In vitro Phosphorylation of NS Protein by the L Protein of Vesicular Stomatitis virus

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SUMMARY

The structural proteins L and NS of vesicular stomatitis virus were obtained from purified viral ribonucleoprotein complex followed by phosphocellulose column chromatography and assayed for protein kinase activity using [γ-32P]ATP as the phosphate donor. The fractions containing purified L protein phosphorylated NS protein in vitro. 8-Azido-ATP, a photoreactive analogue of ATP, was also used as the phosphate donor for phosphorylation of NS protein by the L protein. In the presence of ultraviolet light, only L protein was specifically cross-linked with 8-azido-[γ-32P]ATP. In the absence of u.v. light 8-azido ATP did not inhibit RNA transcription in a reconstituted reaction or substitute ATP for RNA synthesis in vitro. The above results, taken together, suggest that 8-azido-ATP was bound to the kinase site and phosphorylation of NS protein was mediated by the L protein. Exogenous phosphate acceptor proteins such as phosvitin and casein were also phosphorylated by the L protein fraction. However, addition of an excess of phosvitin failed to compete with the phosphorylation of NS by L, indicating that the protein kinase activity possessed higher affinity for NS. The phosphorylation of NS was strongly inhibited by photoreaction of L protein with 8-azido-ATP with concomitant inhibition of transcription in vitro. These results suggest that phosphorylation of NS protein by L may have a role in the regulation of the virus genome transcription in vitro.

INTRODUCTION

The presence of protein kinase activity in purified virions of many enveloped RNA- and DNA-containing viruses has become firmly established (Akusjärvi et al., 1978; Downer et al., 1973; Grubman et al., 1981; Hatanaka et al., 1972; Imblum & Wagner, 1974; Lemaster & Roizman, 1980; Moyer & Summers, 1974; Randall et al., 1972; Silverstein & August, 1973; Snyder, 1982; Tan, 1975) since the first demonstration of protein kinase activity in Rauscher murine leukaemia virus, avian myeloblastosis virus, and vesicular stomatitis virus (VSV) (Strand & August, 1971). In addition, phosphorylated viral proteins are found in both virions and infected cells in a variety of viral systems (Sokol & Clark, 1973; Tan & Sokol, 1972). Viral gene products having autophosphorylating activity specifically at tyrosine residues have been shown in some oncovirus transforming gene products (Eckhart et al., 1979; Hunter & Sefton, 1980; Smith et al., 1979; Witte et al., 1980) and amongst the early proteins of papovaviruses (Eckhart et al., 1979; Schaffausen & Benjamin, 1979; Scheidtmann et al., 1982; Smith et al., 1979). The ubiquitous presence of both virion-associated protein kinases and phosphorylated viral proteins in many non-transforming enveloped RNA viruses have prompted studies to determine the regulatory role, if any, of the phosphorylation process in the life-cycle of these viruses.

VSV, a prototype of membrane-maturing rhabdoviruses, is a model virus which enables aspects of phosphorylation and its possible role in the viral infective process to be studied. VSV contains a protein kinase activity associated with the virion (Imblum & Wagner, 1974; Moyer & Summers, 1974) which phosphorylates serine, threonine, and tyrosine residues in endogenous viral proteins or exogenous phosphate acceptor proteins (Clinton et al., 1982; Clinton & Huang, 1981). In addition, the non-structural protein, NS, and the matrix protein, M, are the major
phosphoproteins found in infected cells (Clinton et al., 1979; Imblum & Wagner, 1974; Moyer & Summers, 1974). Since NS protein, in association with the L protein, constitutes the RNA polymerase complex (Emerson & Yu, 1975; Naito & Ishihama, 1976) that transcribes the nucleoprotein (N)-bound genome RNA template, regulation of phosphorylation of this protein may play a key role in the replicative process of the virus. A number of studies in vitro and in vivo have strongly suggested that the degree of phosphorylation of NS protein plays a regulatory role in transcription processes in vitro (Clinton et al., 1978; Hsu et al., 1982; Kingsford & Emerson, 1980; Sinacore & Lucas-Lenard, 1982; Testa et al., 1980; Watanabe et al., 1974; Witt & Summers, 1980).

Although the virion-associated protein kinase in VSV appears from several studies (Clinton et al., 1982; Harmon et al., 1983; Imblum & Wagner, 1974; Moyer & Summers, 1974; Sinacore & Lucas-Lenard, 1982) to be of cellular origin, the following results lend credence to the idea that part of the protein kinase activity may be of viral origin: (i) the protein kinase activity, like the virion-associated RNA polymerase, is only discernable upon detergent disruption of purified virions (Tan, 1975); (ii) considerably higher specific activity of the enzyme (80-fold) was found in VSV than in enveloped Semliki Forest virus when both viruses were grown in the same host cell (Tan, 1975); (iii) protein kinase activity was always associated with highly purified transcribing ribonucleoprotein (RNP) even after removal of virtually all of the viral membranes and other cellular components (Imblum & Wagner, 1974; Sinacore & Lucas-Lenard, 1982). Therefore, in the present work, we have purified virus structural components, L and NS, in order to investigate the nature and location of the protein kinase. Here, we present evidence that highly purified L protein specifically phosphorylates NS protein in vitro.

**METHODS**

*Virus and cell cultures.* VSV (Indiana serotype, Mudd–Summers strain) was grown in roller bottles containing a monolayer of baby hamster kidney cells (BHK-21, clone 13) and purified as described previously (Banerjee et al., 1974).

**Purification of soluble protein fractions from purified virions.** Purified VSV (500 µg protein/ml) was solubilized in low salt containing 10 mM-Tris–HCl pH 8.0, 5% (v/v) glycerol, 0.4 M-NaCl, 185 mM Trition X-100, and 0.6 mM-dithiothreitol (DTT). The RNP, containing L, NS, and N–RNA complex, was sedimented by centrifugation in an SW60 rotor at 45000 r.p.m. for 2 h, through 20% glycerol onto a 100% glycerol cushion. The RNP was collected from the top of the cushion and diluted fivefold with a buffer containing 10 mM-Tris–HCl pH 8.0, and 1 mM-EDTA. An equal volume of high salt buffer containing 20 mM-Tris–HCl pH 8.0, 1.6 M-NaCl and 10% glycerol was added and the extract held on ice for 1 h with occasional shaking. The extract was then centrifuged through 30% glycerol as described above. The released L and NS proteins (designated low salt–high salt fraction) were recovered from the top (approximately 2 ml) of the centrifuge tube, dialysed against phosphocellulose column buffer (PC buffer) containing 20 mM-Tris–HCl pH 7.4, 10% glycerol, 0.1 Triton X-100, and 0.3 mM-DTT. The dialysate was loaded onto a phosphocellulose column (Whatman) (1.5 x 3.5 cm) equilibrated with PC buffer. The unbound fraction, which polyacrylamide gel electrophoresis revealed to be NS protein, was eluted with 10 ml of PC buffer. The column was then washed with PC buffer containing 0.2 M-NaCl. Elution of L protein was accomplished by using 1 M-NaCl in PC buffer. L and NS proteins were dialysed against 0.05 M-Tris–HCl pH 8.0 containing 20% glycerol, 0.2 mM-NaCl and 0.3 mM-DTT, and concentrated by ultrafiltration in an Amicon cell (Diaflo YM10). To prepare the high salt solubilized protein mixture, purified VSV (500 µg protein/ml) was disrupted with an equal volume of high salt, as described above. The protein fractions were recovered from the top of the centrifuge tube and dialysed as described above. Concentrations of proteins in the low salt–high salt fraction (L + NS), purified L, and NS protein were 340 µg/ml, 150 µg/ml, and 120 µg/ml, respectively. The concentration of protein in high salt fraction was 1.2 mg/ml.

**Polyacrylamide gel electrophoresis.** Proteins were analysed by electrophoresis in 10% polyacrylamide slab gels overlaid with a 5% polyacrylamide stacking gel (Laemmli, 1970). Dried gels were exposed to Kodak X Omat XAR-2 film. The labelled bands were excised from the gels and radioactivity was determined in a Beckman liquid scintillation counter.

**Protein kinase assay.** Protein kinase activity was assayed by measuring the incorporation of $^{32}$P from [γ-$^{32}$P]ATP into the protein bands. A standard incubation mixture contained, in a volume of 0.1 ml, 60 mM-Tris–HCl pH 8.0, 6 mM-MgCl$_2$, 10 mM-DTT, 5 µM-ATP, 20 to 30 µCi [γ-$^{32}$P]ATP (sp. act. 6200Ci/mmol) and variable amounts of viral proteins. Triton X-100 (0.05%) was included when complete virions were used in the assay. Incubations were for 30 min at 37 °C, and the reactions were terminated by addition of 12 µl each of 0.1 M-EDTA and 20 mM-ATP. Proteins were then precipitated with 1.5 vol. of 10% CCl$_3$COOH, washed once with 5% CCl$_3$COOH and three
times with ether. The dried pellets were suspended in gel electrophoresis sample buffer, placed in a boiling water bath for 2 min, and analysed by electrophoresis.

**Labelling of viral proteins with [γ-32P]8-azido-adenosine-5'-triphosphate (8-azido-ATP).** Twenty-five μCi of 8-azido-[γ-32P]ATP was dried and resuspended (10 μM final concentration) in 50 μl water containing the indicated amounts of L and NS proteins. The preparation, at a distance of 6 cm, was exposed to ultraviolet (u.v.) light with a peak wavelength of 364 nm (Ultra-Violet Products, San Gabriel, Ca., U.S.A.) for 30 min. After the incubation, the proteins were precipitated with ice-cold CCl₄:COOH and analysed by polyacrylamide gel electrophoresis.

When non-radioactive 8-azido-ATP was used, preparations of L and NS proteins were irradiated with u.v. light in the presence of 100 μM-8-azido-ATP in a total volume of 30 μl. After irradiation, the other components of the protein kinase reaction mixture were added and the reaction was carried out as described above.

**Immunoprecipitation.** High salt supernatant fraction (10 μl) was treated with 3 μl of monospecific antibody to NS protein (kindly provided by Dr. L. Prevèc, McMaster University, Hamilton, Ontario, Canada) and kept at 4 °C for 14 h. The complex was precipitated by centrifugation, washed twice with buffer containing 0.01 M-Tris-HCl pH 8.0, 0.2 M-NaCl, 20% glycerol and 50 mg/l DTT, and directly assayed for protein kinase activity.

**Materials.** Reagents for PAGE were purchased from Bio-Rad. Phosphocellulose was from Pharmacia. Phosvitin, casein, and 8-azido-ATP were purchased from Sigma. [γ-32P]ATP (6200 Ci/mmol) and [γ-32P]CTP (410 Ci/mmol) were obtained from Amersham. [γ-32P]8-azido-ATP (19.6 Ci/mmol) was purchased from ICN.

**RESULTS**

**Protein kinase activity associated with fractionated VSV structural proteins**

Upon incubation of high salt-solubilized virus proteins with [γ-32P]ATP, as expected, all virus structural proteins were phosphorylated (Fig. 1b) (Clinton et al., 1978; Imblum & Wagner, 1974; Moyer & Summers, 1974). The counts per minute (× 10⁻³)/μg of protein for each of the viral polypeptides were as follows: L, 0.7; G, 4; NS, 38; N, 1; and M, 4. Thus, on a per μg basis, incorporation of ³²P into the NS protein appeared to be highest among all of the viral proteins.

We next used monospecific antibody against NS protein to study whether any protein kinase activity was associated with the NS protein. High salt-solubilized viral proteins were treated with the NS antibody and the purified immunoprecipitated complex was tested for protein kinase activity. As shown in Fig. 1 (a), the NS protein was specifically labelled with ³²P: a trace amount of radioactivity was found with the L protein and in a band migrating faster than the N protein. These results indicated that: (i) the NS protein itself may have an autophosphorylating activity similar to the transforming gene product of oncornaviruses; (ii) the protein kinase activity may be associated with the L protein that phosphorylated the NS protein and was precipitated with the antibody due to its tight association with the NS protein; or (iii) some other cellular contaminant, precipitating with the antibody, phosphorylated the NS protein.

The first indication that one of the viral proteins, L or NS, may have a protein kinase activity associated with it came from the results obtained when a fraction containing the L and NS proteins was tested for phosphorylation of endogenous NS protein. It can be seen in Fig. 1 (c) that the NS protein was almost exclusively labelled. To study whether the L or NS protein was involved in the kinase activity, a mixture of L and NS proteins was separated in a phosphocellulose column (see Methods). The unbound fraction after polyacrylamide gel electrophoresis followed by silver staining showed no L protein but contained the NS protein (Fig. 2a), which was confirmed by Western blot analysis using anti-NS antibody (data not shown). In addition, a protein band migrating slightly faster than the NS protein was found to be N protein by similar analysis using anti-N antibody (data not shown). Possibly a trace amount of N-protein was removed by the treatment of purified RNP with high salt. The L protein, on the other hand, was eluted from the phosphocellulose column with 1 M-NaCl and found to contain a trace amount of NS and N proteins which were only discernible by silver staining (Fig. 2b). The protein kinase activity in each of these two fractions was determined separately and in combination. As shown in Fig. 2(c), the purified NS fraction contained no demonstrable autophosphorylating activity. The L protein fraction incorporated a small amount of radioactivity into the ‘bound’ NS protein (Fig. 2d). However, when equal volumes of NS and L fractions were mixed and protein kinase activity was assayed, a 30-fold increase of ³²P incorporation into the NS protein was found (Fig. 2e). These results suggested that the L protein may possess a protein kinase activity that phosphorylated the NS protein. However, the
Fig. 1. Protein kinase activity in soluble viral protein fractions. (b) High salt-solubilized proteins (10 μl), (c) low salt–high salt fraction (20 μl) and (a) NS–antibody complex were prepared as described in Methods. Protein kinase activity in each fraction was assayed and labelled proteins were analysed by polyacrylamide gel electrophoresis followed by autoradiography. Migration positions of viral structural proteins, L, G, NS, N and M are shown in the figure.

Fig. 2. Protein kinase activity of purified L and NS proteins. (b) L and (a) NS proteins were purified by phosphocellulose chromatography, as described in Methods, and analysed by polyacrylamide gel electrophoresis followed by silver staining. Aliquots of NS (20 μl, c), L (20 μl, d) and L + NS (10 μl each, e) were assayed for protein kinase activity and labelled protein bands were visualized by autoradiography after polyacrylamide gel electrophoresis. Migration positions of L, NS and N are shown. Concentration of L and NS proteins were 150 μg/ml and 120 μg/ml, respectively.
Phosphorylation of VSV NS protein

Fig. 3. Phosphate turnover in kinase reaction. Aliquots of a mixture of L and NS fractions were assayed for kinase activity in the presence of unlabelled ATP (20 μM) (lanes a, b, c). After incubation for 30 min, 30 μCi of [γ-32P]ATP was added and labelled proteins at (a) 15 min, (b) 30 min and (c) 60 min were analysed by polyacrylamide gel electrophoresis followed by autoradiography. Four additional aliquots (lanes d to g) were phosphorylated for 30 min with 20 μM-ATP containing 30 μCi of [γ-32P]ATP, and subsequently an excess of unlabelled ATP (2 mM) was added and the radioactivity incorporated into NS protein was determined after (d) 0 min, (e) 15 min, (f) 30 min and (g) 60 min of incubation and analysed as above. O, Origin.

Presence of a contaminating cellular protein kinase was not ruled out at this stage of L protein purification. The L protein fraction (1 μg) incorporated approximately 1 pmol of 32P/μg of NS protein/30 min. Subsequent analysis indicated that phosphorylation occurred predominantly at serine residue(s) (95%) and a small amount (5%) in threonine residue(s) of the NS protein (data not shown).

To rule out the possibility that the observed protein kinase activity was indeed a kinase reaction and not an exchange of 32P with the unlabelled amino acid phosphates in NS, the following experiments were performed. The NS protein was phosphorylated in the presence of L protein fraction for 30 min with 20 μM-ATP containing 30 μCi [γ-32P]ATP and subsequently unlabelled ATP (2 mM) was added and the radioactivity incorporated in NS protein was determined after 15, 30, and 60 min of incubation. As shown in Fig. 3(e, f, g), the initial 32P incorporated in NS protein remained virtually unchanged following incubation with unlabelled ATP. Similarly, when unlabelled ATP (20 μM) was added to the reaction mixture at the start of the reaction and after 30 min 30 μCi [γ-32P]ATP was added, very little radioactivity was incorporated into the NS protein (Fig. 4a, b, c). These results suggest that phosphorylation of NS by L protein was not due to phosphate exchange but rather to stable linkage of 32P with the amino acid residues of the NS protein.

Further purification of L protein and binding studies with 8-azido-ATP

To confirm that the L protein and not some cellular contaminant might be involved in mediating phosphate transfer from ATP to the NS protein, further purification of L protein was carried out. A mixture of L and NS proteins was first purified by phosphocellulose chromatography as in Fig. 2. The purified L protein was further subjected to two cycles of
Fig. 4. Preparation of highly purified L protein. A mixture of L and NS proteins (lane a) was applied on a phosphocellulose column, as described in Methods. The 1 M-NaCl eluate was dialysed and subjected to two further cycles of chromatography. The final 1 M-NaCl eluate containing the highly purified L protein fraction (lane b) was analysed by polyacrylamide gel electrophoresis followed by silver staining. Lanes (c) and (d) contain NS protein (flow-through fraction) and purified VSV marker, respectively.

phosphocellulose chromatography. As shown in Fig. 4(b), following silver staining, neither NS protein nor any other contaminating protein was discernible in the L protein fraction. The highly purified L protein fraction was reacted with 8-azido-[γ-32P]ATP as a photoaffinity probe (Banerjee & Racker, 1977; Haley, 1983; Haley & Hoffman, 1974) to locate the binding site of ATP. The photoreacted proteins were subsequently analysed by electrophoresis in a polyacrylamide gel. As shown in Fig. 5(a), a distinct labelled band was associated only with the L protein. The label in the band was virtually removed (>90%) when the reaction was carried out in the presence of an excess of unlabelled ATP (Fig. 5b). In contrast, purified NS protein failed to react with 8-azido-[γ-32P]ATP (data not shown). The 8-azido-[γ-32P]ATP was effectively used as a phosphate donor, as shown by the labelling of NS protein in the presence of L protein (Fig. 5c). Since no other protein apart from L protein reacted with 8-azido-ATP, the above results strongly suggest that the L protein possesses a protein kinase activity. It is important to note that in an in vitro reconstitution reaction using purified L, NS, and N–RNA complex, no RNA synthesis occurred when ATP was replaced by 8-azido-ATP. Moreover, the analogue did not inhibit RNA synthesis when added at a concentration of 1 mM (data not shown). Thus, binding of 8-azido-ATP to L protein was directed at the active site of protein kinase and not at the initiation site for RNA synthesis.
Phosphorylation of VSV NS protein

Fig. 5. Labelling of L protein with 8-azido-[γ-32P]ATP. L and NS proteins were obtained as in Fig. 3, except that the L protein was further purified by double chromatography on a phosphocellulose column. Binding of L protein with 8-azido-[γ-32P]ATP was carried out as described in Methods. (a) L protein (15 μl) u.v.-cross-linked with 8-azido-[γ-32P]ATP; (b) L protein (15 μl) u.v.-cross-linked with 8-azido-[γ-32P]ATP in the presence of unlabelled ATP (70 μM); (c) protein kinase activity of L + NS fractions (15 μl each) using 8-azido-[γ-32P]ATP as phosphate donor. Autoradiogram exposure time for lanes (a) and (b) was 24 h, whereas in lane (c) it was 1 h.

Fig. 6. Phosphorylation of exogenous phosphate acceptor proteins. L and NS proteins were obtained as in Fig. 5, and the kinase reaction was carried out as described in Methods. (a) L protein (15 μl); (b) L + NS proteins (15 μl each); (c) L protein (15 μl) + phosvitin (20 μg); (d) L protein (15 μl) + NS protein (15 μl) + phosvitin (20 μg); (e) NS protein (15 μl) + phosvitin (20 μg). The labelled proteins were analysed by polyacrylamide gel electrophoresis followed by autoradiography.

Specificity of the kinase reaction

To test whether the L-associated protein kinase activity was specific for the NS protein, we used phosvitin as a phosphate acceptor protein. As shown in Fig. 6, using highly purified L protein fraction (see Fig. 4), no labelled protein band was detected (a). This result indicated that (i) L protein lacks autophosphorylating activity, and (ii) the fraction seems to be free from non-specific cellular kinase capable of phosphorylating the L protein (see Fig. 1b). However, the L protein fraction effectively phosphorylated the exogenously added phosvitin (c). The phosphorylation of NS protein (b), however, remained virtually unchanged even in the presence of an excess of phosvitin (d). Purified NS protein failed to phosphorylate exogenous phosvitin (e). As shown in Table 1, addition of increasing amounts of phosvitin failed to inhibit phosphorylation of NS by L protein. In contrast, when casein (1 μg) was used as a phosphate acceptor, phosvitin could effectively compete with its phosphorylation. These results suggest that the L protein possesses a protein kinase activity that preferentially phosphorylated the NS protein.

Phosphorylation of NS protein and the transcription process in vitro

Since the degree of phosphorylation of NS protein has been shown to play a role in VSV transcription (Clinton et al., 1978; Hsu et al., 1982; Kingsford & Emerson, 1980; Sinacore &
Fig. 7. Effect of photoreaction of L protein with 8-azido-ATP on protein kinase activity and transcription in vitro. Aliquots of purified L (15 μl) and NS proteins (15 μl) (Fig. 5) were photoreacted with unlabelled 8-azido-ATP, as described in Methods. Protein kinase activity of the mixture of L and NS proteins was assayed and labelled proteins analysed as in Fig. 3. (a) U.v.-irradiated L fractions + NS protein; (b) L protein u.v.-cross-linked with 8-azido-ATP + NS protein; (c) L fraction + u.v.-irradiated NS protein; (d) L fraction + NS protein u.v.-cross-linked with 8-azido-ATP. RNA synthesis in vitro using N–RNA complex reconstituted with L and NS proteins was carried out as described (De et al., 1982). RNA products were labelled with [α-32P]CTP (30 μCi), then purified and analysed by electrophoresis on a 20% polyacrylamide slab gel containing 8 M-urea as described (De et al., 1982). (e) U.v.-irradiated L fraction + NS protein; (f) L protein u.v.-cross-linked with 8-azido ATP + NS protein; (g) L fraction + u.v.-irradiated NS protein; (h) L fraction + NS protein u.v.-cross-linked with 8-azido-ATP. 'Leader' represents leader RNA, and migration positions of NS protein and xylene cyanol (XC) are shown.

Table 1. Effect of phosvitin on the phosphorylation of NS and casein by L protein

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<th>Phosvitin (μg)</th>
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<td>563</td>
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<td>ND†</td>
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*Fractions containing L (15 μl) and NS (15 μl) proteins, prepared as described in Methods, were assayed for protein kinase activity in the presence of casein (1 μg) and different concentrations of phosvitin, as indicated in the table. The labelled proteins were analysed by polyacrylamide gel electrophoresis followed by autoradiography. The bands were excised from the gel and radioactivity determined.
† ND, Not determined.

Lucas-Lenard, 1982; Testa et al., 1980; Watanabe et al., 1974; Witt & Summers, 1980), it was of interest to study whether inhibition of NS phosphorylation has any effect on RNA transcription in vitro. We photoreacted the L protein with unlabelled 8-azido-ATP and studied its effect on phosphorylation of NS protein. As shown in Fig. 7(b), phosphorylation of NS protein was inhibited by more than 90% when L protein was photoreacted, compared to the control reaction (a) where L protein was irradiated in the absence of the analogue. In contrast, photoreaction of
NS protein had no effect on the L-associated protein kinase activity (Fig. 7c, d). Similarly, RNA synthesis virtually ceased when photoreacted L protein was reconstituted with NS and N–RNA complex (Fig. 7f), compared to the control experiment (e). In contrast, photoreaction of NS protein had no effect on RNA synthesis (Fig. 7g, h) when reconstituted with L and N–RNA complex. Since 8-azido-ATP did not substitute ATP for RNA synthesis in vitro or inhibited RNA synthesis in the presence of all four ribonucleoside triphosphates (data not shown), and coupled with the fact that phosphorylation of NS and overall transcription were strongly inhibited by photoreaction of L with 8-azido-ATP, these results suggest that the degree of phosphorylation of NS protein may have a role in the transcription process in vitro.

**DISCUSSION**

The presence of protein kinase activity in detergent-disrupted VSV has been known for some time (Clinton et al., 1978; Imblum & Wagner, 1974; Moyer & Summers, 1974), but the characterization and identification of its role in the viral life-cycle have not been unequivocally established. Despite the fact that the bulk of the enzyme activity could be seen in the detergent-solubilized low salt-washed fraction of the virus (Clinton & Huang, 1981; Imblum & Wagner, 1974; Moyer & Summers, 1974), a consistently low yet saturating level of protein kinase activity could always be found associated with the remaining purified RNP core, the structure known to contain the N–RNA complex in association with the L and NS proteins (Imblum & Wagner, 1974; Harmon et al., 1983; Sinacore & Lucas-Lenard, 1982). These observations raised the possibility that some of the protein kinase activity in VSV might be associated with viral structural proteins.

In the present study we have fractionated the RNP, purified the soluble proteins L and NS and assayed for the protein kinase activity in both preparations separately and in combination. It should be emphasized that our methods of purification of protein kinase activities were different from those reported by others (Imblum & Wagner, 1974; Sinacore & Lucas-Lenard, 1982). We removed all of the G, M, and some L and NS proteins by thoroughly washing the detergent-disrupted VSV with 0.4 M-NaCl. The highly purified RNP (which by silver staining showed only L, NS and N proteins) was used as the starting material for the isolation of the mixture of L and NS proteins. Separation and purification of these proteins were further achieved by phosphocellulose chromatography (Fig. 2). Although in some L protein preparations trace amounts of NS protein were discernible after silver staining (Fig. 2), repeated chromatography of the L protein on a phosphocellulose column removed all contaminating NS protein (Fig. 4). The L or NS proteins purified by this procedure lacked autophosphorylating activity, whereas addition of L fraction to the NS protein resulted in significant phosphorylation of the NS protein (Fig. 5). Furthermore, it was confirmed that the phosphate addition was due to de novo phosphorylation of NS protein rather than phosphate exchange mediated by an associated phosphatase activity (Fig. 3). This result is consistent with the similar observations made by Clinton et al. (1979).

Using photoreactive 8-azido-[γ-32P]ATP, we have demonstrated that only L protein reacts specifically with this ATP analogue. No other labelled protein band was discernible in the gel, even after long exposure of the autoradiogram. These results strongly suggest that the L protein and not a non-viral protein component was involved in the phosphorylation of NS. Similar photoreactive probes have been successfully used to locate and characterize cellular protein kinases and ATPases (Banerjee & Racker, 1977; Haley, 1983; Haley & Hoffman, 1974). It should be noted that the L protein, in addition to possessing a protein kinase activity, is also involved in the transcription process (Emerson & Yu, 1975) and requires ATP for initiation of RNA synthesis. However, 8-azido-ATP did not substitute for ATP in RNA synthesis in vitro or inhibit transcription at a concentration of 1 mM (data not shown). Moreover, the concentration of 8-azido-[γ-32P]ATP was kept at 10 μM, which was considerably lower than the optimum concentration of ATP (the natural substrate for RNA synthesis) required for transcription in vitro (500 μM, Testa & Banerjee, 1979). Thus, it is reasonable to assume that the L protein possesses no binding affinity for 8-azido-ATP at its transcription initiation site. On the other hand, 8-azido-ATP has been shown to be an excellent substrate for protein kinase enzyme.
Therefore, the observed phosphorylation of NS by the L protein and the photoreaction of 8-azido-[^γ-32P]ATP with the L protein strongly suggest that the analogue was binding to the kinase activity site rather than at the RNA initiation site of the L protein.

In addition to phosphorylating the NS protein, purified L protein was also capable of phosphorylating exogenously added phosphate acceptor proteins such as phosvitin (Fig. 6) and casein (Table 1). Efficient phosphorylation of casein and phosvitin by the L protein is consistent with the previous observation of Tan (1975), who showed similar phosphorylation of these proteins by RNA viruses, including VSV, which contain virion-associated RNA polymerase activity. Purified Semliki Forest and Sindbis viruses, on the other hand, phosphorylated casein and phosvitin quite inefficiently. Addition of a tenfold excess of phosvitin effectively competed with the phosphorylation of casein but failed to compete with the phosphorylation of NS protein even in 50-fold excess (Table 1). These results indicated that the L protein has high affinity binding for the NS protein and subsequent phosphorylation occurs at phosphate acceptor sites on the latter protein. The high affinity of NS for L protein is not unexpected since L and NS constitute the RNA polymerase complex that transcribes the viral genome RNA

in vitro

(Emerson & Yu, 1975). Furthermore, we have routinely observed that antibody against NS protein (Fig. 1a) precipitated, in addition to NS protein, a significant amount of L protein. The protein kinase activity was always discernible in this complex. Similar results were also obtained recently by Bell et al. (1984). It is interesting to note that the phosphorylation of NS protein was predominantly at the serine residues with no detectable phosphorylation at tyrosines (data not shown). These results indicate that the previously reported tyrosine-specific protein kinase(s) (Clinton et al., 1982; Clinton & Huang, 1981) and the bulk of threonine-specific protein kinase detected in the detergent disrupted virions are not associated with the L protein. Most probably, these kinases are of cellular origin.

We have also shown that photoreaction of L protein with 8-azido-ATP caused inhibition of both protein kinase activity and the transcriptase activity (Fig. 7). Since 8-azido-ATP is not a substrate for RNA synthesis

in vitro

(data not shown), it appears that 8-azido-ATP may indeed have inactivated the protein kinase site which, in turn, inactivated the transcription initiation site of the L protein. These results are consistent with the previous observations that various phosphorylated states of NS have differential effects on transcription (Clinton et al., 1978; Hsu et al., 1982; Kingsford & Emerson, 1980; Sinacore & Lucas-Lenard, 1982; Testa et al., 1980; Watanabe et al., 1974; Witt & Summers, 1980). Future experiments along these lines will shed light on the role of NS phosphorylation on the transcription process

in vitro.

Finally, the association of protein kinase activity with purified L protein suggests that protein kinase activity associated with the VSV RNP is virus-coded. The remainder of the activity found associated with the mature virion may be cellular in origin. Several recent reports (Clinton et al., 1982; Clinton & Huang, 1981; Harmon et al., 1983; Moyer & Summers, 1974; Sinacore & Lucas-Lenard, 1982) are consistent with this contention, in particular the incorporation of src protein into mature VSV grown in baby hamster kidney cells transformed with avian sarcoma virus (Clinton et al., 1982). Although the majority of cellular protein kinases could be removed by extensive purification of RNP, the L-associated protein kinase activity remains strongly bound to the RNP. Further studies on this L-associated protein kinase activity would certainly help in understanding its function in the life-cycle of VSV.

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