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La Crosse Virus Nucleocapsid Protein Controls Its Own Synthesis in Mosquito Cells by Encapsidating Its mRNA

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Within 24 to 48 h of La Crosse virus infection of mosquito cells, >75% of the S mRNA was found to band in CsCl density gradients at the position of genome or antigenome nucleocapsids. The encapsidation of the S mRNA correlates with the repression of N protein synthesis in vivo, and the encapsidated S mRNA cannot be translated in vitro. Unlike genome and antigenome assembly, S mRNA assembly is a relatively slow process, which is not coupled to its synthesis. Within the encapsidated S mRNA population, three forms could be distinguished, those with intact primers which were or were not also assembled with N protein and those in which the primer and up to 3 template bases had been lost. We suggest that genome replication, but not transcription, is down regulated with time in mosquito cells for reasons that are unclear. The pool of unassembled N protein then increased to the point at which it began to interact with its own mRNA, as this mRNA also contains what is considered to be the assembly site, i.e., the conserved sequences at the 5′ ends of all genome and antigenome chains. This lead to the assembly of the entire mRNA, except for the nontemplate primer. Some of the primers were then also assembled with N protein, whereas others were digested to produce truncated mRNAs.

La Crosse virus (LAC) is a member of the California encephalitis serogroup of the *Bunyaviridae* (19). The genome of these viruses consists of three RNA segments of negative polarity called L (large), M (medium), and S (small), which encode the viral polymerase (L), the surface glycoproteins (G1 and G2), and the nucleocapsid protein (N), respectively (16). The viral genomes (and antigenomes) are found as helical nucleocapsids (NCs), assembled with the N protein, in a structure which is sufficiently stable to survive CsCl density gradient centrifugation. The minus-strand genomes are templates for two kinds of RNA synthesis, that of mRNAs and antigenomes. mRNAs are initiated on capped primers derived from host mRNAs by a cap-snatching mechanism (2, 17), whereas antigenomes (and genomes) initiate with ATP at the precise 3′ end of the template.

These viruses productively infect both mammalian and insect cells, but the infection in these two cell types is quite different (15). The infection of mammalian cells is highly cytopathic and leads inevitably to cell death, whereas that of mosquito cells is asymptomatic and becomes persistent. To investigate the reasons for the very different fates of these infections, we have compared BHK and C6/36 cell infections under otherwise identical conditions of cell growth (24). In BHK cells, the infection is relatively rapid, and the levels of viral mRNAs and genomes reach a maximum around 6 h postinfection (p.i.). At this point, the infection induces a general mRNA instability, to the extent that both host and viral protein synthesis are severely reduced (22). New genome synthesis is also reduced at this time, and cytopathic effect becomes visible by 14 h. In the infection of mosquito cells, on the other hand, replication takes place more slowly and the intracellular levels of viral RNAs and proteins reach a maximum around 24 h. However, the levels of viral RNAs and proteins in mosquito cells at this time often exceed those in mammalian cells. This suggests that cytopathic effects are not simply related to the accumulation of viral macromolecules (24).

All models for RNA virus replication are exponential ones. In these models, primary transcription and translation allow a first round of genome replication, and these amplified genomes lead to an increased rate of viral mRNA and protein synthesis, which leads to further genome replication, and so on. However, a point must be reached at which one or more cellular components become limiting, leading to cytopathic effects. Yet this clearly does not occur during LAC infection of mosquito cells. Instead, LAC appears to limit its own replication in going from an acute (up to 24 h) to a persistent phase (after 24 h) of the infection. The synthesis of N protein and genome replication were found to be markedly reduced around 24 h (24). Since a continuous supply of unassembled N protein is thought to be required for genome synthesis, the LAC infection may become self-limiting through the unavailability of N protein for assembly. The mechanism by which N protein synthesis is repressed would then appear to be central to our understanding of how the mosquito cell infection becomes self-limiting.

The decline in N protein synthesis at around 24 h is not due to a reduction in the S mRNAs present, since their steady-state levels remain high until 72 h. In fact, N synthesis begins to decline at a time when the maximum levels of S mRNA are just being reached (24). It therefore appears that translational control of S mRNA takes place in mosquito cells. This report sets out to examine this control by monitoring the fate of the S mRNA during infection. We have found that N protein controls the translation of its own message by assembling it into a NC. In addition, we have investigated this assembly as a way to better understand the encapsidation of viral RNA.

MATERIALS AND METHODS

Analysis of cytoplasmic RNAs. LAC infections of C6/36 *Aedes albopictus* (8) and BHK-21 cells were carried out at

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33°C (24). The isolation of NC RNA and unencapsidated RNA from infected cells has also been described previously (20). For the isolation of intact NCs, the visible NC band from a CsCl gradient was removed through the side of the centrifuge tube with a syringe. Recovered, NCs were dialyzed for 3 h in the presence of 50 μg of tRNA per ml against 10 mM Tris hydrochloride (pH 7.5)–100 mM NaCl–1 mM EDTA. The NCs were preserved in 10% glycerol at −20°C.

For Northern (RNA) blot analysis, 25 μg of RNA that had been pelleted through CsCl (CsCl-pelleted RNA) or the cell-equivalent amount of NC RNA was heated for 2 min at 90°C in a solution containing 80% formamide, 0.1% xylene cyanol FF, and 0.1% bromophenol blue and then cooled quickly on ice. Electrophoresis on 2.8% polyacrylamide–8 M urea gels (1.5 mm thick) in TBE (89 mM Tris-borate, 89 mM boric acid) was followed by electroblotting to Hybond-N (Amersham) in TAE (40 mM Tris-acetate [pH 7.8], 2 mM EDTA).

For slot blot analysis, 1, 5, or 25 μg of CsCl-pelleted RNA or the cell-equivalent amount of NC RNA was made 1 M in ammonium acetate and then blotted to Hybond-N, using a Schleicher and Schuell Minifold II.

Hybridizations were carried out in a solution consisting of 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and 1× Denhardt solution with 5×10^6 cpm of probe. Hybridizations were conducted at 50°C when the S minus-strand riboprobe was used and at 42°C when the cDNA probe was used.

Preparation of cDNA probe. C6/36 cells from 20 100-mm-diameter culture dishes were lysed in 4 ml of a solution consisting of 10 mM Tris hydrochloride (pH 7.5), 100 mM NaCl, 1 mM EDTA, and 0.5% Nonidet P-40. Nuclei and cellular debris were removed by centrifugation at 4,000 × *g* for 5 min. Total cytoplasmic RNA was isolated by centrifugation through a 20 to 40% CsCl gradient. After suspension in TE (10 mM Tris hydrochloride [pH 7.5], 1 mM EDTA), poly(A)$^+$ RNA was isolated by chromatography on oligo(dT)-cellulose as previously described (14).

For the synthesis of a cDNA probe, 1 μg of poly(A)$^+$ RNA was mixed with 6 μg of random deoxynucleotides (6-mers), denatured at 90°C for 2 min, and precipitated with ethanol. The following reagents were used in the reverse transcription reaction: 25 mM Tris hydrochloride (pH 8.3), 10 mM $MgCl_2$; 75 mM KCl, 30 mM β-mercaptoethanol, 500 μM of dGTP, dATP, and TTP; 10 μCi [α-^{32}P]dCTP (Amersham; 3,000 Ci/mmol; 10 mCi/ml); and 10 U of Moloney murine leukemia virus reverse transcriptase. The reaction mix was incubated for 30 min at 37°C. After the addition of cold dCTP to a concentration of 250 μM, the reaction was allowed to proceed for another 30 min. RNA was then hydrolyzed by heating at 65°C for 1 h in the presence of 50 mM NaOH. After neutralization, the reaction mix was extracted with phenol, and unincorporated nucleotides were removed by gel filtration.

Immunoselection of RNA. For each sample to be analyzed, 40 μl of a 50% slurry of protein A-Sepharose was incubated with 10 μl of either anti-NC serum or a control serum for 2 h at 4°C. After three washes with cold NET (150 mM NaCl, 50 mM Tris hydrochloride [pH 8.0], 0.1% Nonidet P-40), the beads were incubated with 100 μl of cytoplasmic extract (equivalent to half of one 100-mm-diameter dish of C6/36 cells) for 1 h at 4°C. After three washes with NET, the beads were suspended in 0.3 M ammonium acetate (pH 5.4) and 0.5% sodium dodecyl sulfate. After phenol and chloroform extractions, the RNA was precipitated with ethanol. Northern analysis was carried out as described above.

In vitro translation. In vitro translation was performed in rabbit reticulocyte lysates (Promega) as recommended by the supplier. The reaction products were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and viewed by fluorography.

Primer extension. A gel-purified oligonucleotide complementary to positions 44 to 57 of the S antigenome was 5′ end labeled with [γ-^{32}P]ATP (Amersham; 5,000 Ci/mmol; 10 mCi/ml) by using T4 polynucleotide kinase (Genofit). The labeled primer was extracted once with phenol-chloroform, and 5 to 10 pmol of labeled primer was mixed with either 20 μg of CsCl-pelleted RNA or the cell-equivalent amount of NC band RNA, precipitated with ethanol, suspended in 6 μl of TE, heated for 2 min at 90°C, and brought to 0.2 M in NaCl. Annealing was carried out at 29°C for 5 min, and reverse transcriptase buffer was added to the following reagents: 50 mM NaCl, 50 mM Tris hydrochloride (pH 8.3), 8 mM $MgCl_2$, 35 mM β-mercaptoethanol, 500 μM deoxynucleoside triphosphates, and 100 U of Moloney murine leukemia virus reverse transcriptase per ml in a final volume of 30 μl. The reaction was carried out at 43°C for 1 h and stopped by the addition of EDTA to 10 mM. After ethanol precipitation, the reaction products were suspended in a solution containing 80% formamide, 0.1% xylene cyanol FF, and 0.1% bromophenol blue, heated at 90°C for 2 min, cooled on ice, and analyzed by electrophoresis on a 10% polyacrylamide–8 M urea gel (0.3 mm thick).

RESULTS

Translational control of S mRNA by N protein. Translational control can be exerted at almost any level of the translation process. For LAC S mRNA, it is unlikely to be ribosomal loading on the mRNA, since the 5′ ends of the viral mRNAs are derived from host mRNAs (2, 17). The control mechanism also appears to be specific for viral mRNA, since cellular protein synthesis remains unchanged during the period in which N synthesis declines (24). One possible mechanism for this specific control is that N protein interacts with its own mRNA to prevent its translation.

The S genome segment is 983 nucleotides (nt) long. Its mRNA starts on a primer of around 15 nt and terminates at position 886 and is therefore around 900 nt long. In infected BHK cells, a minor transcript which starts at position +1 and terminates at 886 (hereafter referred to as the 1-886 transcript) has also been detected (20). However, unlike the S mRNA, the 1-886 transcript is found only in the form of a assembled NC and must therefore contain the site for the initiation of NC assembly. As the S mRNA contains all the sequences of the 1-886 transcript, it should also contain this site. This situation is unique to the segmented minus-strand RNA viruses, since their nonsegmented counterparts have the site of NC assembly on a leader sequence which is absent from the viral mRNAs (3).

To determine whether S mRNA is assembled into NCs in mosquito cells, we separated NCs from unencapsidated RNA by sedimentation in CsCl density gradients. The distribution of S mRNA between these two fractions was determined by Northern (RNA) blotting (Fig. 1A). Remarkably, by 24 h, 75% or more of the intracellular S mRNA was found to band in CsCl at the density of NCs. The ratio of unencapsidated to encapsidated mRNA then remained constant to at least 72 h. In contrast, less than 10% of the S mRNA was encapsidated at 12 h. In a separate experiment in which shorter time intervals were used, the majority of the S mRNA became encapsidated in a relatively short time,

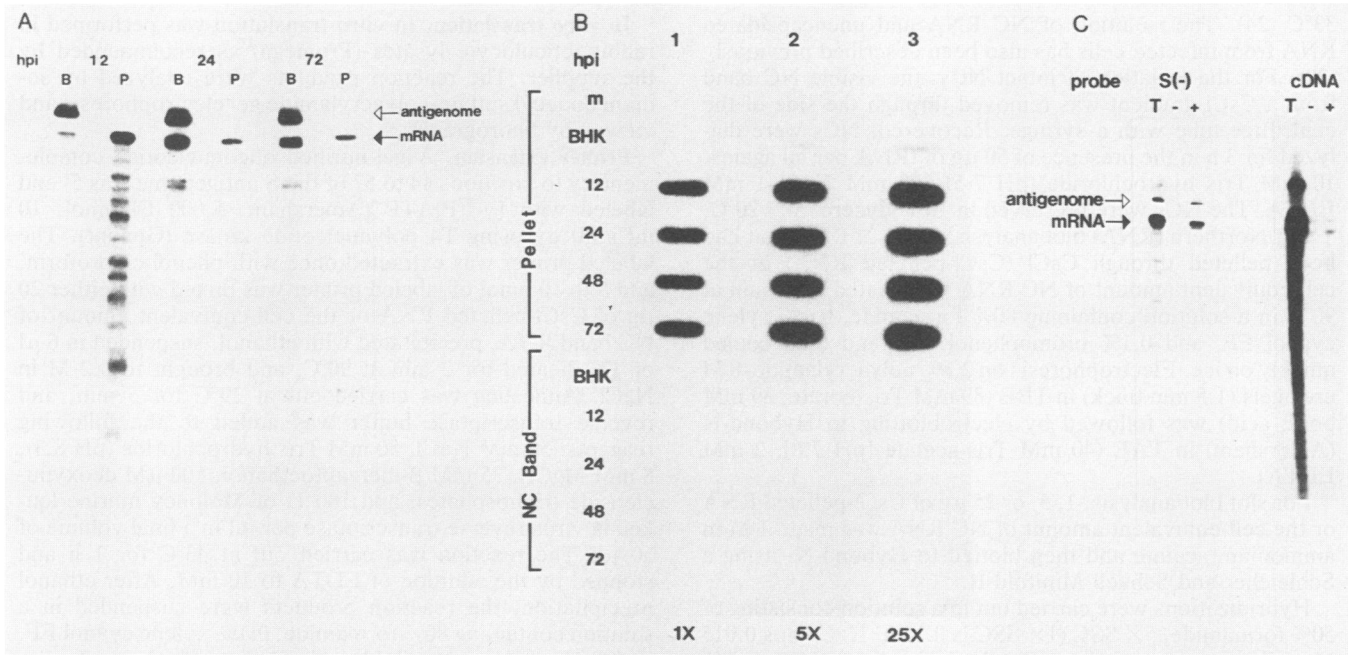

FIG. 1. Analysis of encapsidated RNA from C6/36 cells. (A) CsCl-pelleted (P) and band (B) RNAs from LAC-infected C6/36 cells were isolated at the times indicated (hours p.i. [hpi]). Either 25 μg of pellet RNA or the cell-equivalent amount of band RNA was analyzed by electrophoresis on a denaturing 2.8% polyacrylamide gel. An S minus-strand riboprobe was used for hybridization. The positions of the S antigenome and mRNA are indicated. (B) Either 1, 5, or 25 μg of CsCl-pelleted RNA (lanes 1, 2, and 3) or the cell-equivalent amount of CsCl band RNA (of the same samples used above) were blotted onto a Hybond N membrane and hybridized with a cDNA probe to uninfected C6/36 poly(A)$^+$ RNA. BHK refers to RNA from LAC-infected BHK cells which was isolated at 18 h p.i.; m refers to RNA from mock-infected BHK cells. (C) Immunoprecipitations of N protein-RNA complexes from cytoplasmic extracts (18 h p.i.) were carried out as described in Materials and Methods, using antiserum to the LAC N protein (+) or a control serum (−). Immunoselected RNA was analyzed on a denaturing 2.8% polyacrylamide gel along with total cytoplasmic RNA (T) from an equivalent volume of the extract used for immunoselection. RNA was blotted onto Hybond N, and hybridization was carried out with either an S minus-strand [S(−)] riboprobe or cDNA to C6/36 poly(A)$^+$ RNA.

between 20 and 24 h (results not shown). The timing of S mRNA encapsidation thus correlates with the inhibition of N protein synthesis seen earlier (24).

To determine whether, in fact, encapsidation of S mRNA prevents its translation, we compared the level of N protein synthesis in a reticulocyte lysate programmed with the encapsidated RNA before or after deproteinization (Fig. 2). The deproteinized RNA was capable of synthesizing N protein, whereas the assembled RNA could not be translated at all. The N protein therefore appears to regulate its own synthesis in mosquito cells, by encapsidating its mRNA and preventing its translation.

Specific encapsidation of viral mRNA. Host protein synthesis is not affected by LAC infection of C6/36 cells (24). The assembly of mRNA into NCs would then presumably be specific for viral mRNA. To determine whether cellular mRNAs were encapsidated, ^{32}P-cDNA was made from uninfected mosquito cell poly(A)$^+$ RNA and used as a probe against CsCl-pelleted and band RNA from infected cells. The cDNA probe hybridized only to CsCl-pelleted RNA in slot blots (Fig. 1B), and identical results were obtained by Northern blotting (results not shown).

From the above experiment, however, it could be argued that N protein might have interacted with host mRNA but that the assembly was incomplete. Partially assembled mRNAs would not have banded at the position of NCs (and might even have pelleted) and would not have been detected in the experiment shown in Fig. 1B. We therefore used an alternative approach which did not depend on complete encapsidation. N protein-RNA complexes were immunoselected from infected cell lysates with anti-NC antibodies (Materials and Methods). As a control, immunoselection was also carried out by using an antiserum to a Sendai virus protein. Northern blots of immunoselected and total cellular RNA were probed with either an S minus-strand riboprobe or the mosquito cDNA probe (Fig. 1C). As expected, viral S antigenomes and mRNA were selected by the anti-NC serum, but not by the control serum. Immunoselection was not complete, however, as can be seen by comparing the level of antigenome in the total fraction with that of the immunoselected RNA. In contrast to S mRNA, little cellular RNA was found in the immunoselected fraction (Fig. 1C). A very small amount of RNA was immunoselected by both antisera, which probably represents interactions with the protein A-Sepharose. As our cDNA probe may only have been able to detect the more abundant mRNAs, we cannot rule out the encapsidation of some rare mosquito cell mRNAs. Within this limitation, however, the above results demonstrate that mostly viral mRNAs interact with N protein or are assembled into NCs.

A significant pool of unassembled N protein in C6/36 cells. Since <10% of the S mRNAs are encapsidated at 12 h whereas the majority are encapsidated at later times, we investigated whether this difference was associated with the intracellular level of unassembled N protein. If genome synthesis and assembly are coupled, this level can be estimated by measuring the fraction of genome synthesis which continues after the inhibition of further protein synthesis. During BHK cell infection, this level appears to be very low, in that no genome synthesis can be detected by [^{3}H]uridine labeling after inhibition of protein synthesis (18). When similar experiments are carried out during C6/36 cell infec-

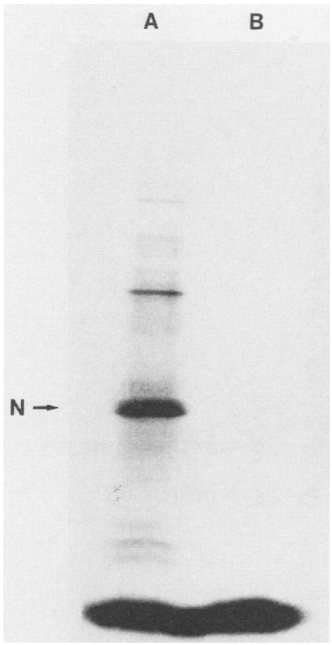

FIG. 2. In vitro translation of encapsidated RNA. NCs were isolated from infected C6/36 cells at 24 h and dialyzed to remove the CsCl. Rabbit reticulocyte lysate was programmed with either 250 ng of the encapsidated RNA (B) or 250 ng of the same RNA after deproteinization (A). The [^{35}S]methionine-labeled translation products were separated by 15% polyacrylamide gel electrophoresis. The position of N protein, as determined by Coomassie blue staining of a parallel nucleocapsid sample, is indicated.

tion, the results are quite different. At 16 to 17 h, in cells pretreated for 10 min with either anisomycin, cycloheximide, or pactamycin, and in which [^{35}S]methionine incorporation was reduced by >97% for either 15 or 60 min (results not shown), S genome synthesis continued at 22 to 40% of the untreated control level (Fig. 3A). In these experiments, all of the pulse-labeled S mRNA pelleted through the CsCl gradient, whereas all the genomes were found as assembled NCs (results not shown). When labeling was carried out at 20 to 24 h, 33 to 51% of S genome synthesis continued (results not shown). However, when the experiment was carried out at 5 to 7 h p.i., genome synthesis was now strongly reduced (3 to 6% of control [Fig. 3B]) but still clearly detectable. This experiment appears to measure the preexisting pool of unassembled N protein. When the [^{3}H]uridine was added at 1 h and 10 min after drug addition rather than 10 min later and the cells were labeled at 6 to 8 h (Fig. 3B), new genome synthesis was undetectable. The ability of C6/36 cells to synthesize S genomes in the absence of on-going protein synthesis thus appears to be due to their levels of unassembled N protein, which increase with time. We note that these experiments do not estimate absolute levels of N protein, but rather the levels required to support a given rate of genome synthesis, and that this rate is much higher in BHK than in C6/36 cells (24).

We also note that synthesis of the M and L genomes, which are respectively 4.6 and 7 times longer than S, is slightly more sensitive to the translational block than that of S (Fig. 3). This suggests that the larger genome segments may replicate more slowly than S when the level of unassembled N has been reduced. It also offers a possible explanation for the curious finding that only the S segment

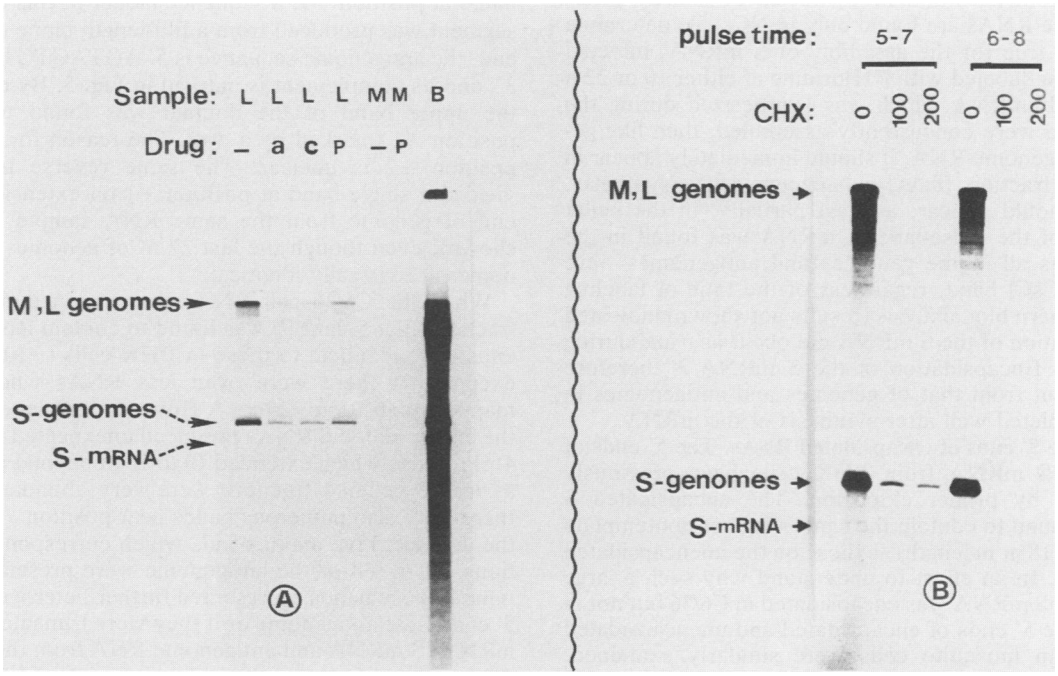

FIG. 3. A significant pool of unassembled N protein in C6/36 cells. (A) Infected C6/36 cultures were either not drug treated (–) or were pretreated for 10 min with a concentration of 100 μg/ml of cycloheximide (c) or anisomycin (a), or with 5 μg of pactamycin (p) per ml and then labeled with 300 μC of [^{3}H]uridine (26 Ci/mmol) for 1 h. The visible NC band was isolated, and its RNA was separated on a denaturing 4% polyacrylamide gel. Samples marked M are from mock-infected cultures; those marked L are LAC infected. The sample marked B is total cytoplasmic RNA from infected BHK cells labeled 4 to 5 h p.i., run as markers. (B) Infected C6/36 cultures were either not treated or treated with 100 or 200 μg of cycloheximide (CHX) per ml at 4 h and 50 min. [^{3}H]uridine was then added at either 5 to 7 or 6 to 8 h, as indicated.

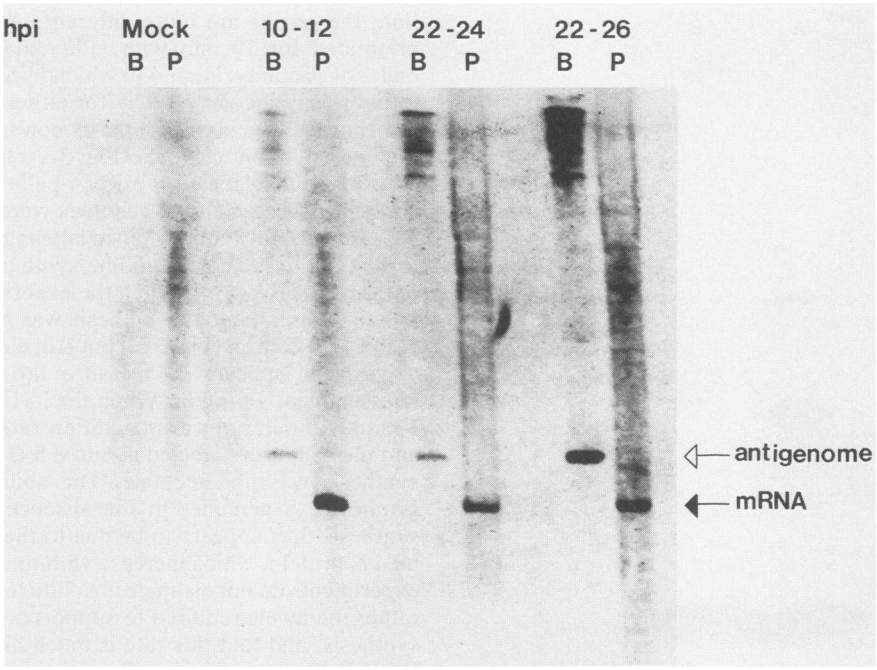

FIG. 4. Pulse-labeling of LAC RNA in C6/36 cells. LAC-infected C6/36 cells were labeled with 300 μCi/ml of [^{3}H]uridine (26 Ci/mmol) at the times (hours p.i. [hpi]) indicated. CsCl-pelleted (P) and band (B) RNAs were prepared and analyzed on a denaturing 2.8% polyacrylamide gel. After fluorography, the gel was visualized by autoradiography. The positions of S antigenomes (and genomes) and S mRNA are indicated. The bands at the top presumably represent the M and L genomes and antigenomes.

can be detected in long-term persistently infected mosquito cells or the particles they shed (7, 24).

Synthesis and assembly of S mRNA are not coupled. Assembly of viral antigenomes and genomes into NCs occurs either concurrently with their synthesis or shortly afterwards, as these RNAs are found only in NCs. To determine if the same is true for the assembly of S mRNA, infected cells were pulse-labeled with [^{3}H]uridine at either 10 or 22 h p.i. (Fig. 4). If mRNA which was synthesized during the labeling period were concurrently assembled, then like genome and antigenome RNA, it should immediately appear in the CsCl band fraction. If assembly occurred after synthesis, the mRNA should appear, at least partially, in the pellet fraction. All of the pulse-labeled mRNA was found in the pellet, whereas all of the genomes and antigenomes were found in the CsCl band, regardless of the time of labeling (Fig. 4). Northern blot analysis (results not shown) indicated that encapsidation of the S mRNA did occur as usual during this infection. Encapsidation of the S mRNA is therefore clearly different from that of genomes and antigenomes in that it is completed well after synthesis of the mRNA.

Mapping the 5′ ends of encapsidated RNAs. The 5′ ends of encapsidated S mRNA from BHK cells have previously been mapped by primer extension. The encapsidated S mRNA was found to contain the same range of nontemplate primers (10 to 18 nt in length) as those on the unencapsidated S mRNA (20). In an effort to understand why such a large fraction of the S mRNA was encapsidated in C6/36 but not in BHK cells, the 5′ ends of encapsidated and unencapsidated S mRNA from mosquito cells were similarly examined, along with RNA from BHK cells (Fig. 5). The results from the BHK cell RNA were similar to those found previously. The CsCl-pelleted fraction (lane 8) contained predominantly RNAs whose 5′ ends were mapped to position −10 to −18, with a minor amount of RNA whose ends were at position −1 to +4. The CsCl band RNA (lane 5), on the other hand, contained only a minor amount of mRNA with nontemplate primers as well as antigenomes. However, two bands appeared at the position corresponding to the 5′ end of the antigenome. To determine which extension product terminated at position +1, a sequence ladder of this end of the S segment was produced from a full-length clone (4). At its 5′ end, the antigenome sequence is 5′ AGTAGTGTACTCCAC 3′, and its complement is marked in Fig. 5. By comparison, the upper band of the doublet was found to represent position +1 (marked by a dot). The reason for the band at position +2 is unclear. The same reverse transcriptase yielded a single band at position +1 on extension to the 5′ end of genome from the same RNA sample (results not shown), even though the last 27 nt of genomes and antigenomes are virtually identical.

When the C6/36 cell RNA was examined, the pelleted fraction (Fig. 5, lane 7) was found to contain RNA whose 5′ ends were identical to those in BHK cells (−10 to −18 nt), except that there were even less RNAs whose 5′ ends mapped to position −1 to +4. However, primer extension on the encapsidated S RNAs produced unexpected results (lane 4). Products which extended 10 to 18 nt beyond position +1, as in the pelleted fraction, were very abundant here, but there were also numerous bands near position +1 instead of the doublet. Five major bands which corresponded to positions −1 to +4 of the antigenome were present. To determine if these bands represented further heterogeneity at the 5′ end of the antigenome or if they were truncated species of mRNA, S mRNA and antigenome RNA from the CsCl band were isolated from a 2.8% polyacrylamide gel (cf. Fig. 1A) and analyzed separately. Extension on the antigenome-length RNA (lane 1) produced the same two bands (+1 and +2) as observed for antigenome RNA from BHK cells. Extension on the isolated mRNA (lane 2) revealed that the heterogeneity around position +1 resulted from the presence

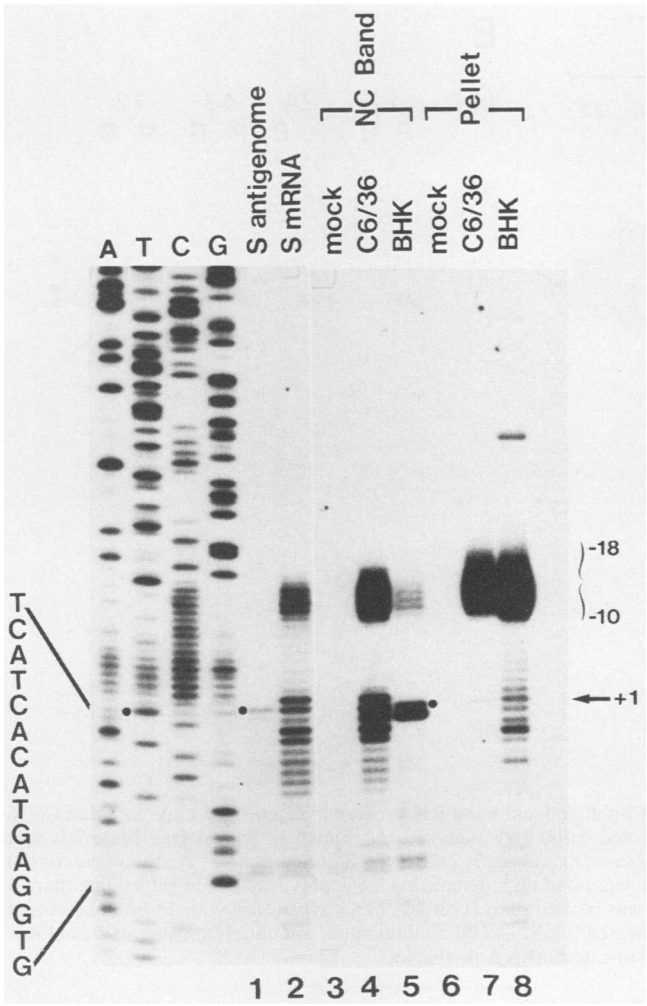

FIG. 5. Mapping the 5′ ends of encapsidated S RNAs. CsCl-pelleted and band RNAs from LAC infections of BHK (8 h p.i.) (lanes 5 and 8) and C6/36 cells (24 h p.i.) (lanes 4 and 7) were examined by primer extension (see Materials and Methods). The reaction products were analyzed on a 10% sequencing gel. CsCl band RNA from C6/36 cells was also separated on a denaturing 2.8% polyacrylamide gel. After ethidium bromide staining, S antigenome (and genome) RNA and S mRNA were isolated separately and analyzed by primer extension (lanes 1 and 2). On the left is shown the sequence produced by the dideoxynucleotide triphosphate method from a full-length S clone, using the same oligonucleotide. The sequence complementary to the 5′ end of the S antigenome is indicated, with the first virally encoded nucleotide (position +1) marked with a solid circle. The poly(C) tail used in the original cloning is visible just above. The positions of the 5′ ends of the nontemplate primers from S mRNA (−10 to −18) and the position of the 5′ end of the S antigenome (+1) are indicated. Position +1 of the S antigenome is also indicated by a solid circle in lanes 1 and 5.

of truncated mRNA species within the encapsidated mRNA population.

Truncated mRNAs. We next investigated when the truncated mRNAs appeared during the infection, by primer extension on RNAs from various time points (Fig. 6A). For the CsCl band RNA, the doublet band from the antigenome (position +1 is marked by a solid circle) was the major species present at the earliest time when viral RNA was visible (11 h). A very low level of intact mRNA was also just visible, but we were unable to detect bands at positions −1, +3, and +4, i.e., evidence of the truncated mRNAs. With time, the levels of encapsidated mRNA, both intact and truncated, increased. However, the level of the truncated mRNA continued to increase up to 72 h, while that of the intact mRNA remained constant after 48 h. For the CsCl-pelleted RNA, the level of S mRNA reached a maximum at 24 h and then fell sharply by 72 h. As before, the truncated mRNAs were very minor constituents of this fraction. Thus, not only are the truncated mRNAs found almost exclusively in NCs, but the time course of their appearance suggests that they are derived from the encapsidated mRNAs whose primers are intact.

When these same RNA samples were analyzed by Northern blotting, it could also be seen that the RNA species present in the CsCl band had changed with time (Fig. 6B). At 11 h, only antigenomes (marked by a solid triangle) were visible, and by 24 h, a very small amount of mRNA (marked by a solid diamond) also appeared in the CsCl band. However, at 48 and 72 h, besides a considerable amount of mRNA, a third species (marked by a solid circle) just below that of the intact mRNA appeared. This species, whose migration is consistent with an RNA that is 15 to 20 nt shorter than intact S mRNA, is presumably the truncated mRNA observed by primer extension (Fig. 6A). A similar RNA species has also been seen relatively late in BHK infections, but its 5′ end was mapped to position +1 without heterogeneity, and it was referred to as the 1-886 RNA (20). We also note that the encapsidation of the majority of the S mRNA in this infection occurred slightly later (between 24 and 48 h) than that shown in Fig. 1, possibly because a lower multiplicity of infection was used.

The primer on viral mRNA can also become encapsidated. The finding that only viral RNAs are assembled into NCs suggests that a specific RNA sequence or structure is required. If only a limited sequence were involved, the most likely assembly site would be the highly conserved 11 nt (AGTAGTGTACT) at the 5′ ends of all three genomes and antigenomes (20). As there is also clearly an assembly site on the S mRNA, the question then arises whether the 5′ nontemplate primer can also become encapsidated. The interest here concerns whether encapsidation can be bidirectional, or whether it occurs only in the 5′-to-3′ direction, having initiated at the conserved 5′ ends of the chains.

If the primer region of the mRNA were also encapsidated, then it would be more resistant to nuclease digestion than the same sequences on unencapsidated mRNA. NCs were therefore isolated from mosquito cells by CsCl banding, dialyzed, and treated with increasing concentrations of either micrococcal nuclease (MNase) or RNase A in the presence of 400 μg of pelleted RNA from uninfected cells per ml. Similar results were obtained with both nucleases, and only those with MNase are shown in Fig. 7. On the right are shown the extension products from untreated BHK cell CsCl-pelleted and band RNAs for reference. MNase treatment of the mosquito cell pelleted RNA showed that >90% of this mRNA was digested with 5 μg of MNase per ml. At higher concentrations, all the mRNA was digested. In contrast, 25 to 35% of the nontemplate primers on the capsidated mRNA were insensitive to as much as 25 μg of MNase per ml. There also appeared to be a population of encapsidated mRNA which was sensitive to MNase and was cleaved by concentrations of 5 μg/ml or more. Also present in the CsCl band fraction were the truncated mRNAs (positions −1 to +4) and antigenomes (+1 and 2). None of these bands decreased in intensity throughout the range of MNase or RNase A concentrations but instead increased slightly. This increase may

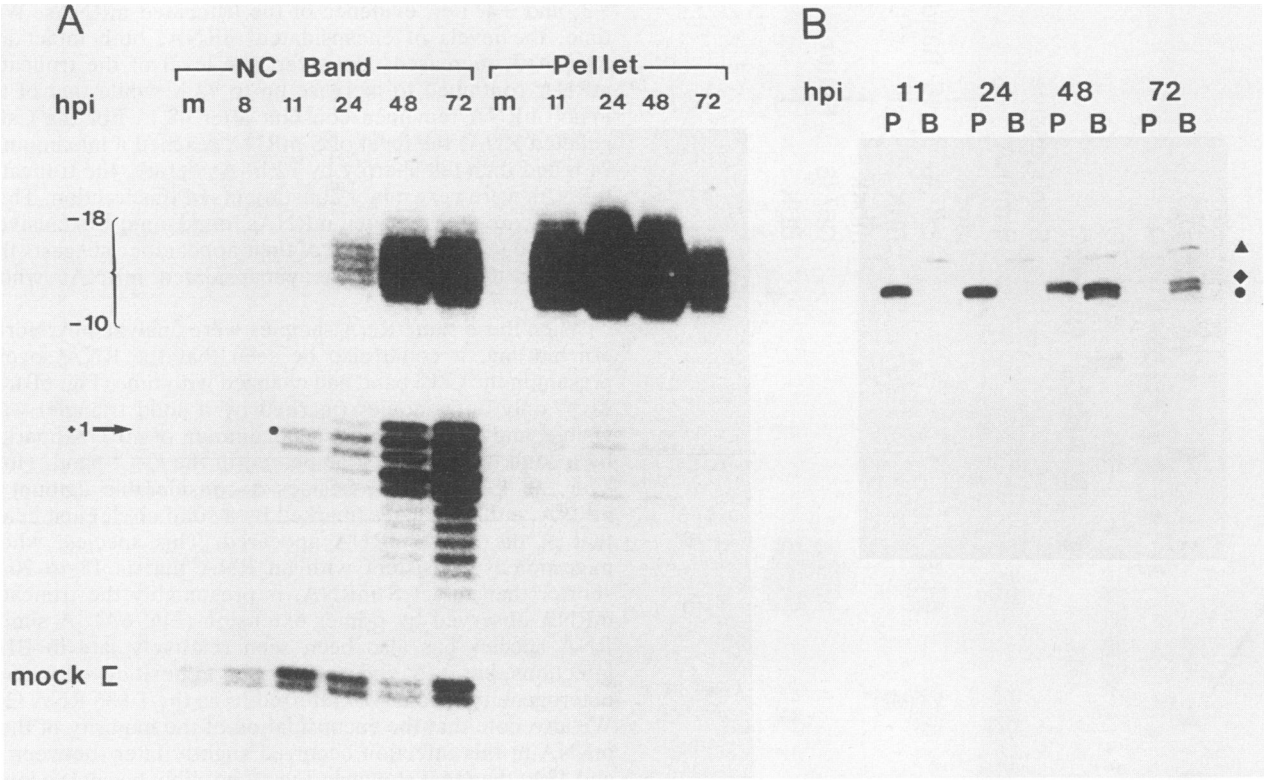

FIG. 6. Time course of appearance of the truncated S mRNA. (A) CsCl-pelleted and band RNAs were isolated from LAC-infected C6/36 cells at the times (in hours p.i. [hpi]) indicated (m refers to mock-infected cells) and analyzed by primer extension (see Materials and Methods). The positions of the nontemplate primer on the S mRNA (−10 to −18) and the 5′ end of the S antigenome (+1) are indicated. (B) The CsCl-pelleted (P) and band (B) RNA from the same experiment were separated on a denaturing 2.8% polyacrylamide gel using either 25 μg of pellet RNA or the cell-equivalent amount of band RNA. The RNA was blotted onto Hybond N and hybridized with an S minus-strand riboprobe. The positions of the S antigenome (▲), S mRNA (◆), and truncated S mRNA (●) are indicated. An underexposure of this blot is shown so that the difference in mobility between the S mRNA and the truncated mRNA is visible.

have resulted from some of the intact mRNAs which specifically lost their primers.

In summary, we found that in a sizeable proportion of the mRNAs which band at the density of genome NCs in CsCl (30 to 50% as judged by MNase and RNase A resistance), the nontemplate primers were as resistant to nuclease attack as the first 55 nt of the template sequence. On these S mRNAs, NC assembly must have occurred bidirectionally.

DISCUSSION

During LAC infection of mosquito cells, an initial acute phase of virus replication evolves into a noncytopathic persistent infection, as the virus down regulates its replication (24). One aspect of this control is the inhibition of N protein synthesis, by the binding of N protein to its own mRNA. The N protein-mRNA interaction was found to be highly specific for viral mRNAs, and assembly occurred on performed mRNAs, rather than concurrently with their synthesis. The encapsidated mRNA was comprised of two populations, those whose primers were intact and those whose 5′ ends were located at positions −1 to +4. Examining mRNAs with intact primers, we were surprised to find some in which these sequences were also encapsidated. These results are discussed below in terms of the persistent infection and their relevance to the NC assembly process.

Translational control of N synthesis. Several examples of translational control by mRNA-binding proteins have been described. Ribosome scanning of ferritin mRNAs is attenuated by the specific binding of a cytosolic protein to a 35-nt sequence in the 5′ untranslated region (13, 25). Stored mRNA in *Xenopus* oocytes are silent due to interactions with oocyte-specific mRNA binding proteins (9, 23). In procaryotes, the T4 gene 32 protein (12, 26) and several of the ribosomal proteins of *Escherichia coli* (5) are part of self-regulatory systems in which the protein binds with high affinity to its primary target (single-stranded DNA for gene 32 protein and rRNA for ribosomal proteins) and with lower affinity to its own mRNA. For the ribosomal protein S4, the site in the 5′ untranslated region of the mRNA may have a similar structure to the binding site on the rRNA (6), while the site on gene 32 mRNA is an unstructured region near the start codon (11).

The control of LAC N protein synthesis is somewhat similar to the latter two examples. N protein recognizes a specific binding site on its own mRNA, and once the initial interactions have taken place, further N binding would presumably become cooperative due to N-N interactions, and assembly would ensue. Ribosomes, as expected, could not translate this complex in vitro. Because the N binding site is highly specific, this control is limited either entirely or mostly to viral mRNAs. One other example of a viral NC protein interacting with viral mRNA has been reported recently (27). The leader RNA of mouse hepatitis virus, which acts as a primer for mRNA synthesis, is specifically

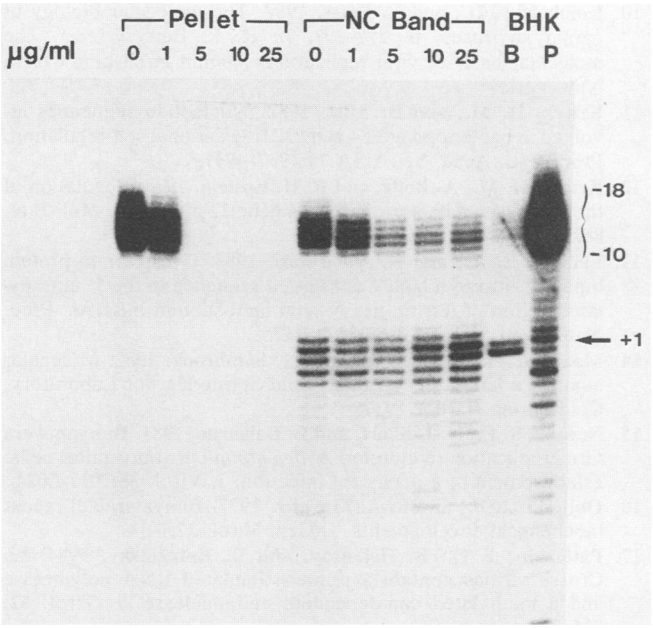

FIG. 7. Micrococcal nuclease sensitivity of encapsidated S RNA. NCs (NC band) and CsCl-pelleted RNA were isolated from LAC-infected C6/36 cells at 24 h p.i. Before nuclease treatment, 0.4 mg of mock-infected C6/36 pellet RNA per ml was added to the NCs. When infected pelleted RNA was analyzed, it was also present at 0.4 mg/ml. The RNA samples were treated with increasing concentrations of micrococcal nuclease as indicated in the presence of 2 mM $CaCl_2$. After the reaction was stopped with 10 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′,-tetraacetic acid (EGTA), the RNAs were extracted and analyzed by primer extension. On the right are shown in primer extension products from untreated CsCl-pelleted (P) and band (B) RNAs from LAC-infected BHK cells. The positions of the 5′ ends of S mRNA (−10 to −18) and the 5′ end of the S antigenome (+1) are indicated.

bound by the NC protein. It has not been determined, however, if this interaction affects translation.

mRNA encapsidation. The encapsidation of viral mRNA has provided an unexpected way in which NC assembly can be examined. Previously, it was inferred that N protein interacted specifically with viral RNAs, as only genomes and antigenomes are found in NCs. The basis for this specificity, however, was unclear. It could not be ruled out, for example, that N simply recognized RNAs with a 5′ triphosphate, there being no other triphosphorylated RNAs in the cytoplasm with which to compete. We have shown here that under conditions in which >75% of the viral S mRNA was encapsidated, little or no cellular mRNA was found to be even partially assembled. In this case at least, N protein must recognize a specific viral sequence or structure.

It seems likely that the N protein assembly site on mRNA is the same as that on genomes and antigenomes, which is thought to be the conserved sequences at the 5′ ends of these chains. If so, this sequence does not have to be located at the precise 5′ end of the RNA to be recongized. However, recognition of the assembly site is much less efficient for mRNA than for genomes and antigenomes, since encapsidation of mRNA occurs at least 4 h after its synthesis. This suggests that N protein binds with a much lower affinity to mRNA than to antigenomes. The difference is presumably due to the nontemplate primer on the mRNA. This primer separates the conserved 5′ end sequences from the triphosphate group, and this 5′ end group is now also blocked with a m^7G.

Among the encapsidated S mRNA, defined by their gel migration (900 nt as opposed to 983 nt), we find two populations: those whose primers are intact and those whose primers and up to 3 template bases appear to have been lost. The time course of the appearance of the truncated mRNAs and their relative absence among the unencapsidated mRNAs suggests that they arise from encapsidated mRNAs which are intact. Nuclease treatment of the encapsidated RNA eliminates only some of the intact mRNAs and slightly increases the population whose 5′ ends map at position −1 to +4. The simplest explanation for the truncated mRNAs is that the mRNA is first assembled completely except for its 5′ primer. In some of these mRNAs, the primer would then be encapsidated by assembly in the 3′-to-5′ direction, and so become resistant to nuclease attack. Assembly in this direction would be less efficient than in the 5′-to-3′ direction. In the others, nuclease would attack the unassembled primer and digest the 5′ end to positions −1 to +4. This scheme is consistent with the assembly site being the conserved 5′ ends of the genome and antigenome chains.

Establishing a persistent infection in mosquito cells. mRNA encapsidation is highly cell-type dependent, since it occurs to very different extents in BHK and C6/36 cells, and the reasons for this difference appear important for the self-limiting infection of mosquito cells. During replication of nonsegmented minus-strand RNA viruses such as vesicular stomatitis virus and Sendai virus, the level of genome replication is controlled by that of unassembled N protein, as genome replication and assembly are coupled (for recent reviews, see references 1 and 10). This also appears to be true for LAC in BHK cells (18, 21) and at early times (5 to 8 h p.i.) in mosquito cells (Fig. 3B). By 16 h p.i. in mosquito cells, however, 22 to 40% of genome replication still occurred in the absence of new protein synthesis, yet the genomes which were made here were all encapsidated. These results indicate that a pool of unassembled N protein exists in C6/36 cells and that this pool increases with time. When this pool increases, several dramatic changes take place within the infected cells. These include the repression of genome replication and N protein synthesis and the encapsidation of most of the S mRNA.

It is paradoxical that the rate of genome replication is maximal early in the infection when the pool of unassembled N protein is low and begins to decline at a time when this pool has increased. One possible explanation for this is that the S mRNA becomes the preferred target for NC assembly over genomes and antigenomes with time. However, newly synthesized mRNA remains unencapsidated up to 4 h after its synthesis, even at times when the level of mRNA encapsidation is high. If its affinity for N protein had changed, we would have expected that the newly synthesized mRNA would have been more quickly encapsidated. We also know that S mRNA synthesis remains relatively constant up to 5 days p.i., and during this period a low level of N protein synthesis is observed (24).

Given these findings, it is difficult to accept a model in which the encapsidation of S mRNA and a subsequent depletion of unassembled N protein are responsible for the shutoff of genome replication. Instead, it would appear that genome replication is down regulated as a result of the absence of a factor other than unassembled N protein. In this model, it is the lack of new genome synthesis which allows the pool of unassembled N protein to grow, so that it now interacts with its lower affinity target, the viral mRNA. This

leads to a decline in N synthesis, which can be viewed as a subsequent level of control in the persistent infection. If this secondary target for encapsidation did not exist, one would expect that the unassembled N would eventually interact with host mRNAs to a greater extent or become insoluble, leading to cytopathic effects.

The arrest of genome replication in mosquito cells occurs in the presence of both functional N and L proteins. Enough N protein is available to encapsidate a large fraction of mRNA, and L protein is active, as demonstrated by the continued synthesis of viral mRNA. From these observations, another factor may be required for genome replication, and it is the absence of this factor which limits genome replication in mosquito cells. Such a factor would presumably be required for the initiation of genome replication (i.e., initiation with ATP, rather than a primer) and could interact with the L protein to modify its activity from a transcriptase to a replicase.

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