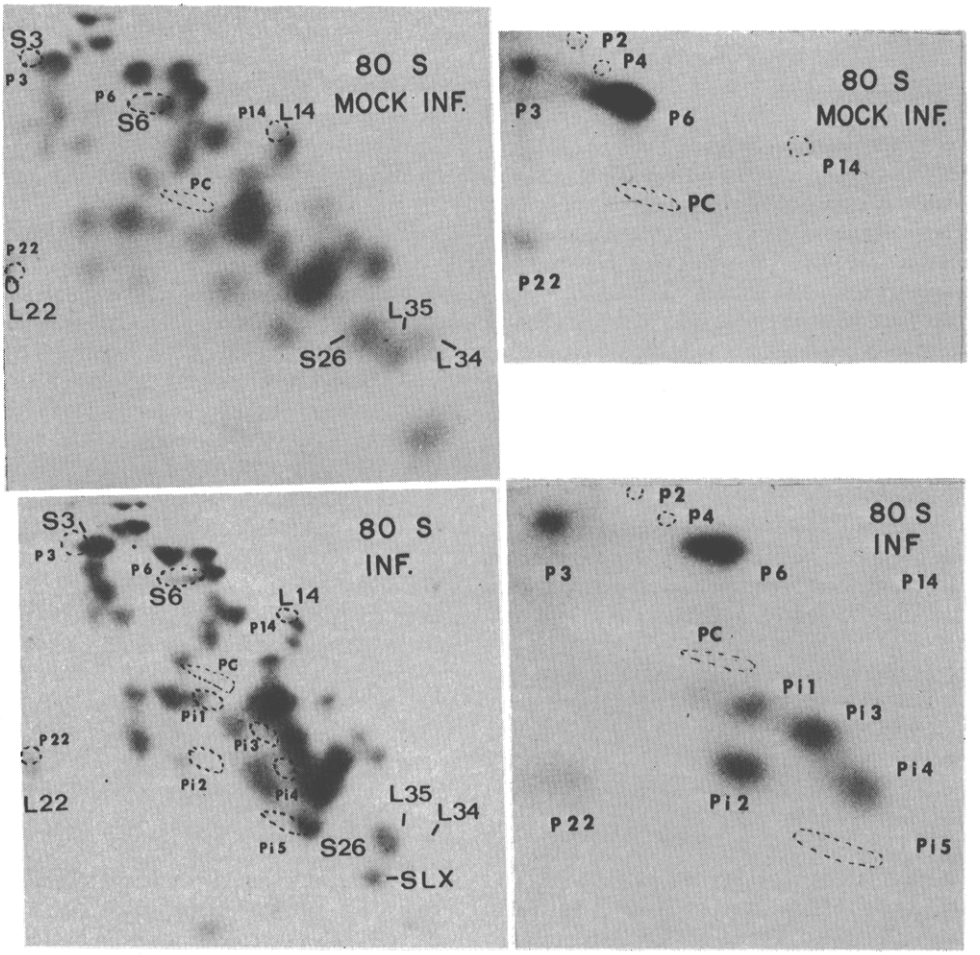

FIGURE 1: Separation by two-dimensional electrophoresis of 80S ribosomal proteins phosphorylated in vivo. Proteins were extracted from 80S washed ribosomes and analyzed by two-dimensional electrophoresis under conditions which resolved only the basic proteins (see Methods). The dried slab gels were exposed for 48 h against x-ray film. The left side of the figure shows the amido black stained pattern of 80S ribosomal proteins from mock infected or infected cells. The circles indicate the radioactive area from the corresponding autoradiogram depicted on the right side of the figure. Spots visible in the original autoradiograms but too faint to be seen in the published photograph are enclosed by dashed lines.

was clarified by centrifugation at 1000*g* for 10 min. The supernatant fraction was made 67 mM in magnesium acetate and 2 volumes of glacial acetic acid (67% final concentration) was added. All subsequent steps were the same as for the extraction of the ribosomal proteins described above. The lyophilized proteins were analyzed by polyacrylamide slab gel electrophoresis in sodium dodecyl sulfate according to the technique of Laemmli (1970), using the apparatus described by Studier (1973). Three concentrations of acrylamide were used per gel: 7.5%, 10%, and 12.5%. Electrophoresis was carried out for 5 h at a constant voltage of 60 V.

Autoradiographic Conditions. (1) Detection of [^{32}P]*Orthophosphate by Autoradiography.* After destaining, the slab gel was soaked in a solution of 50% acetone in water for 1 h and then dried on a dialysis membrane in a Hoeffer gel dryer apparatus. The dried gel was exposed for 24–48 h against a Kodak No Screen film.

(*2*) *Detection of* ^{3}H *Label by Autoradiography* (*Fluorography*). The slab gel was treated with a solution of 16% 2,5-diphenyloxazole in dimethyl sulfoxide according to the technique of Bonner and Laskey (1974) and dried as described for the detection of [^{32}P]phosphate. The dried gel was exposed for 24–48 h at −70 °C against a Kodak RP Royal film.

Results

Analysis of the Protein and Phosphorylation Patterns of the Basic 80S Ribosomal Proteins. In order to study possible virus-induced modifications of cellular ribosomal proteins, two-dimensional gel electrophoresis was used for the separation and identification of the proteins. The basic procedure in these studies involved infecting monolayer cultures of mouse L cells with wild type VS virus (or the various ts mutants) as described in Methods. For phosphorylation studies, [^{32}P]orthophosphate was added at the end of the adsorption period. At 4.5 h after infection the cells were harvested and the 80S ribosomes prepared as described earlier. No unlabeled cells were added as carrier in the [^{32}P]orthophosphate labeling experiments. The ribosomes were washed two times with 0.5 M KCl in the presence of low concentrations of magnesium and incubated at 37 °C in the presence of 0.5 mM puromycin. The proteins were extracted with acetic acid and analyzed by two-dimensional polyacrylamide gel electrophoresis as described in Methods. In the phosphorylation studies, the radioactivity was localized by subjecting the dried gel from the second dimension to autoradiography.

Figure 1 (left) shows that the stained ribosomal protein patterns from uninfected (control) and infected cells are similar with the exception of a new spot (labeled SLX) present on the gel from infected cells. This spot is localized below the three ribosomal proteins S26, L35, L34, as shown in the figure. (The nomenclature of Sherton and Wool (1972) is used throughout this report.) It also appears after infection of L cells with mengovirus (Abreu and Marvaldi, unpublished observations). The presence of this extra spot associated with the total basic

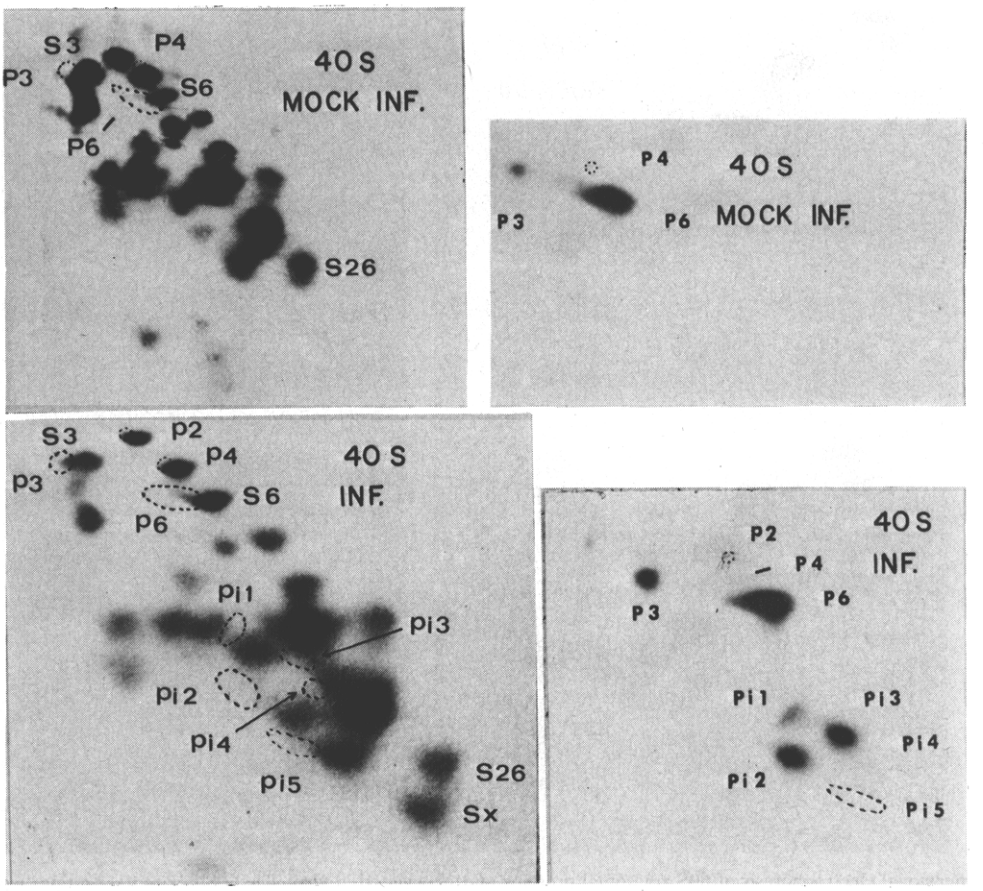

FIGURE 2: Separation by two-dimensional electrophoresis of 40S ribosomal proteins phosphorylated in vivo. The 40S ribosomal proteins were extracted from 40S subunits and analyzed by two-dimensional electrophoresis, and the dried slab gels were submitted to autoradiography for 48 h as described in Methods. The left side of the figure shows the stained pattern derived from mock infected or infected cells. The circles indicate the radioactive areas found on the corresponding autoradiogram (right). The radioactive spots P2, P4, and P_i5 are circled because they were only visible on the autoradiogram and not the published photograph of the autoradiograph.

TABLE I: Appearance of Protein SLX and Phosphorylated Components Pi1-5 after Infection of L Cells with Different Ts Mutants.[a]

	Multiplicity of infection			
	10 Modification		50 Modification	
Viruses	Pi1-5	SLX	Pi1-5	SLX
Wt HR-C	+	+	+	+
G114 (I)	−	−	−	−
G22 (II)	−	−	Not tested	Not tested
G41 (IV)	−	−	+	+
W10 (IV)	+	+	+	+

[a] In each case the experiments were carried out at the nonpermissive temperature (40 °C).

ribosomal proteins from infected cells is multiplicity dependent. With wild type VS virus it was detected at m_{pfp} = 10, 50, 100, and 200, but not at m_{pfp} = 3.

The presence or absence of spot SLX seems also to be dependent on the type of mutation on the VS virus genome (Table I). When the cells were infected at the nonpermissive temperature (40 °C) with a m_{pfp} = 10, only wild type and mutant W10 (IV) induced the appearance of SLX (data not shown). Normal patterns were obtained with mutants G114 (I), G22 (II), and G41 (IV). However, when the cells were infected at a m_{pfp} = 50 at the nonpermissive temperature, the mutant G41 (IV), as well as wt and W10 (IV), but not G114 (I) were able to promote the appearance of SLX (see Table I).

The autoradiograms corresponding to the stained ribosomal protein patterns are shown in Figure 1 (right). [^{32}P]-orthophosphate was present in four major spots in ribosomes derived from uninfected cells. One spot (P6) (P for phosphorylated) was very intense and overlapped the stained, elongated part of protein S6; another spot (P22) partially overlapped protein L22; another spot (P3) lay in the diagonal of protein S3; and a less intense spot (P14) lay in the diagonal of L14. Three additional spots (PC, P2, and P4) were also present in varying intensities from one experiment to another, but always very faint. P2 and P4 partly coincided with proteins S2 and S4. PC (C for control) migrated among several proteins and is not distinctly associated with a specific ribosomal protein. All these spots could be detected after a 48-h exposure of the gel to x-ray film. No additional spots were observed in the other parts of the slab gel, even after an exposure period of 1 week.

The autoradiograms derived from infected cells show the appearance of five distinct additional spots, Pi1, Pi2, Pi3, Pi4, and Pi5 (Figure 1, right). The presence of these spots is characteristic of the infective state and is multiplicity dependent. The spots were present when the cells were infected with a m_{pfp} = 10, 50, 100, or 200, but were absent when the m_{pfp} = 3. The extra spot, SLX, did not appear to be phosphorylated.

Protein and Phosphorylation Profiles of the 40S and 60S Ribosomal Subunits. To determine if the extra stained spot (SLX) and the phosphorylated species (P_i1-5) in the ribosomal protein preparation from infected cells associated with the small or large subunit, subunits were isolated from the twice salt washed 80S ribosomes by centrifugation through a 5-20% sucrose gradient (see Methods). The fractions corresponding

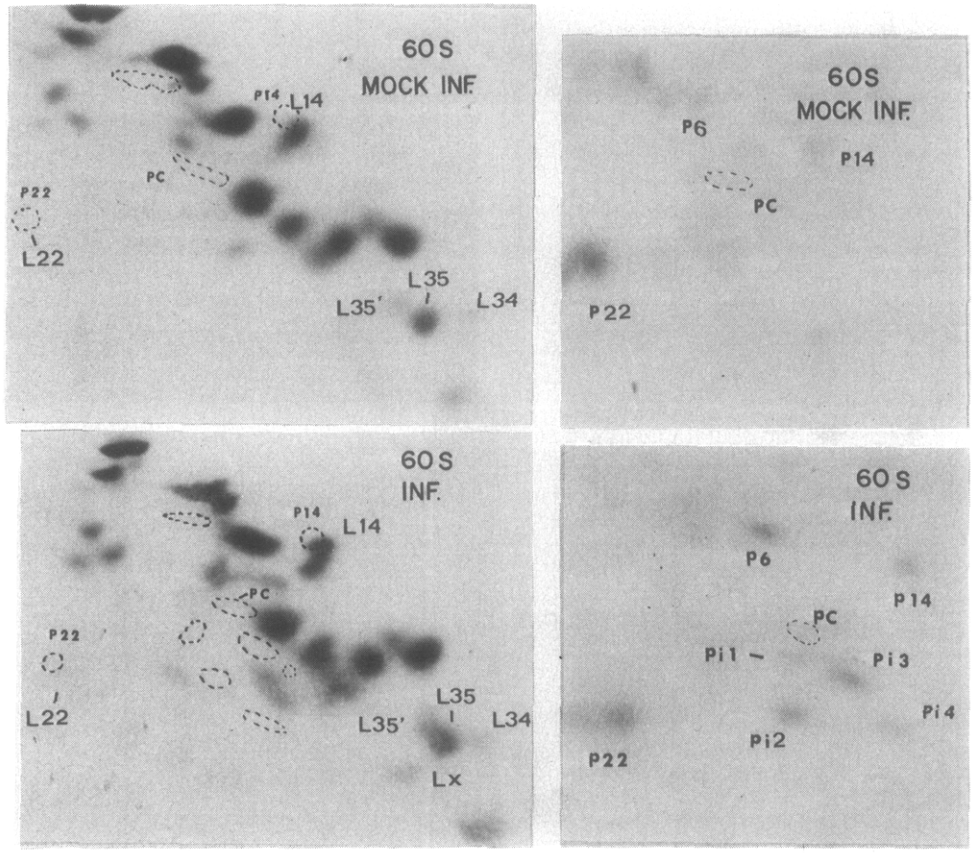

FIGURE 3: Two-dimensional electrophoresis of 60S ribosomal proteins phosphorylated in vivo. The 60S ribosomal proteins were separated by two-dimensional electrophoresis and the gels subjected to autoradiography for 48 h as described in Methods. The left half of the figure shows the pattern of 60S ribosomal proteins stained with amido black and the circles indicate the position of the radioactive spots found on the corresponding autoradiograms (right half). The position of spot PC, not visible on the published photograph of the autoradiogram, is indicated by a circle.

to the 40S and 60S subunits were pooled, the subunits pelleted by centrifugation, and their proteins extracted and subjected to two-dimensional gel electrophoresis as indicated for the 80S ribosomes.

The 40S and 60S subunit stained protein patterns (Figures 2 and 3, left) from control, uninfected cells are identical with those of rat liver subunit ribosomal proteins (Wool and Stoffler, 1974). The 40S subunit stained protein pattern (Figure 2, left) from infected cells clearly shows the presence of the spot (SX) below protein S26. The 60S subunit pattern (Figure 3) derived from infected cells also shows the presence of the extra spot (LX) below the protein L35′. When the patterns of both subunits were superimposed, these two extra spots coincided exactly, suggesting that they may be present on both subunits. However, since spot LX was more faint than spot SX, the possibility that LX is a contaminant on the 60S subunit cannot be excluded.

The radioactivity pattern of the 40S subunit proteins is shown in Figure 2 (right). When the cells were mock infected, two main spots were present on the autoradiograms, P3 and P6. Spot P4 was barely visible. When the subunits were derived from infected cells, five additional spots appeared on the autoradiograms. These spots are localized in the same position as those obtained using the 80S ribosome preparation (Pi1, Pi2, Pi3, Pi4, Pi5).

The radioactivity pattern of the 60S subunit proteins, shown in Figure 3 (right), was quite different from that of the 40S subunit proteins. Spots P22, P14, PC, and P6 were observed in the 60S subunit preparation from uninfected cells. In addition to these proteins, the 60S subunit ribosomal protein preparation from infected cells also contained a small amount of the five additional spots (Pi1–5) and coincided with those on the 40S subunit. The presence of spot P6 on the 60S subunit suggests the possibility of contamination by 40S subunits since this protein is native to 40S subunits. The occurrence of spots Pi1–5 in minor amounts may reflect this contamination.

The Association of Phosphorylated Components with Ribosomal Proteins from Cells Infected with ts Mutants of VS Virus. As mentioned above we found that ts mutants of groups I, II, and IV of VS virus do not inhibit cellular protein synthesis or kill cells at the nonpermissive temperature (Marvaldi et al., 1977). To determine if the phosphorylated components are involved in these functions, the capacity of these mutants to induce phosphorylation at the nonpermissive temperature was studied. Three different multiplicities of infection were used: 3, 10, or 50. The 80S ribosomes were prepared and their proteins analyzed as discussed above. Figure 4 demonstrates that spots Pi1–5 were present when the cells were infected with wild type VS virus (m_{pfp} = 10 or 50 (not shown)), with mutant ts W10 (IV) (m_{pfp} = 10 or 50 (not shown)), or with mutant G41 (IV) at a m_{pfp} = 50. When the cells were infected with mutant ts G22 (II) or ts G41 (IV) at a m_{pfp} = 10 or with G114 (I) at a m_{pfp} = 50, no spots appeared and the usual control (uninfected) pattern was observed (see summary in Table I). When cells were infected with wild type VS virus at a m_{pfp} = 3 at 40 °C, the corresponding autoradiogram was identical with that obtained from mock infected cells (results not shown). The autoradiograms of ribosomal proteins derived from control cells labeled at 40, 37, or 30 °C were identical.

In some experiments the cells were infected with wild type VS virus (m_{pfp} = 50) at 30 °C for 4.5 or 7 h. At 4.5 h after infection, the radioactivity pattern was similar to that derived

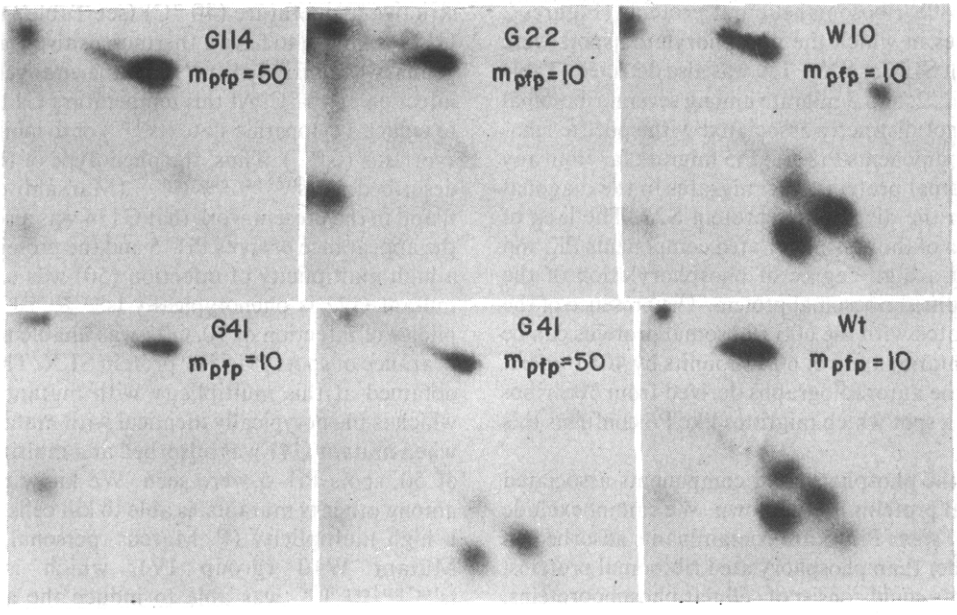

FIGURE 4: Autoradiographs of 80S ribosomal proteins derived from cells infected with different ts mutants. L cells were infected at the restrictive temperature (40 °C) with different ts mutants and multiplicities of infection as indicated in the figure. The analysis of the 80S ribosomal proteins by two-dimensional electrophoresis and their autoradiography were performed as described in Methods and in the legend of Figure 1.

from control cells; at 7 h post infection spots appeared in the same positions as Pi1–5 (data not shown). Similar results were obtained with mutant ts G41 (IV) at m_{pfp} = 50 at 30 °C for 4.5 h.

The Effect of Increasing the Salt Concentration of the Growth Medium on the Appearance of the Phosphorylated Components. It is known that, when the salt concentration is increased in the growth medium, cellular protein synthesis is rapidly inhibited (Saborio et al., 1974). To see if the phosphorylated components appear only after virus infection or after any perturbation resulting in cellular protein synthesis inhibition, the effect of increasing the salt concentration of the growth medium on these components was studied. Monolayer cultures of mouse L cells were incubated for 40 min with MEM supplemented with 0.1 M NaCl in the presence of [^{32}P]orthophosphate. Under these conditions, cellular protein synthesis was inhibited at least 50% (M. Jaye, unpublished data). Control cells were labeled with [^{32}P]phosphate for 40 min under the normal, isotonic, salt conditions. The radioactivity patterns obtained were identical in both the control and "salt shifted" experiments. The usual spots observed in control cells were present (data not shown).

Two-Dimensional Electrophoresis of Viral Markers. It was possible that the additional spot or one of the phosphorylated components may be a viral protein. To determine if viral proteins comigrate with the basic ribosomal proteins, the VS viral proteins were labeled with a mixture of ^{3}H-labeled amino acids as described in Methods. This viral marker preparation was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis or by two-dimensional electrophoresis under the same conditions used to separate the ribosomal proteins. The one-dimensional pattern showed the presence of the five viral components. The two-dimensional pattern demonstrated the presence of radioactive components only in a region of the slab gel corresponding to proteins which migrate toward the anode during the first dimension. Only a faint spot was detected in the region where the basic ribosomal proteins migrate, and this did not correspond to any of the spots observed in Figure 1 (data not shown).

Discussion

In this report we have examined the question of whether infection of mouse L cells with VS virus induces any modifications of host ribosomal proteins. To carry out these studies, the basic ribosomal proteins from uninfected and infected cells were isolated and identified by two-dimensional gel electrophoresis. The gels were either stained for protein or subjected to autoradiography for phosphorylation analysis.

The stained ribosomal protein gel patterns (Figures 1, 2, 3) from uninfected cells were identical with those of rat liver ribosomes (Wool and Stoffler, 1974). The pattern from infected cells was similar except for the presence of a new protein (or a modification of an existing ribosomal protein) SLX, which migrated below S26 (see Figure 1). This spot appeared to associate with both the 40S and 60S ribosomal subunits, since it was detected on the ribosomal protein gels from both subunits (Figures 2 and 3).

In the autoradiographic analyses (Figures 1, 2, 3), the major phosphorylated component associated with the 40S subunit from control, uninfected, cells, P6, is clearly the phosphorylated form of protein S6. Another component, P3, migrates in part with protein S3 and we believe this is the phosphorylated form of protein S3. The very faint spots P2 and P4 (not always present) are also associated with proteins S2 and S4.

Two phosphorylated components P14 and P22 were also found to associate with 60S subunit proteins. These were always very faint compared with spots P6 and P3 on the 40S subunit. From their positions on the autoradiograms, they may correspond to the phosphorylated forms of proteins L14 and L22, respectively. The phosphorylation in vivo of S3 or of the basic proteins of the 60S subunit has not previously been reported. It is possible that we detect the phosphorylation of several components because of our use of high specific activity [^{32}P]orthophosphate in the absence of carrier cells.

After infection, in addition to the phosphorylated components just mentioned, five new phosphorylated components (P_i1–5) appear which comigrate with the basic ribosomal proteins extracted from high salt washed 80S ribosomes (Figure 1). These phosphorylated components associate spe-

cifically with the 40S ribosomal subunit proteins (Figures 2 and 3). In all cases in which the phosphorylated spots were present, component SLX or SX + LX was also detected (Table I). Components Pi1, 2, and 3 migrate among several ribosomal proteins and are not distinctly associated with specific ribosomal proteins. Components Pi2 and Pi5 migrate far from any identifiable ribosomal proteins. Pi2 migrates in the diagonal of S22 and Pi5 in the diagonal of protein S25. The lack of staining in the area of the phosphorylated components Pi2 and Pi5 could suggest a high degree of phosphorylation of the corresponding modified ribosomal protein. The presence of the same spots associated with the 60S ribosomal proteins can be explained by a contamination of 60S subunits by 40S dimers. The presence on the autoradiographs derived from 60S ribosomal proteins of a spot which migrates like P6 confirms this hypothesis.

The nature of the phosphorylated components associated with the ribosomal proteins is not known. We cannot exclude the possibility that spots Pi1-5 are contaminants adsorbed on the ribosomes rather than phosphorylated ribosomal proteins. These contaminants could consist of cellular phosphoproteins, viral phosphorylated proteins, and viral or cellular RNA or phospholipids. In order to eliminate the possibility of adsorption of nonribosomal proteins on the ribosomes, 80S ribosomes were washed twice with 0.5 M KCl in the presence of low concentrations of magnesium. After each high speed centrifugation, the aggregates were removed to eliminate unwashed aggregated ribosomes. The ribosomal subunits were prepared and pelleted in the presence of 0.5 M KCl. After analysis by two-dimensional electrophoresis of the proteins extracted from the subunits, the phosphorylated components still migrated with basic ribosomal proteins.

Contamination by viral phosphoproteins also does not seem likely, since the only viral protein phosphorylated is NS (Sokol and Clark, 1973) and there are five, not one, phosphorylated components found among the ribosomal proteins. Furthermore, the tritiated viral markers do not comigrate with ribosomal proteins when subjected to two-dimensional electrophoresis.

Contamination by viral RNA is also not a likely possibility. When cells were infected with wild type virus at 30 °C for 5 h, there was considerable primary transcription, but components Pi1-5 were absent. Also, ts mutant G41 (IV) allows primary transcription at restrictive temperatures, but was unable to induce the appearance of spots Pi1-5 at a multiplicity of infection of 10. Finally, ts mutant W10 (IV), which produces only a small amount of RNA at 40 °C compared with the amount produced by wild type virus, was able to promote the appearance of components Pi1-5 as efficiently as the wild type virus. Contamination by viral induced cellular RNA or phospholipid cannot be ruled out. The isolation and purification of these components in greater quantities will be necessary to be able to identify them and to learn more about the nature of the association of these components with the ribosomal proteins.

Even though the nature of the components Pi1-5 is not known, it is clear that they are only present after viral infection. Furthermore, their appearance is multiplicity dependent. When L cells were infected at 40 °C with wild type VS virus at a multiplicity of infection of 3, spots Pi1-5 were absent. At all other multiplicities tested (10 or 50 at 40 °C or 10, 50, 100, or 200 at 37 °C), characteristic autoradiographic patterns containing spots Pi1-5 were observed.

Studies with ts mutants of VS virus indicate that the presence or absence of spots Pi1-5 depends on the type of mutation present in the VS virus genome during infection at the restrictive temperature (40 °C) (see Table I). Group I mutant G114 is known to have a thermosensitive transcriptase and no primary transcription (ts^{tra^-}) of the viral genome occurs after infection at 40 °C. At this temperature G114 was also unable to induce a cytopathic state (ts^{ckp^-}) or to inhibit cellular protein synthesis (ts^{psi^-}). Thus, the phenotype of this mutant can be described as $ts^{tra^-psi^-ckp^-pfp^-}$ [2] (Marvaldi et al., 1977). We found in the present work that G114 was also unable to induce the appearance of spots Pi1-5 and the protein SLX even when a high multiplicity of infection (50) was used. The group II mutant G22 is phenotypically $ts^{tra^+psi^-ckp^-pfp^-}$. At a multiplicity of infection of 10, G22 was unable to promote the appearance of spots Pi1-5 or protein SLX. The same result was obtained at this multiplicity with mutant G41 (group IV) which is phenotypically identical with mutant G22. However, when mutant G41 was adsorbed at a multiplicity of infection of 50, spots Pi1-5 were seen. We know that mutant G41, among other ts mutants, is able to kill cells when adsorbed at a high multiplicity (P. Marcus, personal communication). Mutant W10 (group IV), which is phenotypically $ts^{tra^+psi^+ckp^+pfp^-}$ was able to induce the appearance of the phosphorylated components even at a multiplicity of infection of 10.

Based on the results discussed above, two hypotheses can be proposed: (1) the phosphorylated components and/or the protein SLX are responsible for the decrease of cellular protein synthesis and/or the induction of a cytopathic state in the cell. The results obtained with the ts mutants of VS virus using a multiplicity of infection of 10 show a complete correlation between the presence of the phosphorylated components and SLX protein and the presence of the tra^+psi^+ and ckp^+ phenotype. The results found after infection of the cell with wild type virus or mutant G41 at 30 °C, however, are in contradiction with this interpretation. After 4.5 h of infection at 30 °C, no components associating with ribosomal proteins were detected. Yet, we know that within 5 h after infection host protein synthesis inhibition occurs at 30 °C (Marvaldi et al., 1977). To reconcile the results obtained at 30 °C in terms of protein synthesis inhibition, we can propose that only a small amount of the components is synthesized after infection at 30 °C, and that this level would be sufficient to inhibit host protein synthesis but insufficient to be detected under our experimental conditions. Cellular protein synthesis inhibition per se is not sufficient to induce the appearance of spots Pi1-5 or protein SLX. When the initiation of protein synthesis was inhibited by high salt concentrations (salt shift experiment), no components associating with the ribosomal fraction could be detected.

The second hypothesis is that the appearance of both the components Pi1-5 and the protein SLX is a consequence of the establishment of a cytopathic state in the cell rather than the cause. The results obtained with the ts mutants at restrictive temperatures at high multiplicities of infection or at 30 °C with the wild type virus are in agreement with this hypothesis. Compared with 40 °C the induction of a cytopathic state is significantly reduced at 30 °C (P. Marcus, personal communication) and no spots are apparent. Late in infection (7 h), however, when the cytopathic state becomes evident, spots Pi1-5 also begin to appear on the autoradiograms. Thus, it is probable that the components observed are a consequence of virus infection and the establishment of the cytopathic state rather than the cause.

[2] All the ts mutants should be designated ts^{pfp^-} because they do not form plaques at the nonpermissive temperature.

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References

Bonner, W. M., and Laskey, R. A. (1974), *Eur. J. Biochem. 46,* 83–88.

Eil, C., and Wool, G. G. (1973a), *J. Biol. Chem. 248,* 5122–5129.

Eil, C., and Wool, G. G. (1973b), *J. Biol. Chem. 248,* 5130–5136.

Gordon, J. (1971), *Biochem. Biophys. Res. Commun. 44,* 579–586.

Gressner, A. M., and Wool, J. G. (1974), *J. Biol. Chem. 249,* 6917–6925.

Gressner, A. M., and Wool, J. G. (1976a), *J. Biol. Chem. 251,* 1500–1504.

Gressner, A. M., and Wool, J. G. (1976b), *Nature (London) 259,* 148–150.

Holloway, A. F., Wong, P. K. Y., and Cormack, D. V. (1970), *Virology 42,* 917–926.

Hunt, D. M., and Wagner, R. R. (1974), *J. Virol. 13,* 28–35.

Kaerlein, M., and Horak, I. (1976), *Nature (London) 259,* 150–151.

Kaltschmidt, E., and Wittmann, H. G. (1970), *Anal. Biochem. 36,* 401–412.

Krystosek, A., Bitte, L. F., Cawthon, M. L., and Kabat, D. (1974), in Ribosomes, Nomura, M., Tissieres, A., and Lengyel, P., Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., pp 855–870.

Laemmli, V. K. (1970), *Nature (London) 227,* 680–685.

Lafay, F. (1974), *J. Virol. 14,* 1220–1228.

Marcus, P. I., and Sekellick, M. J. (1974), *Virology 57,* 321–338.

Marcus, P. I., and Sekellick, M. J. (1975), *Virology 63,* 176–190.

Marcus, P. I., and Sekellick, M. J. (1976), *Virology 69,* 378–393.

Martin, E. M., and Kerr, I. M. (1968), in The Molecular Biology of Viruses, Crawford, L. V., and Stoker, M. G. P., Ed., London, Cambridge University Press, pp 15–46.

Marvaldi, J. L., Lucas-Lenard, J., Sekellick, M. J., and Marcus, P. I. (1977), *Virol. 79,* 267–280.

Pringle, C. R. (1970), *J. Virol. 5,* 559–567.

Rahmsdorf, H. J., Herrlich, P., Pai, S. H., Schweiger, M., and Wittmann, H. G. (1973), *Mol. Gen. Genet. 127,* 259–271.

Saborio, J. L., Pong, S.-S., and Koch, G. (1974), *J. Mol. Biol. 85,* 195–211.

Sherton, C. C., and Wool, I. G. (1972), *J. Biol. Chem. 247,* 4460–4467.

Sherton, C. C., and Wool, I. G. (1974), *Mol. Gen. Genet. 135,* 97–112.

Sokol, F., and Clark, H. F. (1973), *Virology 52,* 246–263.

Studier, F. W. (1973), *J. Mol. Biol. 79,* 237–248.

Szilagyi, J. E., and Pringle, C. R. (1972), *J. Mol. Biol. 71,* 281–291.

Traugh, J. A., and Porter, G. G. (1976), *Biochemistry 15,* 610–616.

Wagner, R. R. (1975), in Comprehensive Virology, Fraenkel-Conrat, H., and Wagner, R. R., Ed., New York, N.Y., Plenum Press, p 1.

Wertz, G. W., and Youngner, J. S. (1970), *J. Virol. 6,* 476–484.

Wertz, G. W., and Youngner, J. S. (1972), *J. Virol. 9,* 85–89.

Wool, I. G., and Stoffler, G. (1974), in Ribosomes, Nomura, M., Tissieres, A., and Lengyel, P., Ed., Cold Spring Harbor, N.Y., Cold Spring Harbor Laboratory, pp 417–460.

Yamazaki, S., and Wagner, R. R. (1970), *J. Virol. 6,* 421–429.

Zinker, S., and Warner, J. R. (1976), *J. Biol. Chem. 251,* 1799–1807.