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Rabies Virus Protein Synthesis in Infected BHK-21 Cells

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Rabies virus-specific polypeptide synthesis was examined under hypertonic conditions, which selectively inhibit cellular protein synthesis. The rabies virus proteins (L, G, N, M_1, M_2) were synthesized throughout the course of infection, with little change in their relative rates of synthesis. The rates of synthesis of the G and M_1 polypeptides were more sensitive to increasing osmolarity than those of the L, N, and M_2 polypeptides. Extrapolation to isotonicity of the results obtained under hypertonic conditions indicated that the molar ratios of the polypeptides synthesized under normal conditions were 0.4 (L), 64 (G), 100 (N), 75 (M_1), and 35 (M_2). A high-molecular-weight polypeptide (~190,000), designated polypeptide L, was repeatedly detected both in infected cells and in extracellular virus. The estimated number of L polypeptide molecules per virion was 33. The synthesis of a viral glycoprotein precursor, designated gp78, preceded the appearance of the mature viral glycoprotein in infected cells labeled with [^{3}H]glucosamine under isotonic conditions. In cells labeled under hypertonic conditions, little or no mature viral glycoprotein was detected, but a virus-specific glycoprotein with an electrophoretic mobility similar to that of gp78 was observed. This glycoprotein could be chased into mature viral glycoprotein when the hypertonic conditions were made isotonic. These results suggest that a reversible block of viral glycoprotein synthesis occurs under hypertonic conditions.

Rabies virus, a rhabdovirus, is a negative-strand virus that contains single-stranded RNA with a molecular weight of approximately 4.6×10^6 (40, 43). The RNA is associated in the virion with phosphorylated nucleoprotein (N), forming a nucleocapsid core (40, 41). A viral envelope, which contains two non-glycosylated membrane proteins (M_1 and M_2) and a glycoprotein (G), surrounds the nucleocapsid (40). The viral glycoprotein is responsible for eliciting the production of neutralizing antibody against the virus (53).

Rabies virus has an eclipse period of 6 to 12 h in BHK-21 cells and produces virus for several days without appreciable inhibition of cellular DNA, RNA, or protein synthesis (12, 27, 47, 52). Evidence of viral polypeptide synthesis in rabies virus-infected cells has been limited to the detection of virus-specific polypeptides in the cytoplasm of infected cells by immunofluorescent techniques (47, 52) and by the isolation of nucleocapsid cores after cell fractionation (8, 39). Recently, we reported that the synthesis of the major rabies structural proteins (G, N, M_1, M_2) could be detected in infected cells labeled with radioactive amino acids under hypertonic conditions (26).

The RNA of this negative-strand virus would appear to require a virion-associated RNA transcriptase for transcription (2, 21). The L protein, which is present in small amounts in intact VSV virions (16, 50, 51), has been shown to be necessary for RNA transcriptase activity (5-7, 10, 11, 31). In contrast, the presence of L protein in rabies virions has not been clearly established (40, 42), and RNA transcriptase activity has not been detected in purified rabies virions (41, 48), except possibly at extremely low levels (D. Bishop, personal communication). Although virus-specific RNA transcriptase activity has been detected in rabies-infected cells (48; H. P. Madore and F. Sokol, unpublished observations), the virus-specific polypeptide (L), which may be necessary for viral RNA transcriptase activity, had not been detected in our previous analysis of infected cells labeled under hypertonic conditions (26).

In this report, hypertonic labeling conditions were employed to characterize rabies-specific polypeptide synthesis during the course of BHK-21 cell infection. In addition to the major viral structural polypeptides, a high-molecular-weight polypeptide (L) has been clearly identified both in infected cells and in extracellular

virus. The effect of increased osmolarity on rates of synthesis of individual viral polypeptides and on glycosylation of viral glycoprotein was also examined.

MATERIALS AND METHODS

Cells and virus. BHK-21/C13 cells (25) were serially passaged in Blake bottles and maintained in minimal essential Eagle medium (MEM), supplemented with 10% fetal calf serum (growth medium) at 37°C (18). Rabies virus (ERA strain) and vesicular stomatitis virus (VSV) (Indiana strain) were obtained from Tadeusz Wiktor of the Wistar Institute. Stocks of rabies virus and VSV (10^8 to 10^9 PFU/ml) were propagated in BHK-21 cells in a manner similar to that described for the preparation of labeled virus. Both cells and virus stock were shown to be mycoplasma-free by the assay of Sedwick and Wiktor (38).

Infection and mock infection of cells. The growth medium was removed from confluent monolayers of BHK-21 cells in 60- or 100-mm tissue culture dishes, and the cells were infected with 50 to 100 PFU of rabies virus (ERA strain) per cell in 1 ml or mock-infected with an equal volume of MEM supplemented with 0.2% bovine serum albumin (0.2% BSA-MEM). Virus was adsorbed for 30 min at 37°C before unadsorbed virus and mock infection medium were removed. Both cell culture types were incubated in growth medium at 37°C in a 5% CO_2 atmosphere.

Labeling of virus-infected and mock-infected cells. Growth medium was removed, and each culture was incubated in Earle salt solution plus 10% fetal calf serum at 37°C for 15 min to deplete amino acid pools. Cells labeled under isotonic conditions were incubated for various times at 37°C in Earle salt solution plus 10% fetal calf serum with 40 μCi of ^{3}H-labeled amino acid mixture per ml (NET-250, 15 amino acids, New England Nuclear Corp. [NEN]) or 10 μCi of [^{3}H]leucine per ml (TRK 170, 50 Ci/mmol, Amersham/Searle). Cells labeled under hypertonic conditions were incubated for 15 min at 37°C in various hypertonic media containing different amounts of excess NaCl (to allow runoff of cellular mRNA from polysomes [35]) in Earle salt solution plus 10% fetal calf serum and then labeled for various times with one of the above isotopes at 37°C in hypertonic media. Identical results were obtained when labeling was done under hypertonic conditions with [^{3}H]leucine or ^{3}H-amino acid mixture (after correcting for differences in the relative leucine content of the individual viral polypeptides). Cells were labeled with 50 μCi of [^{3}H]glucosamine per ml (NET-190, 12 Ci/mmol, NEN) in isotonic or hypertonic 0.2% BSA-MEM according to the procedure previously described for amino acid labeling. The tonicity (milliosmolarity) of the various media was determined with an automatic osmometer (Osmette-A, Precision Systems, Inc., Sudbury, Mass.).

In pulse-chase experiments, cells in 60-mm tissue culture dishes were pulse-labeled with 100 μCi of [^{3}H]leucine in hypertonic medium (550 mOsM) for 2 h and then washed twice with 5 ml of 0.2% BSA-MEM (290 mOsM), followed by a chase with 2 ml of 0.2% BSA-MEM (290 mOsM) for 24 h at 37°C.

Preparation of cellular fractions. Cells labeled under hypertonic or isotonic conditions were washed twice with ice-cold NT buffer (0.13 M NaCl, 0.05 M Tris-hydrochloride, pH 7.8). Whole-cell lysates were prepared by scraping the cells from the tissue culture dish with 2 ml of ice-cold NT buffer and sonicating (Accousticon) for 1 min. Nuclear and cytoplasmic fractions were prepared after Dounce homogenization, as previously described (26). Total protein content was determined by a modified Lowry procedure (30), and trichloroacetic acid-precipitable radioactivity was determined by precipitation of samples in ice-cold 10% trichloroacetic acid, followed by washing with ice-cold 5% trichloroacetic acid on glass-fiber filters (Whatman GF/C).

Preparation of labeled virus. Roller bottles of confluent BHK-21 cells (1.5×10^8 cells/bottle) were infected with 5 PFU of rabies virus (ERA strain) per cell or 0.01 PFU of VSV (Indiana strain) per cell. Virus was adsorbed for 30 min at 33°C in 10 ml of inoculum, and then 100 ml of 0.2% BSA-MEM was added to each bottle for incubation at 33°C. A ^{14}C-labeled amino acid mixture (0.5 μCi/ml, NEC-445; 15 amino acids, NEN) was added to the rabies-infected cells, or [^{14}C]leucine (0.5 μCi/ml, NEC-279, 270 mCi/mmol, NEN) was added to the VSV-infected cells. The rabies-infected cells were harvested after 4 to 5 days of incubation at 33°C; the VSV-infected cells were harvested after 12 to 16 h of incubation at the same temperature. The culture medium containing labeled virus was clarified by centrifugation at $450 \times g_{av}$ for 15 min at 4°C, and the virus was purified by a modification of the procedure described by B. Dietzschold, J. H. Cox, and L. G. Schneider (manuscript in preparation). In brief, the virus was pelleted from the clarified supernatant by centrifugation in a Beckman type 19 rotor at 19,000 rpm for 120 min at 4°C. The virus pellet was resuspended with a Pasteur pipette in NTE (0.13 M NaCl, 0.05 M Tris, pH 7.8, 0.001 M EDTA) buffer and layered on a 10 to 60% sucrose gradient in NTE buffer. The gradient was centrifuged in a Beckman SW27 rotor at 22,000 rpm for 90 min at 4°C. The virus band was collected, diluted in NTE buffer, and repelleted by centrifugation in an SW27 rotor at 20,500 rpm for 90 min at 4°C. The virus pellet was resuspended in a small amount of NTE buffer overnight at 4°C, and then clarified by centrifugation at $800 \times g_{av}$ for 10 min at 4°C. Labeled virus released into the culture medium during the 24-h chase (Fig. 6c) was purified by layering the supernatant (2 ml) directly onto a 10 to 50% sucrose gradient in NTE buffer and centrifuging as described above.

Polyacrylamide gel electrophoresis (PAGE). Cell lysates and virus samples were prepared for PAGE after trichloroacetic acid precipitation, as described by Sokol et al. (44), or precipitation in 5 volumes of absolute ethanol. The samples (in 8 M urea) were subjected to electrophoresis in continuous 7.5% polyacrylamide gels in 0.1 M phosphate buffer (pH 7.2) containing 0.1% sodium dodecyl sulfate (SDS) (44), or on discontinuous 10% SDS-polyacrylamide gels

(23). The gels were fixed in 25% isopropanol-10% acetic acid, cut into 1-mm slices, and then incubated overnight at 33°C in 5 ml of a 5% Protosol-liquid scintillation fluid mixture.

RESULTS

Relative rate of viral polypeptide synthesis in infected BHK-21 cells under hypertonic conditions (550 mOsM). The incorporation of ^{3}H-labeled amino acids into viral polypeptides was measured under hypertonic conditions (550 mOsM) in infected cells during 1-h labeling periods at 6, 12, 24, 36, 48, and 72 h after infection. Analysis of the cytoplasmic and the nuclear fractions on PAGE revealed that about 85% of the labeled rabies-specific proteins were in the cytoplasmic fraction, as previously described (26). From these gels, the relative rate of ^{3}H-labeled amino acid incorporation into the viral polypeptides per milligram of total cellular (nuclear + cytoplasmic) protein per hour was calculated (Fig. 1). During the 1-h labeling period, ^{3}H-labeled amino acid incorporation into individual viral polypeptides was linear. Only 1.5 to 3.0% of the labeled polypeptides were found in the medium. Analysis of these released polypeptides by PAGE revealed that less than 10% of these polypeptides co-migrated with viral proteins and that there was no preferential release of any viral polypeptide into the medium. In addition, the medium used for cells labeled for 2 h with [^{3}H]leucine at 550 mOsM did not contain detectable labeled virus particles (detection limit, 5% of 290 mOsM control), as determined by sucrose gradient analysis (10 to 50% sucrose).

^{3}H-labeled amino acid incorporation into G, N, and M_1 polypeptides was detected by 12 h after infection, and incorporation into M_2 polypeptide was noted by 24 h after infection (Fig. 1). The rate of ^{3}H-labeled amino acid incorporation into the major viral polypeptides (G, N, M_1, M_2) increased gradually for at least 72 h after infection. If amino acids for viral polypeptide synthesis are available from a single amino acid pool and the individual polypeptides have comparable stabilities, the relative rate of amino acid incorporation into these polypeptides is most likely a measure of their relative rate of synthesis. In Fig. 2, the relative rate of amino acid incorporation into viral polypeptides under hypertonic conditions (550 mOsM) obtained from several experiments was used to determine the relative number of viral protein molecules synthesized per hour at 550 mOsM. These data show that individual viral polypeptides are not synthesized in equimolar amounts under hypertonic conditions. The L polypeptide, especially, is synthesized at a rate approximately 170-fold less than that of the N polypeptide. The presence of this polypeptide in infected cells is at times obscured by other high-molecular-weight polypeptides at the top of SDS-polyacrylamide gels.

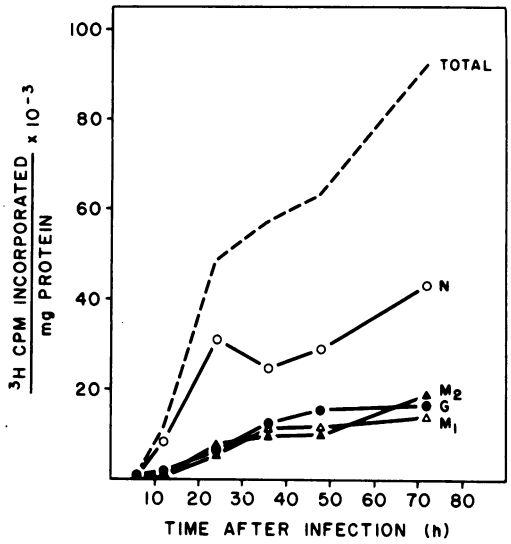

FIG. 1. *Rate of ^{3}H-labeled amino acid incorporation into rabies virus polypeptides under hypertonic conditions (550 mOsM). Confluent monolayers of BHK-21 cells (5 × 10^6 cells per dish) infected with 50 PFU of rabies virus per cell were labeled for 1 h at 37°C with a ^{3}H-labeled amino acid mixture (40 μCi/ml) in 550 mOsM medium at 6, 12, 24, 36, 48, and 72 h after infection. The cells were fractionated into nuclear and cytoplasmic fractions, and 400 μg of protein from each fraction was run on continuous 7.5% SDS-polyacrylamide gels. Radioactivity incorporated into the viral polypeptides in each cellular fraction was obtained by subtracting radioactivity due to residual host polypeptide synthesis from the peaks representing the viral polypeptides. Finally, the sum of radioactivity incorporated into the viral polypeptides in the nuclear plus cytoplasmic fractions was divided by the total protein in the nuclear plus cytoplasmic fractions to give the counts per minute incorporated per milligram of total cellular protein. Co-electrophoresis with ^{14}C-amino acid-labeled rabies virus proteins identified the viral-specific peaks.*

Effect of increasing tonicity on viral polypeptide synthesis. The rates of N and M_2 polypeptide synthesis were enhanced relative to the ratios of L, G, and M_1 polypeptide synthesis with increasing tonicity (Fig. 3). The incorporation of radioactive leucine into all viral polypeptides was inhibited as tonicity increased (e.g., incorporation into viral polypeptides at 550 mOsM is 25% of incorporation at 290 mOsM [26]). Therefore, the enhancement in rate of synthesis of N and M_2 (as compared with the other

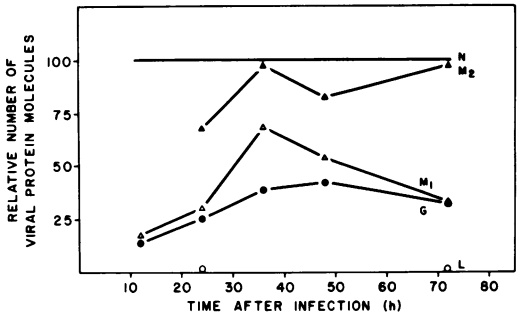

FIG. 2. *Relative number of viral polypeptide molecules synthesized under hypertonic conditions (550 mOsM) at different times after infection. Radioactivity (^{3}H-labeled amino acid or [^{3}H]leucine) incorporated into individual viral polypeptides per hour was determined in several experiments by 7.5% SDS-polyacrylamide gel analysis of cell extracts as described in Methods. Corrections were made for differences in the relative leucine content of steady-state-labeled viral polypeptides (the ratio of ^{3}H-labeled amino acid mixture/[^{3}H]leucine is: L, 0.78; G, 1.03; N, 1.21; M_1, 0.80; M_2, 0.72). The percentage of radioactivity incorporated into individual viral polypeptide peaks (counts per minute incorporated into individual viral polypeptide peaks/total counts per minute incorporated into viral polypeptides × 100) was converted to molecules of protein from their respective molecular weights and normalized to 100 molecules of N polypeptide. The molecular weights used for the calculation were: L, 190,000 (see text); G, 80,000; N, 62,000; M_1, 40,000; M_2, 25,000 (40). Except for the 12-h and the 36-h time points, the values obtained are derived from two to four separate experiments.*

viral polypeptides) reflects a relative, rather than an absolute, increase in the rate of synthesis of these proteins.

Over the range of tonicities examined (450 to 650 mOsM), the plots of the relative rate of synthesis versus tonicity of the individual viral polypeptides were linear. If we assume that this linear relationship extends to isotonic conditions, as has been shown for VSV polypeptide synthesis (32), the data in Fig. 3, when extrapolated to isotonicity, give an estimate of the relative rates of synthesis of individual rabies virus polypeptides under isotonic conditions (Fig. 3, dotted lines). The percentage of total virus-specific radioactivity incorporated into individual viral polypeptides at isotonicity (290 mOsM) was estimated to be: L = 0.5%, N = 34%, G = 33%, M_1 = 25%, and M_2 = 8%. These values were used to calculate the relative number of viral protein molecules synthesized under isotonic conditions (Table 1) and show that, under isotonic conditions, the L, G, N, M_1, and M_2 polypeptides were not synthesized in equimolar amounts. Furthermore, the relative number of polypeptide molecules synthesized was not comparable to the relative number of polypeptide molecules present in extracellular virus (Table 1).

Virus-specific high-molecular-weight polypeptide (L) in infected cells and in purified virus. Synthesis of a high-molecular-weight polypeptide was repeatedly detected by PAGE in infected (Fig. 4a) but not in uninfected cells (Fig. 4b). This polypeptide, designated the L polypeptide, was also present in purified rabies virions (Fig. 5a) and co-migrated with L polypeptide of VSV. Analysis of purified rabies virions labeled with [^{3}H]glucosamine and ^{14}C-labeled amino acids indicated that the L polypeptide of rabies virus was not glycosylated and

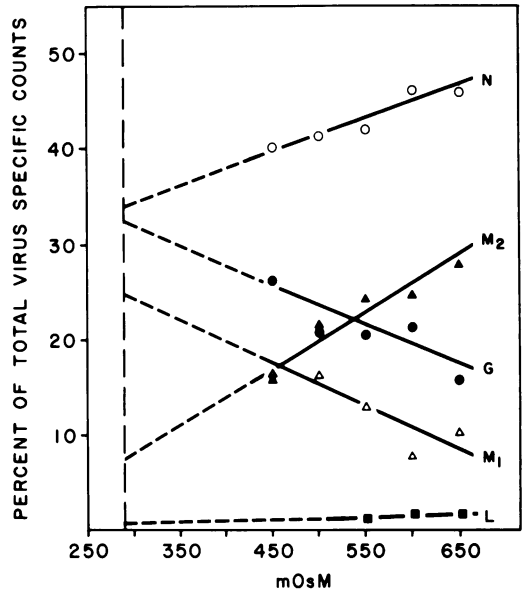

FIG. 3. *Effect of increasing tonicity on relative rates of rabies virus polypeptide synthesis. Confluent monolayers (5 × 10^6 cells/60-mm dish) of rabies-infected (50 PFU/cell) and mock-infected BHK-21 cells were labeled at 24 h after infection for 1 h with [^{3}H]leucine (10 μCi/ml) under isotonic (290 mOsM) or hypertonic (450, 500, 550, 600, and 650 mOsM) conditions. Whole-cell lysates (400 μg of protein) of infected and mock-infected cells were run with ^{14}C-amino acid-labeled protein markers on continuous 7.5% SDS-polyacrylamide gels. The [^{3}H]leucine incorporation into the viral polypeptide peaks attributable to residual cellular protein synthesis was subtracted after normalizing the polypeptide pattern of the mock-infected to the rabies-infected cells. The net [^{3}H]leucine incorporation into virus-specific polypeptides (after correction for differences in relative leucine content of the viral polypeptides) was converted to the percentage of total virus-specific counts in the individual viral polypeptides.*

TABLE 1. *Relative number of viral polypeptide molecules synthesized*

Conditions	L	G	N	M_1	M_2
Hypertonic (550 mOsM)[a]	0.60	32	100	31	79
Isotonic (290 mOsM)[b]	0.40	64	100	75	35
Relative no. of viral polypeptide molecules per virion[c]	1.9	101	100	52	86

[a] Percentage of ^{3}H-labeled amino acid incorporated into virus-specific polypeptides at 550 mOsM (Fig. 3) converted to relative number of viral polypeptide molecules synthesized per hour, as described in Fig. 2. The number of N polypeptides was set equal to 100.

[b] Extrapolated percentage of ^{3}H-labeled amino acid incorporated into virus-specific polypeptides at 290 mOsM (Fig. 3), converted to relative number of viral polypeptide molecules synthesized per hour. The number of N polypeptide molecules was set equal to 100.

[c] Derived from gel analyses of three separate preparations of purified rabies virus (ERA strain), uniformly labeled with ^{14}C-amino acids (see text). The value for N polypeptide was set equal to 100.

thus did not appear to be a dimer of the viral glycoprotein (Fig. 5b).

Number of L protein molecules per virion. An estimate of the number of L protein molecules per virion was made from data derived from PAGE analysis of three different preparations of purified rabies virus uniformly labeled with ^{14}C-labeled amino acids (Table 2). Approximately 33 L protein molecules were estimated to be present per rabies virion. The number of each of the other major structural protein molecules per virion estimated from these data corresponded to the values reported by Sokol et al. (40).

Analysis of viral glycoprotein biosynthesis. A virus-specified glycopolypeptide labeled for 2 h with [^{3}H]leucine in rabies-infected cells under hypertonic conditions consistently migrated in PAGE faster than the marker virion glycoprotein (Fig. 6a). Since this component was not detected in uninfected cells labeled under simi-

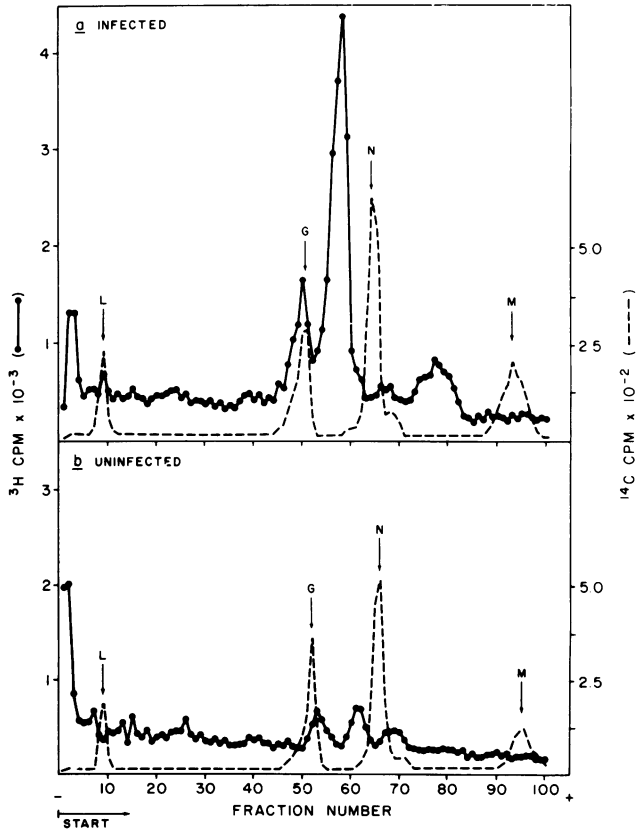

FIG. 4. *Virus-specific high-molecular-weight polypeptide (L) in infected cells. (a) Rabies-infected (50 PFU/cell) or (b) mock-infected BHK-21 cell monolayers were labeled for 1 h with [^{3}H]leucine (10 μCi/ml) under hypertonic conditions (550 mOsM) 24 h after infection at 37°C, and 400-μg portions of protein of the whole-cell lysates were analyzed on discontinuous 10% SDS-polyacrylamide gels. [^{14}C]leucine-labeled VSV was subjected to co-electrophoresis with the cell lysates. The arrows refer to the VSV marker proteins.*

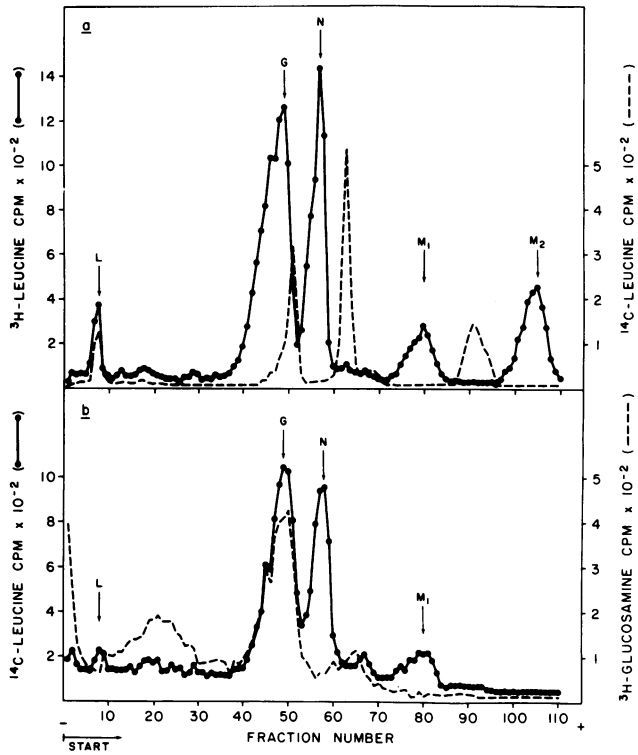

FIG. 5. *Virus-specific high-molecular-weight polypeptide (L) in purified rabies virus. Roller bottles of BHK-21 cells (1.5 × 10⁸ cells/bottle) were infected with rabies virus at 5 PFU/cell; the infected cells were grown in 0.2% BSA-MEM for 4 days at 33°C in the presence of [³H]leucine (2 μCi/ml) alone, or [³H]glucosamine (10 μCi/ml) and [¹⁴C]leucine (2.5 μCi/ml). The virus was harvested and purified as described in Materials and Methods. (a) Analysis of [³H]leucine-labeled rabies virus with [¹⁴C]leucine-labeled VSV (12.5-cm gel). (b) Analysis of [³H]glucosamine- and [¹⁴C]leucine-labeled rabies virus (11-cm gel).*

TABLE 2. *Number of polypeptide molecules per rabies virion*[a]

Polypeptide species	% Total radioactivity	Mol wt	Daltons (polypeptide/virion)	Molecules (no./virion)
L	1.85 ± 0.94	190,000	6 × 10⁶	33
G	42.67 ± 1.30	80,000	145 × 10⁶	1,815
N	32.94 ± 0.43	62,000	112 × 10⁶	1,806
M_1	11.17 ± 0.19	40,000	38 × 10⁶	951
M_2	11.36 ± 0.73	25,000	39 × 10⁶	1,547

[a] Percentage of total virion-associated ¹⁴C-labeled amino acid incorporated into the individual viral polypeptides was determined. The number of daltons of each viral polypeptide per virion was determined by multiplying the percentage of each viral polypeptide in the virion times the molecular weight of the total polypeptide (340.4 × 10⁶ daltons) in the virion (calculated from the ratio of polypeptide to RNA [74:1] in the virion, assuming the RNA molecular weight to be 4.6 × 10⁶ daltons [40]). The number of each polypeptide molecule per virion was calculated by dividing the number of daltons of each polypeptide per virion by the molecular weight of that polypeptide. The molecular-weight values for the G, N, M_1, and M_2 are from Sokol et al. (40). The molecular weight of the L protein was estimated at 190,000 on the basis of co-electrophoresis with the VSV L protein (Fig. 5a) (49).

lar conditions (not shown) and could be quantitatively precipitated with monospecific antiserum directed against purified virion glycoprotein (B. Dietzschold, H. P. Madore, and J. M. England, unpublished observations), it was considered to be virus specific. When rabies-infected cells were labeled for 2 h with [³H]leucine under hypertonic conditions and chased for 24 h in isotonic medium, a large proportion of the radioactivity that was previously associated with the faster-migrating intracellular glycopolypeptide shifted to the slower migration rate of the virion glycoprotein (Fig. 6b). A small proportion of the radioactivity associated with the intracellular glycopolypeptide still migrated more rapidly than the virion glycoprotein (Fig. 6b). The [³H]leucine-labeled glycopolypeptide that was synthesized under hypertonic conditions was incorporated into virus released into the medium during the 24-h chase in isotonic medium, as shown by comigration with the rabies ¹⁴C-labeled virion glycoprotein that was labeled in isotonic me-

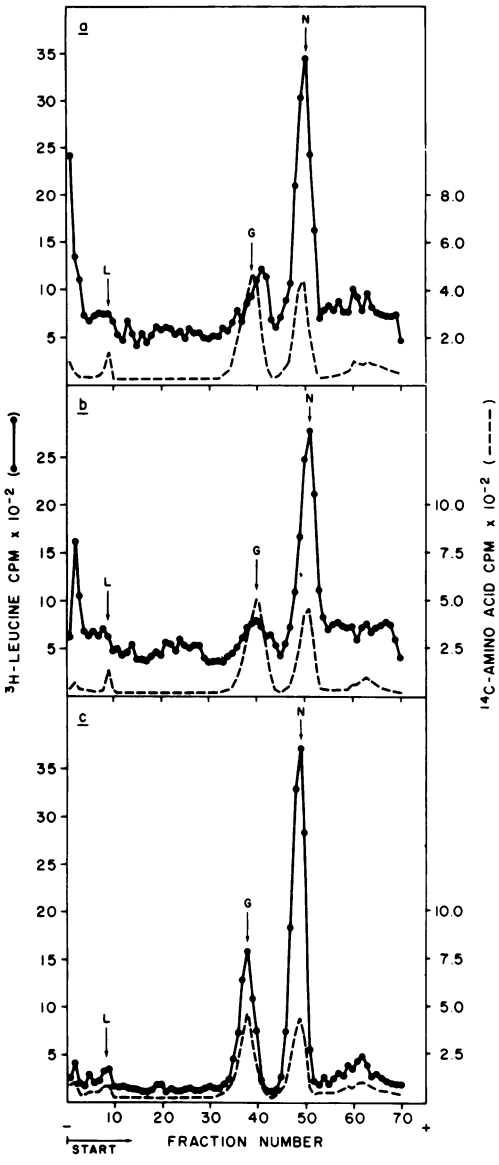

FIG. 6. *Pulse-chase analysis of intracellular viral glycoprotein synthesized under hypertonic conditions. Cultures (5 × 10^6 cells/60-mm dish) of rabies-infected (50 PFU/cell) BHK-21 cells grown at 37°C for 24 h were either pulse-labeled with [^{3}H]leucine (100 μCi/ml) for 2 h at 550 mOsM at 37°C (a) or pulse-labeled for 2 h at 550 mOsM and then chased for 24 h at 37°C in 290 mOsM medium (b). Whole-cell lysates containing 50,000 trichloroacetic acid-precipitable cpm (a) and (b) and extracellular virus from (b) above containing 30,000 trichloroacetic acid-precipitable cpm (c) were analyzed on discontinuous 10% SDS-polyacrylamide gels. The arrows indicate ^{14}C-amino acid-labeled rabies virus marker proteins subjected to co-electrophoresis with the samples.*

dium (Fig. 6c). These results indicate that hypertonic conditions cause the accumulation of a more rapidly migrating form of the viral glycoprotein. During an isotonic chase, however, this more rapidly migrating form of glycoprotein is converted to a form that co-migrates with virion glycoprotein and becomes associated with extracellular virus.

In efforts to detect the synthesis of a faster-migrating form of viral glycoprotein under isotonic conditions (290 mOsM), infected and uninfected cells were labeled with [^{3}H]glucosamine. As shown in Fig. 7a and b, a prominent heterogeneous peak of [^{3}H]glucosamine label was detected in the infected but not in the uninfected cell lysates. This viral-specific glycopolypeptide showed two components on the gel, a major component, which co-migrated with the virion glycoprotein, and a minor component, which migrated more rapidly as a shoulder. Both components could be quantitatively precipitated with antibody directed against virion glycoprotein (B. Dietzschold, H. P. Madore, and J. M. England, unpublished observations). We have designated the more rapidly migrating component gp78 to distinguish it from the glycoprotein present in extracellular virus (gp80). The faster-migrating component exhibited approximately the same electrophoretic mobility as the virus-specific peak labeled with [^{3}H]glucosamine under hypertonic conditions (Fig. 7c and d).

To examine the possibility that gp78 was an intermediate in the biosynthesis of rabies virus glycoprotein (gp80), infected cells were pulse-labeled with [^{3}H]glucosamine under isotonic conditions for intervals from 30 min to 4 h (Fig. 8). After 30 min of labeling, the majority of [^{3}H]glucosamine, which was associated with virus-specific glycopolypeptide, was in the faster-migrating component, gp78 (Fig. 8a). After 1 h of labeling, a portion of the [^{3}H]glucosamine label was in a shoulder of the gp78 peak that migrated with the gp80 marker (Fig. 8b). After 2 h (Fig. 8c) and 4 h (Fig. 8d) of labeling, the majority of the [^{3}H]glucosamine label was associated with gp80. This shift of [^{3}H]glucosamine label from gp78 to gp80 with increased labeling time suggests that gp78 is a precursor of the mature viral glycoprotein (gp80).

DISCUSSION

Using hypertonic conditions to selectively suppress cellular protein synthesis, we have characterized several aspects of rabies virus protein synthesis in BHK-21 cells. The rabies virus structural polypeptides L, G, N, M_1, M_2 are synthesized continually throughout the

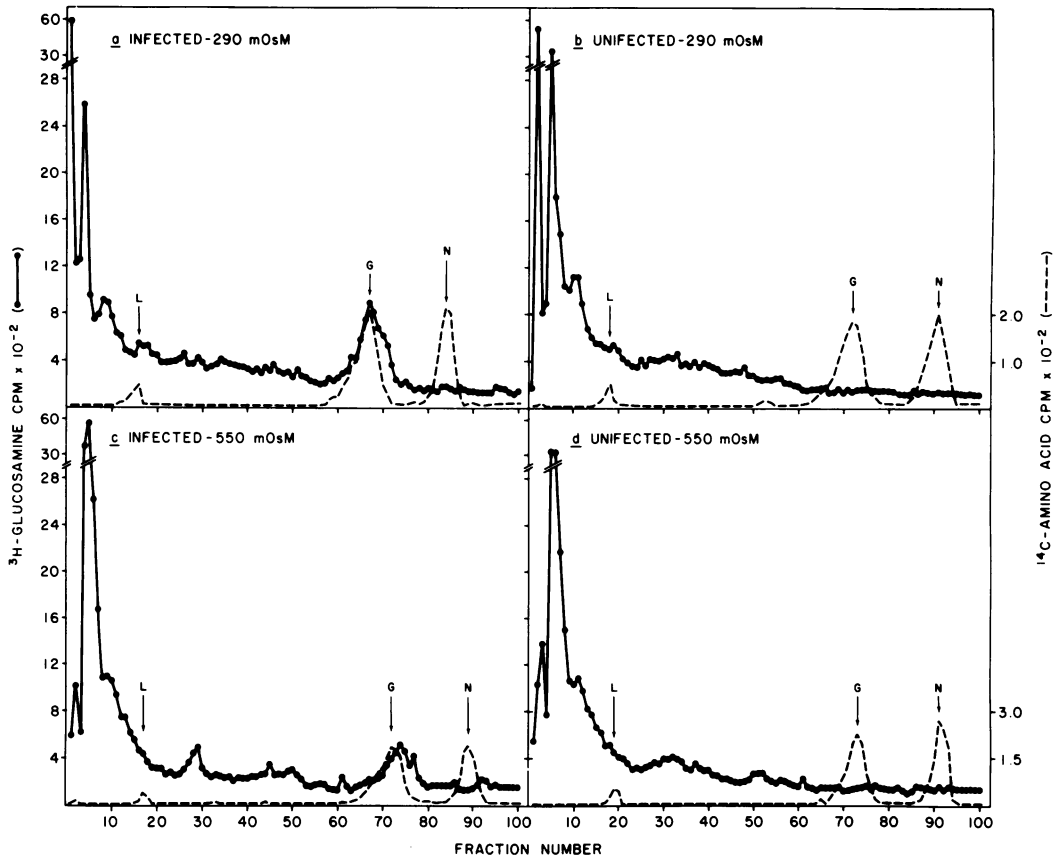

FIG. 7. *Labeling of rabies-infected or uninfected cells with [^{3}H]glucosamine under isotonic (290 mOsM) or hypertonic (550 mOsM) conditions. Confluent monolayers (5 × 10^6 cells/60-mm dish) of BHK-21 cells were either infected with rabies virus (100 PFU/cell) (a and c) or mock-infected (b and d) and incubated at 37°C for 40 h. Cells were then labeled under isotonic conditions (290 mOsM) for 2 h with [^{3}H]glucosamine (50 μCi/ml) (a and b). After pretreatment for 15 min with hypertonic medium (550 mOsM), other cells were labeled for 2 h in the same hypertonic medium containing [^{3}H]glucosamine (50 μCi/ml) (c and d). The whole-cell lysates containing 27,000 trichloroacetic acid-precipitable cpm were analyzed on discontinuous 10% SDS-polyacrylamide gels. Arrows indicate ^{14}C-amino acid-labeled rabies virus proteins.*

course of infection. In contrast to the synthesis of the NS polypeptide of VSV, which has been shown to be preferentially synthesized early in infection (17), none of the rabies-specific polypeptides appeared to be synthesized at a greater relative rate early in infection.

The relative rates of synthesis of the rabies virus polypeptides do not correspond to their relative proportions in the intact virion. This observation indicates that the protein composition of virions is not solely determined by the relative rate at which the viral polypeptides are synthesized and suggests that additional mechanisms underlie viral assembly. In the absence of data that compare the absolute rate of synthesis of a given polypeptide to the rate at which that polypeptide is incorporated into virus, no conclusion can be made concerning the rate of synthesis of a given viral polypeptide and a rate-limiting step in viral assembly.

Lodish (24) has proposed a model for the regulation of α- and β-globin synthesis which predicts that a reduction in the overall rate of peptide chain initiation will cause the preferential translation of mRNA species with higher rate constants of peptide initiation. Since hypertonic conditions have been shown to specifically suppress peptide chain initiation, this model can account for the differential inhibition caused by hypertonic conditions of the synthesis of various cellular (33) and viral (rabies, VSV [32], and MMTV [36]) polypeptides.

If this model is indeed correct, the different sensitivities among the syntheses of L, N, and M_2, as well as G and M_1, polypeptides to hypertonic conditions suggests that at least three

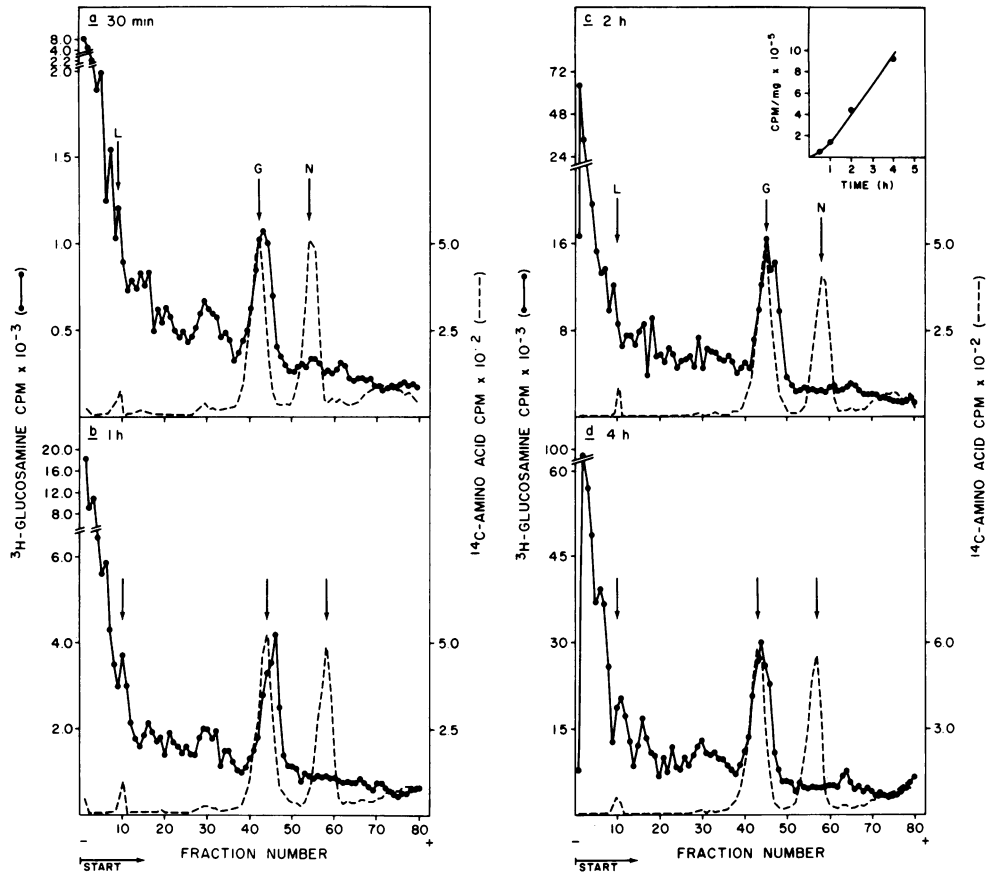

FIG. 8. *Kinetic analysis of intracellular viral glycoprotein synthesis. Confluent monolayers (5 × 10⁶ cells/60-mm dish) were infected with rabies virus (100 PFU/cell), and then incubated for 40 h at 37°C. Cells were exposed to [³H]glucosamine (50 μCi/ml) in isotonic medium for 0.5 h (a), 1 h (b), 2 h (c), and 4 h (d). Whole-cell lysates (450 μg of protein) were analyzed on discontinuous 10% SDS-polyacrylamide gels. ¹⁴C-amino acid-labeled rabies virus proteins were subjected to co-electrophoresis with the cell lysates. Total incorporation of [³H]glucosamine into acid-precipitable material as a function of time is shown in the insert of (c).*

ribosome-binding sites (with different initiation efficiencies) exist in rabies virus mRNA. Since eukaryotic mRNA apparently contains only one functional initiation site (9, 13, 22), these data suggest that there is a minimum of three discrete rabies virus mRNA species. Consistent with this argument are observations that the synthesis of individual poliovirus proteins, which are translated from a single polycistronic mRNA molecule with one initiation site (13, 35), does not show differential inhibition by hypertonic conditions (34), whereas the synthesis of individual VSV proteins, which are translated from several monocistronic mRNA molecules (4, 20, 28, 29, 46), is differentially inhibited by hypertonic conditions (32, 34).

A major difference between rabies virus and VSV has been the inability to detect RNA transcriptase activity in purified rabies virions (41, 48). RNA transcriptase activity could not be detected in purified rabies virions by in vitro assays that were sensitive enough to detect RNA transcriptase with a specific activity 4,000 times less than that present in VSV virions (H. P. Madore and F. Sokol, unpublished observations).

In VSV, the high-molecular-weight L polypeptide has been shown to be necessary for RNA transcriptase activity (5–7, 10, 11, 31). Since a high-molecular-weight polypeptide analogous to the VSV L polypeptide had not been clearly demonstrated in rabies virions (40, 42), it was possible to attribute the deficiency in RNA transcriptase activity in rabies virions to the absence or extremely low number of L polypeptide molecules per virion. In the present work, however, we have detected a high-molecular-weight polypeptide (with an electrophoretic mobility similar to that of the VSV L polypeptide) in rabies virions and in infected cells, and we have estimated that each rabies

virion contains approximately 33 L polypeptide molecules (Table 2). In comparison, a VSV virion contains about 60 L protein molecules (3). Therefore, if we assume that the L polypeptides of rabies virus and VSV are functionally similar, the extremely low level of RNA transcriptase activity in rabies virions does not seem to result from an absence or extremely low number of L polypeptide molecules in the rabies virion.

In rabies-infected cells labeled for 2 h with [^{3}H]glucosamine under isotonic conditions, two forms of virus-specific glycoprotein are observed: the major form, designated gp80, comigrates with the viral glycoprotein; a minor form, designated gp78, is seen as the faster-migrating shoulder of the major viral glycoprotein peak (Fig. 7a). Kinetic analysis of the biosynthesis of these two forms has revealed that the initial appearance of the faster-migrating gp78 and the subsequent accumulation of the gp80 is coupled with the slow disappearance of the gp78 (Fig. 8a–d). These results suggest that the faster-migrating gp78 is an intermediate in the synthesis of gp80. Furthermore, gp78 may represent an incompletely glycosylated form of the viral glycoprotein, since neuraminidase-treated viral glycoprotein migrates on PAGE at a rate that approximates that of intracellular gp78 (B. Dietzschold, unpublished observations). Glycoprotein synthesis of another rhabdovirus, VSV, also appears to involve incompletely glycosylated intermediates that migrate faster than the mature glycoprotein in SDS-polyacrylamide gels (1, 4, 17, 20, 28, 29, 45, 46).

The rabies-specific glycoprotein in infected cells labeled with amino acids (Fig. 6a) or with glucosamine (Fig. 7c) under hypertonic conditions migrates in SDS gels with a mobility similar to that of gp78. The observation that this faster-migrating glycoprotein can be chased under isotonic conditions into an intracellular glycopeptide that co-migrates with gp80 and is subsequently released with extracellular virions (Fig. 6b and c) suggests that hypertonic conditions may reversibly interfere with the biosynthesis of rabies virus glycoprotein. The faster migration rate of the product synthesized under hypertonic conditions suggests that interference occurs at an intermediate that requires supplementary glycosylation to form the mature viral glycoprotein. Whether this component is (i) identical to gp78, (ii) another intermediate not resolved from gp78, or (iii) an aberrant step in viral glycoprotein maturation remains to be determined. A comparison of the size and ion exchange distribution of the oligosaccharides of the viral glycoproteins is being carried out to answer this question.

The reversible block of rabies glycoprotein biosynthesis caused by hypertonic conditions appears to be analogous to the reversible block in Semliki forest virus glycoprotein synthesis, which occurs when infected cells are grown in sugar-free medium (15). In contrast to hypertonic and sugar-free conditions, the impaired glycosylation of influenza (19, 37) and Semliki forest virus (14) glycoprotein intermediates induced by 2-deoxyglucose or excess glucosamine cannot be reversed after removal of these inhibitors. This irreversible blockage in glycoprotein biosynthesis is attributed to incorrect glycosylation induced by the inhibitors (15).

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