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# Inhibition of Vesicular Stomatitis Virus RNA Synthesis by Protein Hyperphosphorylation

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**Vesicular stomatitis virus (VSV) RNA synthesis requires the template nucleocapsid, the polymerase (L) protein, and the cofactor phosphorylated (P/NS) protein. To determine whether the degree of phosphorylation regulated VSV RNA synthesis, infected Chinese hamster ovary cells were treated with okadaic acid (OKA), a serine/threonine phosphatase inhibitor. OKA reduced viral penetration and uncoating but had little or no effect on primary transcription or viral protein synthesis. However, approximately 80% of total viral RNA synthesis was inhibited when 2 μM or more OKA was added to infected cells after viral uncoating had taken place. Analysis of proteins and RNA species in infected cells labeled with $^{32}P$ showed that OKA led to hyperphosphorylation of two viral phosphoproteins, the P/NS protein and matrix protein (M), resulting in inhibition of full-length RNA synthesis and subsequent secondary transcription. Pulse-chase experiments demonstrated that the hyperphosphorylated P/NS species was converted rapidly from the less phosphorylated form. Hyperphosphorylated P/NS as well as the less phosphorylated form, but not M, were found to be associated with nucleocapsids isolated from cytoplasmic extracts. These results suggest that phosphorylation played an important role in the regulation between viral transcription and viral RNA replication as well as the turning off of RNA replication. Thus, phosphatase inhibitors promise to be a valuable tool for dissecting the regulatory mechanisms involving phosphorylated viral proteins.**

Phosphorylated proteins are widely recognized to play a central role in the regulation of macromolecular synthesis. To understand the mechanisms and to determine how phosphorylation affects regulation, simple regulatory systems need to be studied. Viruses, particularly those that replicate only in the cytoplasm, are ideal because they code for a few well-defined phosphoproteins and these proteins can be readily detected by radiolabeling. This investigation combines vesicular stomatitis virus (VSV)-infected cells with the protein phosphatase inhibitor okadaic acid (OKA) to determine whether alterations in phosphoproteins can be correlated directly with important regulatory steps during the synthesis of viral progeny. It is hoped that such studies will lead to an understanding of the detailed mechanisms underlying regulation.

VSV, an enveloped, negative-strand RNA virus, codes for five structural proteins, two of which, P/NS (found predominantly in the cytoplasm and previously considered a nonstructural [NS] protein) and M (matrix protein), are phosphoproteins. Their major phosphoamino acids are phosphoserine and phosphothreonine (9). Both proteins play a role in the regulation of VSV RNA synthesis. Viral transcription and replication require the participation of P/NS, complexed with the RNA-dependent RNA polymerase (13, 14, 31). Phosphorylation of P/NS was found to be essential for transcription in cell-free assays (1, 17, 18). Within infected cells (in vivo), there are two phosphorylated species, separable on urea-containing sodium dodecyl sulfate (SDS)-polyacrylamide gels; the less phosphorylated one binds to VSV nucleocapsid templates (6) which contain full-length RNA covered with nucleoprotein. Studies of RNA synthesis by temperature-sensitive (*ts*) mutants with lesions in the P/NS gene suggest that this protein plays a role in the switch from transcription to replication (20, 30). It has been postulated that this switch is regulated by the dissociation of the less phosphorylated protein from templates and that this dissociation may be triggered by increased phosphorylation (6). M protein, on the other hand, appears to regulate only transcription. This effect of M was determined when transcription was greatly increased at the nonpermissive temperature when cells were infected with *ts* mutants of M (7) and when in vitro transcription was inhibited by the addition of wild-type M protein to a polymerase assay (4). In addition to phosphoserine and phosphothreonine, M protein also contains phosphotyrosine (9); however, when the phosphotyrosine content of M is increased 20-fold, there is no detectable effect on VSV transcription or progeny formation (8).

Although there is increasing in vitro evidence that phosphorylation of P/NS is essential for the regulation of VSV RNA synthesis, it has been difficult to compare the results obtained by cell-free (in vitro) studies directly with those obtained by using infected cells. In part this is because gel systems for separating the different species of P/NS have not been used consistently in different laboratories and because direct comparisons of the in vitro-synthesized P species have not been done with in vivo-synthesized NS species to ensure their identity. Until such comparisons have been made, we shall continue to use the terminology of Clinton and colleagues (6, 7) to refer to the different species synthesized in VSV-infected cells as NS1 for the less phosphorylated one and NS2 for the more phosphorylated one. In distinction to P species synthesized in vitro, the completely nonphosphorylated P is not detectable in infected cells.

OKA is a specific inhibitor of protein phosphatases 1 and 2A, which dephosphorylate serine and threonine residues (reviewed in reference 11). The approach taken here was to add the inhibitor to VSV-infected cells and determine its effect(s) on VSV macromolecular synthesis and progeny formation. Despite pleiotropic effects of the drug, a specific correlation was seen between hyperphosphorylation of NS1 to NS2 and inhibition of full-length RNA replication. In addition,

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binding of NS proteins to RNA templates was reexamined, resulting in a model which helps us to understand the transition from template function to progeny formation. This report contains in vivo data that support the essential regulatory role for phosphorylation of NS during VSV RNA synthesis. Moreover, it shows that hyperphosphorylated NS2, rather than dissociating from templates, remained, and that it may be responsible for inhibition of full-length RNA replication after exposure to OKA.

(This work was done in partial fulfillment of the requirements for the Ph.D. degree by T.L.-Y.C.)

## MATERIALS AND METHODS

**Isotopes, chemicals, and media.** [$^{14}$C]uridine (>50 mCi/mmol), [$^{35}$S]methionine (>1,000 Ci/mmol), and $^{32}P_i$ (8,500 to 9,120 Ci/mmol) were obtained from DuPont/New England Nuclear Corp. OMNISORB cells were from Calbiochem. Joklik's modified minimal essential medium (MEM) without phosphate or methionine, penicillin G-streptomycin sulfate, and OKA were from GIBCO Laboratories. Endoglycosidase H (endo H) was from Boehringer Mannheim Biochemica. Complete Joklik's modified MEM, fetal bovine serum (FBS; type II), and other chemicals were from Sigma.

**Cells and virus.** In general, Chinese hamster ovary (CHO) cells and VSV were propagated as described by Stampfer et al. (28). CHO cells were grown in continuous suspension culture at 37°C and maintained at $4 \times 10^5$ cells per ml in MEM containing 2% FBS, nonessential amino acids, 100 U of penicillin G sodium per ml, and 100 μg of streptomycin sulfate per ml. The Indiana serotype, San Juan strain, of VSV was used. A *ts* mutant of the Indiana serotype, *ts*G114 (complementation group I), was obtained from Craig Pringle.

**Radioactive labeling of cells and virus.** For $^{32}P$ or $^{35}S$ labeling of proteins, MEM depleted of phosphate or methionine was used, and either 75 μCi of $^{32}P_i$ per ml or 110 μCi of [$^{35}$S]methionine per ml was added to each medium. Cells were placed at $2 \times 10^6$/ml in medium containing 5 μg of actinomycin D (ActD) per ml, 25 mM *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.4), nonessential amino acids, and 2% dialyzed FBS. For labelling RNA with $^{32}P$, 300 μCi of $^{32}P_i$ per ml was added to phosphate-depleted MEM, and all other additions remained the same.

**Cumulative uridine incorporation into VSV RNA.** Experiments were carried out as described previously for pilot infections (15). CHO cells ($4 \times 10^6$) were infected with VSV in 2 ml of MEM containing 2% FBS, nonessential amino acids, and 25 mM HEPES (pH 7.4). The multiplicity of infection (MOI) for each experiment is indicated in the figure legends. ActD at 5 μg/ml was added, and viral attachment and penetration was allowed to proceed for 40 min at 37°C before the addition of 0.3 μCi of [$^{14}$C]uridine. To measure primary transcription, infected cells were exposed to 5 μg of ActD per ml, 100 μg of cycloheximide per ml, and 0.6 μCi of [$^{14}$C]uridine. Since primary transcription depends on input virions, MOIs had to be increased in order to conserve radioisotope usage. A sample of uninfected, ActD-treated cells served as a negative control. When OKA was used, the amounts and time of addition are indicated in the figure legends. Incorporation of radioactive uridine into RNA was determined by withdrawing 0.1 ml from each sample and mixing it into 1 ml of 5% trichloroacetic acid at 4°C. The acid-precipitated RNA was concentrated on an HA filter (Millipore HAWP), washed with 15 ml of 5% trichloroacetic acid, dissolved in 5 ml of liquid scintillation cocktail (Ready Protein; Beckman), and counted in a Beckman LS 6000LL scintillation counter.

**Analysis of viral RNA species.** ActD-treated infected cells ($4 \times 10^6$) in the presence or absence of OKA (5 μM) were labeled with $^{32}P$ from 1.5 to 4 h postinfection (p.i.). Total RNA from VSV-infected cells was then extracted by a single-step procedure (5). Cells were resuspended in 400 μl of solution D containing 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarcosyl, and 0.1 M β-mercaptoethanol and mixed in 40 μl of 2 M sodium acetate (pH 4.0). RNA was extracted with 400 μl of saturated phenol and 80 μl of chloroform-isoamyl alcohol (49:1). After 15 min of incubation on ice, RNA in the aqueous phase was collected by centrifugation at $14,000 \times g$ in a microcentrifuge at 4°C for 20 min and precipitated with 1 volume of isopropanol at −20°C overnight. Each sample was resuspended in diethyl pyrocarbonate-treated $H_2O$. Aliquots of each sample were analyzed on a 0.7% agarose gel containing formaldehyde (25). rRNAs were stained with 30 μg of acridine orange per ml in 10 mM sodium phosphate buffer (pH 7.0). The gel was dried and then exposed to Kodak X-Omat AR film.

**Immunoprecipitation.** Proteins from infected cells ($4 \times 10^6$) labeled with [$^{35}$S]methionine were harvested and lysed in NEB buffer (0.01 M Tris [pH 7.4], 0.01 M NaCl, 0.02 M EDTA) containing 1% Nonidet P-40, 1% sodium deoxycholate, and 2 mM phenylmethylsulfonyl fluoride. Samples were incubated with 3 μl of hyperimmune goat anti-VSV at 4°C for 1 h, and the complexes were precipitated with a 10% prewashed cell suspension of heat-inactivated protein G-bearing group G *Streptococcus* sp. (OMNISORB cells) for 1 h at 4°C. The complexes were then washed twice with radioimmunoprecipitation assay buffer (0.01 M Tris [pH 7.2], 0.15 M NaCl, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 5 mM EDTA) containing 0.5 M NaCl and three times with radioimmunoprecipitation assay buffer. Aliquots of each sample were boiled in Laemmli buffer (19) and analyzed in SDS–10% polyacrylamide gels.

**Endo H treatment.** After immunoprecipitation, viral proteins from $4 \times 10^6$ cells were resuspended in endo H incubation buffer (200 mM sodium citrate [pH 5.8], 0.5 mM phenylmethylsulfonyl fluoride 0.1% SDS, 0.1 M β-mercaptoethanol). Half of each sample was boiled for 5 min and then treated with endo H (3 mU) overnight at 37°C. Samples were then boiled in Laemmli buffer and analyzed on SDS–10% polyacrylamide gels.

**Preparation of VSV ribonucleoprotein-associated viral proteins.** VSV-infected cells ($8 \times 10^7$) were labeled with 0.3 μCi of [$^{14}$C]uridine per ml at 0.5 h p.i. OKA at 2 μM was added to half of the cells at 1.5 h p.i., and 75 μCi of $^{32}P$ per ml was added at 2.5 h p.i. At 4.5 h p.i., infected cells were harvested, washed with cold (PBS-A), and lysed in NEB buffer containing 1% Nonidet P-40 and 1% sodium deoxycholate as described by Huang et al. (15). After removal of nuclei by centrifugation, the cytoplasmic extract was fractionated in a 15 to 30% sucrose gradient in NEB buffer. The pattern of optical density at 260 nm was recorded with a Beckman DU640 spectrophotometer. The polyribosomes were dissociated to 50S and 30S subunits in NEB buffer and used as markers for locating the ribonucleocapsids (120S). The location of VSV ribonucleocapsids was determined by the pattern of optical density at 260 nm and the profile of [$^{14}$C]uridine incorporation as analyzed by acid precipitation of 1/10 of each fraction. The volume of each fraction was about 1.3 ml. Fractions containing cores were pelleted at $150,000 \times g$ for 90 min, and the proteins were analyzed in SDS–10% polyacrylamide gels with 5 M urea and 0.19 M Tris-HCl (8). Nucleocapsid (N) proteins, but not M protein, were observed on the gel stained with Coomassie blue.

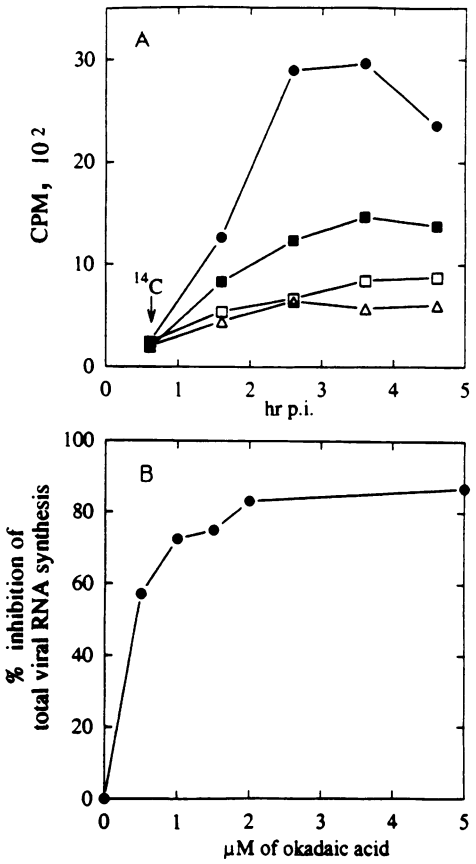


FIG. 1. Cumulative RNA synthesis in VSV-infected cells in the presence of OKA. (A) CHO cells infected with VSV at an MOI of 125 were exposed to OKA (2 μM) at either 0 (□) or 40 (■) min p.i. ActD-treated uninfected (△) and infected (●) cells in the absence of OKA were also established. Cumulative incorporation of [$^{14}$C]uridine was determined as described in Materials and Methods. (B) OKA at different concentrations was added to infected cells at 40 min p.i. in pilot assays as described in Materials and Methods. Results of [$^{14}$C]uridine incorporation at 3 h and 40 min p.i. are shown. After subtraction of the background incorporation by ActD-treated uninfected cells, the results are expressed as the inhibition of VSV RNA synthesis by OKA relative to the control. Similar percentages of inhibition were seen as soon as 1 h and 40 min p.i.

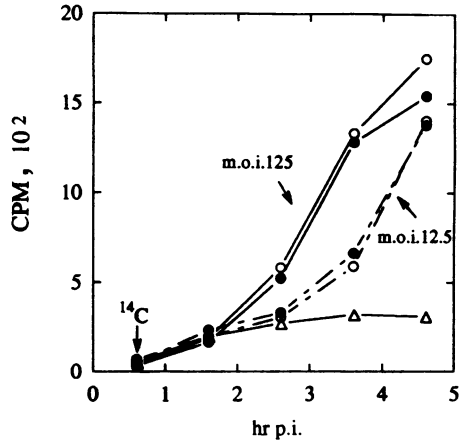


FIG. 2. Cumulative VSV RNA synthesis after exposure of cells to VSV and OKA at 4°C. CHO cells were infected with VSV at MOIs of 125 and 12.5, and viral attachment was allowed to proceed at 4°C for 40 min in the presence (●) and absence (○) of 2 μM OKA. A sample of uninfected, ActD-treated cells served as a negative control (△). After samples were washed with PBS-A, [$^{14}$C]uridine and ActD were added to each sample, and the samples were transferred to 37°C. Cumulative incorporation of radioactive uridine was assayed.

## RESULTS

**Effect of OKA on total VSV RNA synthesis.** To determine if inhibition of serine and threonine phosphatases had any effect on VSV RNA synthesis, OKA was added to cell cultures at the same time as VSV or at 40 min after the initiation of infection. At a concentration of 2 μM OKA, total VSV RNA synthesis was almost completely inhibited when the inhibitor was added at the same time as VSV; with the later time of addition, less inhibition was observed, suggesting that very early events as well as RNA synthesis were affected by OKA (Fig. 1A). To ensure that maximal inhibition of RNA synthesis was being attained, increasing concentrations of OKA were added to the infected cells at 40 min p.i. The dose-response curve in Fig. 1B shows that total viral RNA synthesis was maximally inhibited to 80% with a concentration of 2 μM or greater. Also, as would be expected, progeny formation was decreased to less than 0.011% when infected cells were treated with 2 to 5 μM OKA. Thus, in all subsequent experiments, OKA at concentrations of between 2 and 5 μM was used. The degree of inhibition on RNA synthesis was observed as soon as 30 min after the addition of OKA. Despite reports that the effects of OKA are slowly reversible after cells have been washed (21), this system of VSV-infected CHO cells in suspension did not show any reversibility in RNA synthesis with incubation times of between 15 min to 4 h even after extensive washes and observation times (data not shown).

**Inhibition of penetration and uncoating but not attachment and primary transcription.** Because OKA had a greater inhibitory effect on VSV RNA synthesis when added early during infection than when added later, one can postulate OKA effects on viral attachment, penetration, uncoating, or primary transcription leading indirectly to the overall inhibition of VSV RNA synthesis. To delineate between these steps of the viral life cycle, three different approaches were taken. Viral attachment was allowed to proceed at 4°C for 40 min in the presence and absence of 2 μM OKA. Two different MOIs (125 and 12.5) were used. Cells were then washed, and viral RNA synthesis was analyzed as described previously. If there is an effect on viral attachment at this temperature, a change in viral RNA synthesis should be seen. Since no inhibition was observed (Fig. 2), it can be concluded that at 4°C OKA had no effect on viral attachment at either of the multiplicities tested. Its reversibility indicated that OKA was inactive or did not become cell associated at 4°C.

A second way to define where OKA might act during the early stages of VSV infection was to measure primary transcription. VSV penetrates, uncoats, and carries on primary transcription in cells in the presence of ActD and cycloheximide. In the absence of protein synthesis, only primary transcription from incoming templates occurs in these cells (16). Figure 3A shows that primary transcription was partially inhibited if OKA was added at the same time as virus compared with its being added 40 min later or not at all. This finding suggests that the inhibition probably occurred during penetration or uncoating and not during primary transcription.

To obtain a clearer separation between these early steps and primary transcription, the *ts* mutant *ts*G114 was used. At the

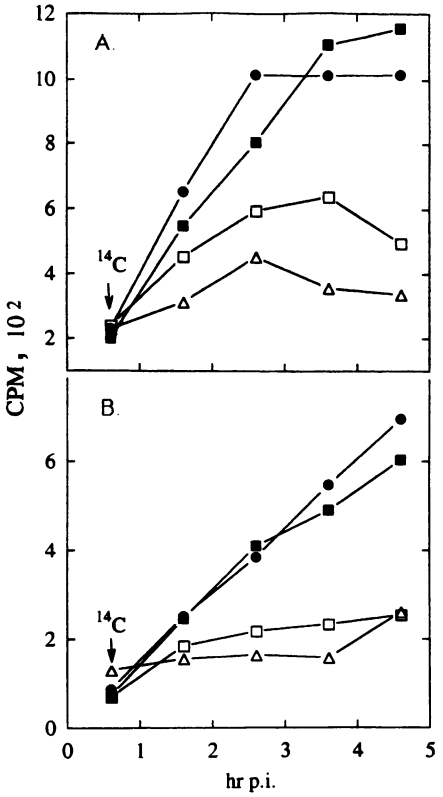


FIG. 3. Primary transcription of VSV in the presence of OKA. (A) CHO cells infected with VSV at an MOI of 250 were exposed to ActD and cycloheximide at 37°C. OKA at 2.5 μM was added at either 0 (□) or 40 (■) min p.i. Uninfected (△) and infected (●) cells in the absence of OKA were also established. Cumulative incorporation of radioactive uridine was determined. (B) CHO cells infected with *ts*G114 at an MOI of 75 were exposed to ActD and cycloheximide at the nonpermissive temperature (39°C). OKA at 2.5 μM was added at either 0 (□) or 40 (■) min p.i. Uninfected (△) and infected (●) cells without OKA were also established. At 40 min p.i., [$^{14}$C]uridine was added to each sample and all samples were shifted to the permissive temperature (31°C). Cumulative incorporation of [$^{14}$C]uridine was assayed.

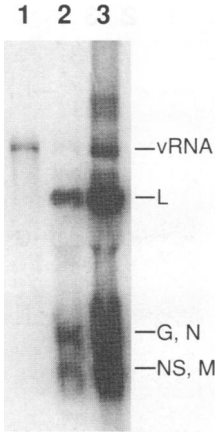


FIG. 4. RNA species synthesized in VSV-infected cells in the presence of OKA. (A) CHO cells infected with VSV at an MOI of 125 at 37°C in the presence of ActD. OKA (5 μM) was added at 40 min p.i. RNA was labeled with $^{32}$P from 1.5 to 4 h p.i. and was extracted as described in Materials and Methods. Equal aliquots of each sample were loaded onto a 0.7% agarose gel containing formaldehyde. Full-length genomic RNA from $^{32}$P-labeled virions was used as a marker (lane 1). Lane 2, RNA from infected cells treated with OKA; lane 3, RNA from infected cells without OKA. vRNA, viral RNA.

nonpermissive temperature (39°C), this mutant penetrates into cells and uncoats but fails to carry out any further steps (23, 24, 29). Therefore, the addition of OKA and cycloheximide after the virus has been incubated at 39°C for 40 min and then shifted to 31°C would be a direct test of whether OKA affects primary transcription. As shown in Fig. 3B, primary transcription by *ts*G114 was not inhibited by OKA at 2.5 μM. Thus, the early effects of OKA on VSV infection must have been on either viral penetration or uncoating. Therefore, to distinguish between early effects of OKA and later, more specific effects on viral RNA synthesis, all further experiments were carried out with the addition of OKA at 40 min after the initiation of infection.

**Effect of OKA on viral RNA replication.** Since there was a graded inhibition on total VSV RNA synthesis dependent on when OKA was added, it was likely that the inhibition occurred at the stage of full-length RNA replication, with indirect effects on secondary transcription. Examination of RNA species from $^{32}$P-labeled infected cells showed that OKA prevented the replication of full-length RNA (Fig. 4). mRNA was also reduced as expected. Comparison of OKA with cycloheximide indicated similar inhibition of RNA replication and reduced transcription without a noticeable effect on the relative molar ratios of viral mRNA species synthesized (data not shown). Examination of lane 2 in Fig. 4 in the original autoradiograph indicates a trace amount of full-length viral RNA in the OKA-treated infected cells. This may represent residual RNA replication in the presence of OKA or a selective inhibition by OKA on minus-strand RNA synthesis. To test whether this was full-length plus-strand RNA, Northern (RNA) blot analysis was performed by probing with $^{32}$P-labeled minus-strand RNA from virions or $^{32}$P-labeled viral mRNAs (plus strands). Only a residual small amount of minus-strand full-length RNA was detected (data not shown). In this hybridization experiment, what was detected may represent RNA derived from input virions. These results indicate that OKA inhibited RNA replication by preventing the accumulation of both minus and plus strands of full-length RNA.

**Effect of OKA on VSV protein synthesis.** Since protein synthesis is required for VSV RNA replication, the effects of OKA on viral RNA replication might have been due indirectly to the complete inhibition of protein synthesis. To rule out such indirect effects, viral proteins from OKA-treated and untreated infected cells were radiolabeled, immunoprecipitated, and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) (Fig. 5). All five viral proteins were synthesized in the infected cells in the presence of OKA at 2 μM. Some reduction of proteins was evident, as expected from indirect inhibition of secondary transcription by OKA, but this partial reduction would not be expected to affect viral RNA replication. Both G and M proteins migrated slightly differently in this gel system, suggesting that OKA had effects other than on viral proteins synthesis per se. Therefore, the large and rapid inhibition of viral RNA replication by the addition of OKA must be due to some other event besides inhibition of protein synthesis.

**Endo H treatment of G synthesized in the presence of OKA.** Parenthetically, examination of lane 2 in Fig. 5 shows that the viral glycoprotein, G, migrated faster and more uniformly when it was synthesized in the presence of OKA. This could be due to cleavage of the glycoprotein or a difference in process-

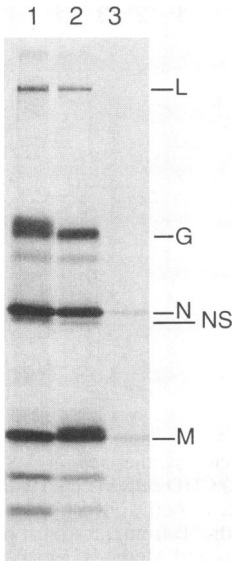


FIG. 5. VSV proteins synthesized in OKA-treated infected cells. CHO cells infected with VSV at an MOI of 125 at 37°C were exposed to OKA at 2 μM (lane 2) or cycloheximide at 100 μg/ml (lane 3) at 3 h p.i. Infected cell in the absence of OKA served as a control (lane 1). Ten minutes after addition of drugs, all samples were labeled with [$^{35}$S]methionine for 30 min. Viral proteins were then immunoprecipitated as described in Materials and Methods and analyzed by SDS-PAGE (10% gel).

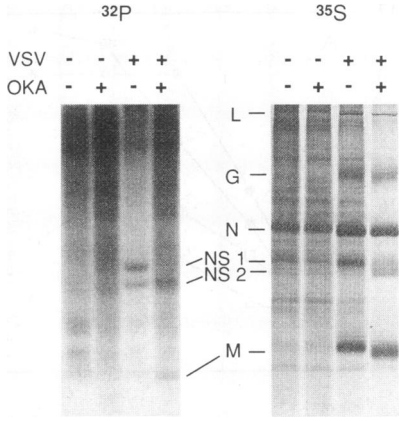


FIG. 7. Urea-polyacrylamide gel electrophoretic separation of proteins from VSV-infected and uninfected cells made in the presence and absence of OKA. CHO cells, uninfected or infected with VSV at an MOI of 125 at 37°C, were radiolabeled between 3 and 4 h p.i. with either $^{32}$P (A) or [$^{35}$S]methionine (B). Half of each sample was also treated with 5 μM OKA at the same time as the labeling period. Cytoplasmic extracts were then prepared by suspending cells in NEB buffer containing 1% Nonidet P-40 and 1% sodium deoxycholate, and nuclei were removed by centrifugation. Aliquots of cytoplasmic extracts from each sample were electrophoresed in an SDS–10% polyacrylamide gel containing urea.

ing of its carbohydrate moiety. To determine whether carbohydrate processing was affected, G protein synthesized in the presence of OKA was digested with endo H prior to separation by SDS-PAGE. Figure 6 indicates that a majority of G synthesized in the presence of OKA was sensitive to digestion by endo H and migrated faster; thus, some G synthesized in the presence of OKA was immature in terms of its carbohydrates, indicating a block in its transport out of the rough endoplasmic reticulum. This observation is in agreement with previously described effect of OKA in blocking transport between the endoplasmic reticulum and the Golgi apparatus (12, 21). In addition, endo H-resistant G, migrating faster than mature G, was found in both OKA-treated and untreated cells and may represent partially mature G.

**Effect of OKA on viral protein phosphorylation.** To determine whether there was a relation between the inhibition of viral RNA replication and inhibition of phosphatases, the effect of OKA on protein phosphorylation was examined. VSV-infected cells were labeled with either $^{32}$P or [$^{35}$S]methionine in the presence and absence of OKA. Total proteins were analyzed in SDS-polyacrylamide gels containing urea as described by Clinton et al. (7). In this gel system, NS proteins of VSV are resolved into NS1 and the more phosphorylated form, NS2. As shown in Fig. 7, when infected cells were treated with OKA, NS2 became the dominant species of NS, suggesting that much of NS1 became hyperphosphorylated when phosphatase activity was inhibited. The other VSV phosphorylated protein, M, was not as dramatically affected, showing only a slight mobility shift indicating possible increased phosphorylation. This is consistent with the mobility shift seen in Fig. 5. However, since there is no evidence to suggest that M plays any role during full-length RNA replication, there was no reason to implicate M protein in any role during RNA replication. Also, in lanes containing $^{32}$P, OKA effected an overall increase in incorporation, whether cells were infected or not, as evidenced by the background radioactivity seen in Fig. 7. This increase in phosphate labeling was not matched by a similar increase in [$^{35}$S]methionine labeling, which suggests that previously synthesized proteins became hyperphosphorylated in the presence of OKA.

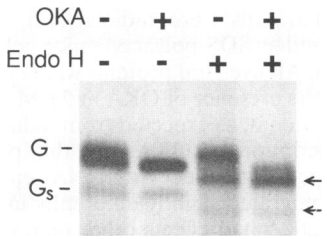


FIG. 6. Endo H-treated VSV glycoproteins synthesized in the presence of OKA. CHO cells were infected with VSV at an MOI of 125. Half of each sample was treated with OKA (2 μM) at 3 h p.i., and all samples were labeled with [$^{35}$S]methionine for 30 min. Viral proteins were immunoprecipitated, and half of each sample was treated with endo H as described in Materials and Methods. Viral proteins were then analyzed by SDS-PAGE (10% gel). Arrows at the right indicate *endo H*-sensitive G protein species.

**Conversion of NS1 to NS2 in OKA-treated infected cells.** Clinton et al. (7) have shown that the two forms of NS proteins are interconvertible in vitro. To determine whether the hyperphosphorylated NS2 in OKA-treated cells was derived from the less phosphorylated NS1, a pulse-chase experiment using [$^{35}$S]methionine and $^{32}$P was performed. As shown in Fig. 8, conversion of NS1 to NS2 did not occur in the absence of the drug even with a chase period of 60 min; in contrast, no matter which radioisotope was used, NS1 converted to NS2 in the infected cells after 15 min of treatment with OKA, but some residual NS1 always remained. In other experiments (data not shown), conversion of NS1 to NS2 occurred within 5 min,

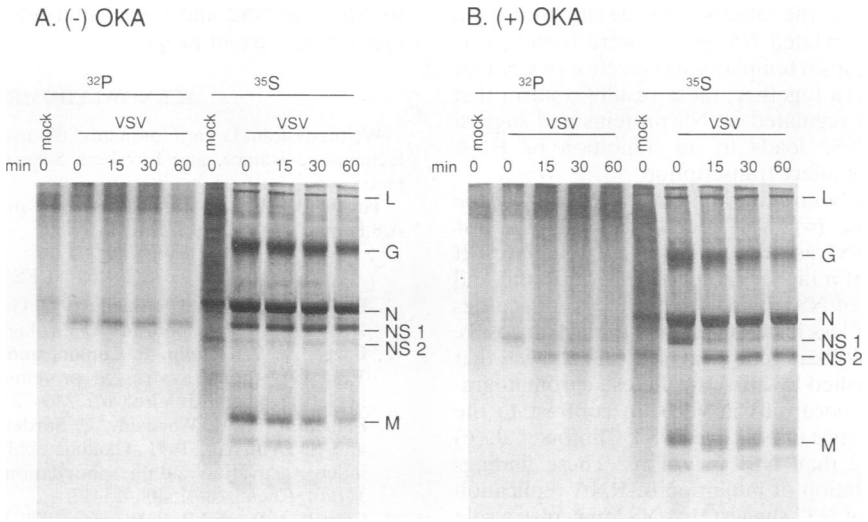


FIG. 8. Proteins from VSV-infected cells from a pulse-chase experiment. VSV-infected cells were labeled with $^{32}P$ or [$^{35}S$]methionine at 2.5 h p.i. At 3.5 h p.i., 4,000-fold unlabeled phosphate or 1,000-fold L-methionine was added to the samples, and one half of each sample was treated with OKA at 5 μM. Aliquots were removed at 0, 15, 30, and 60 min of further incubation. Cytoplasmic extracts were prepared from each sample as described for Fig. 7. Aliquots of cytoplasmic extracts were analyzed in an SDS–10% polyacrylamide gel containing urea.

indicating that hyperphosphorylation was quite rapid. This result showed that NS1 was a precursor of NS2, indicating that hyperphosphorylation of NS protein does not require de novo protein synthesis. Since the inhibition of total viral RNA synthesis could not be reversed in attempts to wash out OKA, NS species were observed under similar conditions to determine whether hyperphosphorylation was similarly not reversed. Although there was a 30% loss of NS2, by [$^{35}S$]methionine quantification, the loss occurred very slowly over a 4-h chase period (data not shown).

**Association of NS protein with nucleocapsids in OKA-treated infected cells.** NS1 is reported to bind to nucleocapsid templates from virions and from infected cells (6). To reexamine this binding now that more detectable NS2 was produced in OKA-treated cultures, infected cells were doubly labeled with $^{32}P$ and [$^{14}C$]uridine in the presence or absence of OKA. Cell extracts were prepared at 4.5 h p.i., and the nucleocapsids were purified by centrifugation through a 15 to 30% sucrose gradient. The fraction containing nucleocapsids was collected and analyzed by urea-SDS-PAGE (Fig. 9). In infected cells not treated with OKA, in which NS1 is usually found in greater abundance than NS2, both NS1 and NS2 were found to be associated with nucleocapsids, but by far the predominant species was NS1. With OKA, both NS1 and NS2 were also bound, with NS2 predominating. Phosphorylated M protein was not detectable in the nucleocapsid fractions, supporting the assumption that hyperphosphorylated M did not play a role in RNA replication.

## DISCUSSION

OKA had a pleiotropic effect on VSV-infected cells. It inhibited an early stage of infection during either penetration or uncoating. Total VSV RNA synthesis was also inhibited maximally by 80% in a dose-dependent manner. The residual RNA synthesis represented products of primary transcription. The inhibition of RNA replication by OKA was not due to a block in protein synthesis. Analysis of viral RNA species showed that RNA inhibition was the result of reduced full-length RNA synthesis, with a subsequent indirect inhibition of secondary transcription and progeny formation. Both plus- and minus-strand RNA syntheses were inhibited. Detailed examination of VSV proteins synthesized in the presence of OKA showed a shift in the migration of three of the five VSV proteins. The glycoprotein migrated faster as a result of a block in carbohydrate processing. M protein changed its migration rate, which was most noticeable in urea-containing SDS-polyacrylamide gels. The most dramatic result of OKA treatment was on NS protein: most of NS1 became hyperphospho-

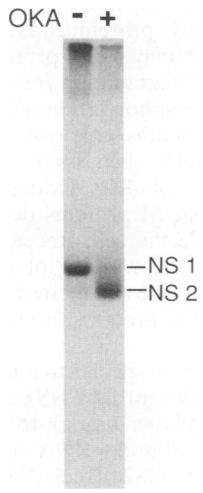


FIG. 9. Nucleocapsid-associated proteins from OKA-treated infected cells. VSV-infected cells were labeled with [$^{14}C$]uridine at 0.5 h p.i., and half of each sample was treated with OKA (2 μM) at 1.5 h p.i. $^{32}P$ was added at 2.5 h p.i. At 4.5 h p.i., cytoplasmic extract was fractionated in a sucrose gradient as described in Materials and Methods. The location of nucleocapsids was determined on the basis of the pattern of optical density at 260 nm and the profile of [$^{14}C$]uridine incorporation. The fractions containing nucleocapsids were collected and $^{32}P$-labeled proteins were analyzed in an SDS–10% polyacrylamide gel containing urea.

rylated to NS2 even in the absence of de novo protein synthesis. Both phosphorylated NS species were found to be associated with nucleocapsid templates irrespective of whether OKA was present. Taken together, these results confirm that VSV RNA synthesis is regulated by NS proteins and suggest that the binding of NS2 leads to an inhibition of RNA replication but does not affect transcription.

The finding that NS2 bound to templates contrasts with the report of Clinton et al. (6), who found that only the less phosphorylated form-NS1 associated with VSV templates. It may be that their use of a higher radioactive background and the very small amount of NS2 found in cells led to difficulties in detecting NS2, much less its association with nucleocapsids. In a later publication, Williams and Emerson (32) showed that both NS1 and NS2, purified by DEAE-cellulose chromatography, rebind to ribonucleocapsids in vitro. In contrast to the intracellular preponderance of NS1 over NS2, Clinton et al. (6) found much more NS2 than NS1 in virions. These findings coupled with the correlation of inhibition of RNA replication with the accumulation of NS2 suggest that NS2 may play a role in progeny maturation by shutting off further use of full-length RNA as a template for replication. This step and the budding of virions with excess NS2 may be in addition to the inhibitory function of M protein on RNA transcription (10) and coalescence of the templates into progeny.

Besides the effect of OKA on VSV RNA replication, there were also effects on viral penetration and/or uncoating and on the transport of newly synthesized glycoprotein. These results may be explained by the many other reported effects of OKA on cellular functions, particularly membranes. Changes in pH occur, since OKA affects cellular cotransport of potassium and chloride (22) and alters the exchange of sodium and hydrogen ions (3, 27). Moreover, OKA induces Golgi apparatus fragmentation (21) and inhibits vesicular fusion of endosomes (33). Our studies on VSV glycoprotein transport are similar to those reported for the *ts*O45 VSV glycoprotein; in that case, transport between the endoplasmic reticulum and Golgi apparatus is blocked in cells treated with OKA (12). Another possibility for the role of OKA in G protein trafficking might be its effect on calcium ions, which may cause proteins to fold incorrectly leading to retention or degradation (reviewed in reference 26).

What role, if any, phosphorylation of M protein plays during the VSV life cycle remains unknown. Hyperphosphorylated forms of M are seldom seen under normal conditions of infection in the absence of OKA. Evidence presented here and elsewhere suggests that M proteins do not bind tightly with ribonucleocapsid inside the cell. Recently, an organizing role for M protein during the winding of ribonucleoprotein into bullet-shaped cores has been suggested (2), and it may be that phosphorylation helps M proteins to recognize each other and coalesce into cores.

Given the ability of both species to bind to templates, we can postulate a simple model for how NS1 and NS2 regulate VSV RNA synthesis. Templates bound to homodimers of NS1 support transcription, whereas RNA replication requires the binding of both NS1 and NS2. Excess NS2 would drive NS1 off, leading to binding of homodimers and inhibition of RNA replication. Transcription remains in the presence of excess NS2 if we postulate that homodimers of NS1 are irreversible.

This model fits with the relative ratios of NS1 and NS2 in cells, in virions, and in OKA-treated cells (6, 7) (Fig. 7). Such a model naturally leads to experiments on coinfections with wild-type VSV and *ts* mutants containing lesions in the P/NS gene, in vitro RNA assays with controlled concentrations of phosphorylated NS species together with viral ribonucleoprotein templates, and characterizations of host proteins that bind to NS1 and NS2 and might alter VSV RNA synthesis. Such experiments are in progress.

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