Identification of Virus-Coded Nonstructural Polypeptides in Bunyavirus-Infected Cells

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Analyses of bunyavirus-infected cell extracts identified at least two virus-induced nonstructural polypeptides. With snowshoe hare (SSH), La Crosse (LAC), and six SSH-LAC reassortant viruses, it was shown that one of these nonstructural polypeptides (NS_S, approximate molecular weight, 7.4×10^3) is coded by the SSH small (S)-size viral RNA species. This nonstructural polypeptide was not detected (at least in the same relative abundancies) in LAC virus-infected cells or in cells infected with reassortants having LAC S RNA. For SSH virus, tryptic peptide analyses of either [³H]leucine- or [³H]arginine-labeled NS_S indicated that it contains unique sequences not present in the SSH nucleocapsid (N) polypeptide (also coded by the S RNA; J. R. Gentsch and D. H. L. Bishop, J. Virol. 28:417-419, 1978). Analyses of SSH virus-infected cell extracts and extracts of cells infected with SSH-LAC reassortants having SSH medium (M)-size RNA species indicated that a nonstructural polypeptide (NS_M; approximate molecular weight, 12×10^3) is coded by the SSH M RNA species. In extracts of LAC virus-infected cells (or cells infected with SSH-LAC reassortants having LAC M RNA), a polypeptide with an electrophoretic mobility slightly faster than that of the SSH NS_M polypeptide was observed (approximate molecular weight, 11×10^3); it has been designated LAC NS_M. The relationships of the NS_M polypeptides to the other M RNA-coded polypeptides (G1 and G2; J. R. Gentsch and D. H. L. Bishop, J. Virol. 30:767-770, 1979) have not been determined. Two additional polypeptides present in both LAC- and SSH-infected cell extracts also appear to be virus induced (one with an approximate molecular weight of 10×10^3 , p10; the other with an approximate molecular weight of 18×10^3 , p18). Whether these polypeptides are virus coded has not been determined.

The Bunyaviridae is a large family of arthropod-borne viruses (1, 2). Members of the *Bunyavirus* genus (Bunyaviridae) have an RNA genome that consists of three single-stranded negative-sense RNA species (5). Their medium-size species, M RNA (apparent size, 1.8×10^6 to 2.3×10^6 daltons), has been shown to code for the two virion glycoproteins, G1 (108×10^3 to 120×10^3 daltons) and G2 (29×10^3 to 41×10^3 daltons) (9, 14). The small-size species, S RNA (apparent size, 0.28×10^6 to 0.50×10^6 daltons), has been shown to code for the nucleocapsid (N) polypeptide (19×10^3 to 25×10^3 daltons) (3, 8, 14). The coding assignment of the largest viral RNA species, L RNA (apparent size, 2.7×10^6 to 3.1×10^6 daltons) (14), has not been determined, although it is assumed to code for a large viral polypeptide (180×10^3 to 200×10^3 daltons) found in small amounts in association with each of the three viral nucleocapsids (2).

The L and M mRNA species of snowshoe hare (SSH) virus are equivalent, or almost equivalent, in size to their corresponding viral

RNA species (3). However, the SSH S mRNA (0.38×10^6 daltons) is smaller than its corresponding viral RNA species (0.5×10^6 daltons) (3). Since the coding capacities of the bunyavirus RNA species do not appear to be saturated by the known structural polypeptides, we initiated a study to identify virus-induced nonstructural polypeptides present in bunyavirus-infected cell extracts. The California group bunyaviruses were selected since California group reassortant viruses are available, having RNA species originating from different parent viruses (6, 7, 10). The results obtained indicate that there are several nonstructural polypeptides induced in bunyavirus-infected cells. One nonstructural polypeptide coded by the S RNA (NS_S) has been shown to differ by tryptic peptide analyses from the viral N polypeptide, indicating that it has a unique amino acid sequence and may be a distinct gene product. Another nonstructural polypeptide (NS_M) appears to be coded by the M RNA; its relationship to the G1 and G2 polypeptides has not been determined.

MATERIALS AND METHODS

Materials. Other than radioactive lysine, all radioisotopes were purchased from New England Nuclear Corp. (Boston, Mass.). Radioactive lysine was purchased from Amersham Corp. (Arlington Heights, Ill.).

Viruses and cells. The origins and passage histories of prototypic SSH and La Crosse (LAC) viruses and of the six SSH-LAC reassortant viruses that were used have been described (6, 7, 10, 11a). Viruses were grown in BHK-21 cells as described previously (6).

Labeling of intracellular viral polypeptides. Monolayers of BHK-21 cells were infected at multiplicities of 5 to 10 PFU per cell, the virus was allowed to adsorb for 30 min at 20°C, and then the infected cell monolayers were incubated at 33°C. At the specified times postinfection, medium was removed, the cells were washed three times with amino acid-free medium, and then labeling medium was added. For the pulse-labeling experiments, the labeling medium consisted of amino acid-free Hams nutrient mixture plus 2.5% (vol/vol) complete Eagle medium containing 20 mM HEPES buffer (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.4) and 100 μ Ci of [3 H]arginine (90 Ci/mmol), [3 H]lysine (19.7 Ci/mmol), [3 H]serine (57 Ci/mmol), [3 H]tyrosine (78.4 Ci/mmol), [3 H]alanine (82.7 Ci/mmol), or [3 H]phenylalanine (100 Ci/mmol) per ml or 25 μ Ci of [3 H]leucine (55 Ci/mmol) per ml or 50 μ Ci of [35 S]methionine (1,315 Ci/mmol) per ml. Cell extracts were resolved by polyacrylamide gel electrophoresis as described previously (10).

Isolation of polypeptides for tryptic peptide analyses. Labeled polypeptide preparations were resolved on 12% polyacrylamide gels with 5-cm loading wells. After electrophoresis a portion of the gel was stained with Coomassie brilliant blue to identify the positions of the polypeptides, and the virus-induced polypeptide bands were excised from the unstained part of the gel and eluted by electrophoresis with an ISCO sample concentrator (Lincoln, Nebr.). The polypeptides were retrieved from the concentrator recovery well and diluted in 25 mM Tris-hydrochloride, pH 7.8, containing 100 μ g of bovine serum albumin per ml and 1 mg of tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin (Millipore Corp., Bedford, Mass.). The polypeptides were then digested overnight at 37°C. Tryptic digests of viral polypeptides were resolved by ion-exchange column chromatography as described by Gentsch and Bishop (8).

RESULTS

Intracellular polypeptide synthesis by SSH and LAC viruses. The synthesis of polypeptides in SSH and LAC virus-infected cells was analyzed to determine whether virus-induced nonstructural polypeptides could be detected in virus-infected cell extracts and whether, for corresponding polypeptides induced by the two parental viruses, differences could be demonstrated. These two viruses were selected since all six genotype combinations of SSH-LAC reas-

sortant viruses are available (6, 7, 10, 11a), and such reassortants could therefore be used to determine which viral RNA species codes for the nonstructural polypeptides.

Shown in Fig. 1 are the polypeptides detected in SSH and LAC virus-infected cell extracts pulse-labeled for 1-h intervals between 13.5 and 18, or 24, h postinfection. By comparison with a mock-infected cell extract, it was evident that, in addition to the virus-induced G1, G2, and N structural polypeptides (11), both LAC and SSH viruses induced polypeptide species that migrated faster than the N polypeptides did. One polypeptide (NS_S; estimated molecular weight, 7.4×10^3), identified in SSH virus-infected cell extracts, was not detected in the same relative abundance in LAC virus-infected cell extracts. A second polypeptide (NS_M; estimated molecular weight, 12×10^3) was detected in SSH virus-infected cells. For LAC virus, a similar polypeptide species was identified that migrated slightly faster (approximate molecular weight, 11×10^3) than the corresponding SSH polypeptide. A third polypeptide found in extracts of both SSH and LAC virus-infected cells (estimated molecular weight, 10×10^3 ; designated p10) has an electrophoretic mobility similar to a polypeptide species found in mock-infected cells. However, the p10, the NS_M, and the NS_S polypeptides were more intensely labeled than the similar-size mock-infected cell polypeptides. A fourth polypeptide species (estimated molecular weight, 18×10^3 ; designated p18), which in this experiment was only clearly seen in the LAC virus-infected cell extracts, migrated faster than the N polypeptide, but slower than NS_M. The p18 polypeptide was occasionally observed in low amounts in SSH virus-infected cell extracts (data not shown).

Coding assignments of SSH and LAC virus nonstructural polypeptides. The question of which viral RNA segment encodes the genetic information for the nonstructural polypeptides was investigated by analyzing the patterns of intracellular polypeptides recovered from cells infected with the various SSH-LAC reassortant viruses compared with those obtained from cells infected with the parental viruses (Fig. 2). As shown previously, the N polypeptides of LAC and SSH viruses have different electrophoretic mobilities and are coded by their respective S RNA species (3, 8). Reassortants with an SSH S RNA direct the synthesis of the 7.4×10^3 -dalton polypeptide (Fig. 2). Since this polypeptide is not found in preparations of purified SSH virus (data not shown), we conclude that it is a nonstructural gene product of the SSH S RNA. Therefore, it is designated NS_S. By similar reasoning, it was concluded that the 12×10^3 -dalton polypeptide induced by SSH virus was encoded

FIG. 1. Time course of LAC and SSH virus polypeptide synthesis in BHK-21 cells. Cultures of BHK-21 cells were infected with virus and labeled with [3 H]leucine for 2-h periods at the times indicated (from 13.5 to 24 h postinfection). The labeled polypeptides were resolved by polyacrylamide gel electrophoresis (10). A mock-infected cell extract (M) was included for comparison. The migration positions of the viral G1, G2, and N polypeptides are indicated. The right-pointing arrow indicates the position of the 12×10^3 -dalton LAC NS_M polypeptide, and the left-pointing arrow indicates the position of the 11×10^3 -dalton SSH NS_M polypeptide. The p10 polypeptide is the labeled species that migrates slightly faster than the SSH or LAC NS_M polypeptide. NS_S is the fastest-migrating labeled polypeptide identified in extracts of SSH-infected cells.

by the SSH M RNA, since it was also observed in extracts of cells infected with reassortants having an SSH M RNA. It is designated NS_M. Likewise, the 11×10^3 -dalton polypeptide is designated LAC NS_M, since it was observed in cells infected with LAC virus and in reassortants having an LAC M RNA. Although the NS_S polypeptide was not identified in extracts of LAC virus-infected cells (or in extracts of cells

infected with reassortants having an LAC S RNA), the results do not exclude the possibility that a similar gene product is coded by the LAC S RNA if, for example, it is metabolically labile.

Since the p18 (or p10) polypeptides identified in extracts of LAC and SSH virus-infected cells exhibit equivalent electrophoretic mobilities, no decision could be reached as to which viral RNA, if any, coded for them.

FIG. 2. Intracellular polypeptides synthesized in LAC, SSH, or LAC-SSH reassortant virus-infected cells. Cultures of BHK-21 cells were either mock infected (M) or infected with LAC, SSH, or LAC-SSH reassortant viruses (6, 7, 10, 11a) and labeled at 16 to 18 h postinfection with [3 H]leucine. The cells were harvested, and extracts were resolved by polyacrylamide gel electrophoresis. The migration positions of the LAC or SSH polypeptides are indicated in the right- or left-hand columns. The reassortant viruses are designated by the origin of their large, medium, and small RNA genotypes (L = LAC, S = SSH). NS_S is identified by asterisks, LAC NS_M is identified by a left-pointing arrow, and SSH NS_M is identified by a right-pointing arrow.

Tryptic peptide analyses of SSH NS_S and N polypeptides. The data indicated that SSH NS_S polypeptide was coded by the viral S RNA segment (Fig. 2). It has been previously demonstrated that SSH N polypeptide is also coded by the viral S RNA (3, 8); therefore, the question was investigated of whether the SSH NS_S polypeptide was a derivative of the viral N polypeptide or a different S RNA gene product. Both SSH N and NS_S polypeptides were isolated from infected cells labeled in the presence of [³H]leucine. The two polypeptides were individually digested with TPCK-trypsin, and the products were resolved by ion-exchange column chromatography (8). The NS_S polypeptide gave a single tryptic peptide (Fig. 3B). The N polypeptide (Fig. 3A) gave approximately 14 labeled tryptic peptides. When both hydrolysates were mixed and cochromatographed (Fig. 3C), it was found that the single labeled NS_S tryptic peptide did not coelute with any of the labeled N tryptic peptides. These results, however, were inconclusive, since if NS_S was a degradation product of N, then a single leucine-labeled NS_S tryptic peptide could represent an amino- or carboxy-terminal portion of a polypeptide derived by cleavage within an N tryptic peptide sequence. For this reason we analyzed the incorporation of various radiolabeled amino acids into N and NS_S to identify a precursor amino acid that, by virtue of its incorporation into an NS_S relative to N, would allow us to undertake a more definitive analysis. By comparison with N (standardized to 1.0), the relative incorporations, of the following radiolabeled amino acids into NS_S were: arginine, 0.39; leucine, 0.36; lysine, <0.05; serine, 0.17; methionine, 0.2; methionalanine, <0.05; tryrosine, <0.03; and tryptophan, 0.11.

Since radiolabeled methionine was incorporated efficiently into the NS_S polypeptide, purified [³⁵S]methionine-labeled N and [³H]methionine-labeled NS_S polypeptides were mixed and digested with TPCK-trypsin, and the digests were resolved by ion-exchange column chromatography. The N polypeptide gave four discrete tryptic peptides in addition to some poorly resolved material in the early fractions of the column. The NS_S polypeptide only gave digestion products that eluted in the void volume of the column (data not shown). These results indicated that the methionine residues of NS_S were probably located in large tryptic peptides that did not bind to the column (data not shown). In an alternate experiment [³H]arginine-labeled NS_S and N polypeptides were recovered. NS_S yielded four [³H]arginine-labeled tryptic peptides (Fig. 4B), and N polypeptide yielded 14 discrete peaks (Fig. 4A). The coelution pattern (Fig. 4C) demonstrated that at least three of the NS_S [³H]arginine-labeled tryptic peptides were

FIG. 3. Tryptic peptide analyses of [³H]leucine-labeled SSH N and NS_S polypeptides. The SSH N and NS_S polypeptides labeled with [³H]leucine were isolated from SSH virus-infected cells as described in the text and digested with TPCK-trypsin, and the peptides were resolved by ion-exchange column chromatography (8). (A) SSH N; (B) SSH NS_S; (C) mixture of SSH N and SSH NS_S. The arrows in (B) and (C) indicate the position of the one NS_S [³H]leucine-labeled tryptic peptide.

unique. Therefore, it was concluded from these studies that the SSH NS_S and N polypeptides were distinct S RNA gene products.

DISCUSSION

The bunyavirus analyses described in this paper provide evidence for at least two non-structural gene products, one coded by the S RNA (NS_S) and the other coded by the M RNA

FIG. 4. Tryptic peptide analyses of [^3H]arginine-labeled SSH N and NS_S polypeptides. The SSH N and NS_S polypeptides labeled with [^3H]arginine were isolated from SSH virus-infected cells as described in the text and digested with TPCK-trypsin, and the peptides were resolved by ion-exchange column chromatography (8). (A) SSH N; (B) SSH NS_S; (C) mixture of SSH N and NS_S. The arrows in (C) indicate the deduced positions of the [^3H]arginine-labeled tryptic peptides identified in (B).

(NS_M). Nonstructural bunyavirus-induced polypeptides have recently been described in analyses of Shark river bunyavirus (Patois serogroup) (13) and for Uukuniemi virus (*Uukuvirus* genus, Bunyaviridae) (12). The p18 and p10 polypeptides that are induced in both SSH and LAC virus-infected cells have electrophoretic mobilities similar to those of host cell polypeptides. Whether they are virus coded polypeptides or

virus-induced host cell polypeptides has not been determined.

To investigate the relationship of the SSH NS_S polypeptide to the N polypeptide, tryptic peptide analyses of [^3H]leucine- and [^3H]arginine-labeled NS_S and N preparations were undertaken. The results indicated that the NS_S is a distinct gene product. Similar studies were not undertaken with the NS_M, p18, or p10 polypeptide, since we have encountered difficulties in recovering sufficient amounts of these species from SSH-infected cell extracts.

The fact that NS_S is not observed as a major labeled species in LAC virus-infected cell extracts is surprising in view of the close serological and sequence relationships of LAC and SSH viruses (2, 4). Recent studies of the S RNA sequences of LAC and SSH viruses have identified two overlapping open reading frames (3a). Such results, although in agreement with the evidence described in this manuscript for the SSH S RNA coding for two distinct gene products, emphasize the anomaly between the LAC and SSH data with reference to NS_S. It is possible that the SSH NS_S polypeptide is a cleavage derivative of a p18 (or another) induced polypeptide, a cleavage that does not occur on a similar precursor formed in LAC virus-infected cells due to sequence differences between the corresponding SSH and LAC S RNA gene products. A resolution of this issue will first require proof that NS_S and N represent the products of the two SSH S RNA open reading frames and that the p18 (or another) polypeptide is the precursor of the NS_S sequence.

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