Sequential translation of nonstructural proteins in cells infected with a Semliki Forest virus mutant

(synchronized initiation/pactamycin/alphaviruses/polyproteins)

BAT-EL LACHMI AND LEEVI KÄÄRINEN

Department of Virology, University of Helsinki, Haartmaninkatu 3, 00290 Helsinki 29, Finland

Communicated by David Baltimore, April 1, 1976

ABSTRACT Four nonstructural proteins with apparent molecular weights of 70,000 (ns-70), 86,000 (ns-86), 78,000 (ns-78), and 60,000 (ns-60) were translated in cells infected with Semliki Forest virus ts-1 mutant and maintained at the restrictive temperature. After synchronization of the initiation of protein synthesis these proteins were synthesized in the above order, suggesting that they are translated as a polyprotein starting from one initiation site. Two short-lived intermediates with apparent molecular weights of 155,000 and 135,000 were regularly detected. The former is presumably the precursor of proteins ns-70 and ns-60 and the latter of ns-78 and ns-60.

Semliki Forest virus is an alphavirus and as such it contains a nucleocapsid surrounded by a lipoprotein envelope. The nucleocapsid consists of a single-stranded RNA, the 42S RNA, with a molecular weight of 4 to 4.5 X 10^6, which is associated with the capsid protein of molecular weight 33,000 (1, 2). The envelope contains three glycoproteins: E-1, E-2, and E-3 with molecular weights of 49,000, 52,000, and 10,000, respectively (3). The structural proteins are synthesized as a polyprotein which has a molecular weight of about 130,000 (4). The order of synthesis of the individual protein has been recently established as capsid protein-E-2 (and E-3)-E-1 (5, 6).

The main template for the synthesis of the structural polyprotein in the infected cells is a virus-specific messenger, the 265 RNA (1.6 X 10^6 daltons) (7), which is identical to the 3' terminal end of the 42S RNA (8). The structural proteins, without carbohydrate, account for about 130,000 daltons of the total coding capacity of the 42S RNA genome, and so leave roughly 300,000 daltons for the nonstructural proteins.

In the middle of the growth cycle, when host cell protein synthesis is effectively inhibited, mainly structural proteins are synthesized (4, 9). This preferential synthesis of the structural proteins has made the identification of the nonstructural proteins, such as the enzyme(s) responsible for RNA replication, unsuccessful so far (1).

We have recently shown that one of our temperature-sensitive mutants of Semliki Forest virus, ts-1, directs the synthesis of two nonstructural proteins with apparent molecular weights of 78,000 (ns-78) and 86,000 (ns-86) (4, 5). These two proteins account for 15-20% of the total labeled proteins compared to about 5% in the cells infected by wild type. We have taken advantage of the ts-1 mutant to answer the following question: Are the ns-78 and ns-86 proteins initiated independently or are they translated sequentially as a polyprotein? Synchronization of the initiation of protein synthesis and preferential labeling of COOH-terminal proteins after treatment with pactamycin, an inhibitor of initiation, were used as tools to answer this question.

MATERIALS AND METHODS

Virus and Cells. The origin and cultivation of the cloned wild type of Semliki Forest virus prototype strain and the temperature-sensitive mutant ts-1 have been described previously (10). The permissive temperature was 27° and the restrictive one, 39°. Secondary special pathogen-free chick embryo fibroblasts and hamster BHK21 cells were grown in plastic dishes as described (4, 10, 11).

Labeling of Virus-Specific Proteins. Confluent monolayers were infected at 50 PFU per cell in the presence of actinomycin D (1 μg/ml) as described (5). The cells were labeled at the indicated times with [35S]methionine (210 Ci/mmol, Amersham) 10-50 μCi per dish in Eagle's minimum essential medium from which methionine was omitted, incubated in the presence of excess of unlabeled methionine to chase out the labeled material, and then collected into 2% sodium dodecyl sulfate as described (4, 5). The only difference was that the samples were boiled for 3 min immediately after processing, to inactive proteases.

Synchronization of Initiation of Protein Synthesis. The medium of infected cultures was replaced at 4 hr 20 min postinfection by the same medium containing 335 mM NaCl (6). At 5 hr postinfection the high salt medium was sucked off and normal medium containing [35S]methionine was added. The cultures were then chased as indicated and the proteins were isolated as above.

Polyacrylamide Gel Electrophoresis. The discontinuous polyacrylamide gel system of Neville (12), modified as described previously (5), was used. The slab gels containing 5% or 7.5% acrylamide in the separating gel were either autoradiographed or fluorographed as described (13, 14). In one of the experiments 7.5% cylindrical gels were used. They were halved longitudinally, dried, cut into 1 mm slices, treated with NCS (Nuclear Chicago solvent) overnight, and counted in a toluene-based scintillation fluid. Molecular weights were determined in 7.5% and 5% slabs using the following markers: iodinated fibroblast surface antigen SF 210 (210,000) (15), 14C-labeled phosphorylase A (98,000), human serum albumin (68,000), and ovalbumin (43,500), and nonlabeled dimethyl-suberimidate and dimethyladipimidate polymers of ovalbumin and glutamate dehydrogenase (16). The known Semliki Forest virus precursor proteins with molecular weights of 130,000, 97,000, 86,000, and 62,000 were used as additional internal markers.

Tryptic Peptide Analysis. Proteins were separated, digested with trypsin, and analyzed by high voltage paper electrophoresis first at pH 6.5 and then at pH 3.5 as described (5).
RESULTS

Proteins synthesized in cells infected with ts-1 mutant

Chick embryo cells infected with ts-1 mutant and maintained at the restrictive temperature of 39° were labeled for 10 min with $^{35}$Smethionine, 5 hr postinfection, i.e., in the middle of the virus growth cycle. The pulse was followed by chase periods of different lengths. At the end of each chase period the cells were collected in 2% sodium dodecyl sulfate, and the proteins were resolved by electrophoresis in a discontinuous polyacrylamide slab gel and visualized by autoradiography (Fig. 1).

The typical pattern of ts-1-induced proteins detected after a 60 min chase is shown in Fig. 1, lane D. The fastest migrating band is the capsid protein, followed by envelope protein E-1, p-62 (precursor of E-2 and E-3) and the nonstructural proteins ns-78 and ns-86. The largest proteins close to the top of the gel are host cell proteins, which are synthesized at about 10% of the level found in uninfected cells (9). One of them is the fibroblast surface antigen SF-210, which has a molecular weight of 210,000 (15). The proteins detected after a 5 min chase are shown in lane A of Fig. 1. In addition to the proteins already described there are two proteins, A and B, which, unlike the large host cell proteins, disappear gradually as the chase is prolonged (Fig. 1). This suggests that A and B may be precursors of virus-specific structural or nonstructural proteins.

Our previous results suggested that the B protein and the polyprotein precursor of the structural proteins, p-130, were identical, due to their comigration on 11% discontinuous polyacrylamide gels (4). When the molecular weights of the proteins A and B were determined on 5% slab gels they were found to be about 155,000 and 135,000, respectively. Comparison of these proteins with p-130 from ts-3-infected cells on both 7.5% and 5% slab gels reveals that there is also a slight difference in the mobilities of p-130 and the B protein (data not shown). Preliminary tryptic peptide analysis of proteins A and B, which were not adequately separated, was carried out. Fig. 2I shows an autoradiograph of the tryptic peptides of proteins A and B after electrophoresis at pH 6.5. Digests of the structural proteins were included as controls. Reelectrophoresis of the specific capsid peptide and a specific envelope E-1 peptide are demonstrated (II and III, respectively). Both peptides are absent from proteins A and B, indicating that capsid and E-1 proteins are not constituents of A and B. This was confirmed by analysis of other peptide bands, including those specific for the envelope protein E-2 (data not shown). These results exclude the possibility that A and B are precursors of the structural proteins. Their disappearance during the chase period (Fig. 1) rather suggests that they are precursors of the nonstructural proteins specified by the virus.

Proteins synthesized after synchronous initiation

To see whether ns-78 and ns-86 are formed from a polyprotein or are possibly primary gene products, initiation of protein synthesis in the ts-1-infected cells was synchronized according to Saborio et al. (17). The NaCl concentration of the medium was increased to 335 mM (6) and incubation was continued for 40 min. Under these conditions initiation of protein synthesis is inhibited but elongation continues until the ribosomes are released (17). The treatment results in 95% inhibition of protein
Materials and Methods. Pulse of 30 sec (A and B), 4 min (C and D), and 10 min (E, F) were followed by 15 (A, C, E) and 60 min (B, D, F) chase. The absence of excess of unlabeled methionine.

synthesis. The NaCl concentration was restored to normal at 5 hr postinfected and, simultaneously, [35S]methionine was added for various periods of time, followed by 15 and 60 min chases in the presence of unlabeled methionine. After restoration of isotonicity, protein synthesis starts immediately and synchronously (17, 18). Also in the case of Semliki Forest virus, viral protein synthesis recovers more quickly than host protein synthesis (data not shown).

The rate of elongation has been estimated to be 200 amino acid residues per minute under these conditions (17, 19, 20). We selected a chase period of 15 min to allow the detection of short-lived proteins such as A and B (see Fig. 1). A longer chase of 60 min was also used to enable us to detect the stable nonstructural and structural proteins.

The results obtained from one such experiment are shown in Fig. 3. Pulse lengths of 30 sec, 4 min, and 10 min were used. The first should result in labeling of only those proteins which, like capsid, are adjacent to initiation sites for protein synthesis. As can be seen in lanes A and B, the only structural protein labeled is the capsid. After the 60 min chase one other protein is clearly seen (lane B), which has a different mobility from both ns-78 and p-62. Its molecular weight has been determined to be about 70,000. We interpret this result as indicating that this protein is the NH2-terminal nonstructural protein, just as capsid is the NH2-terminal structural protein, and have designated it ns-70. After a 15 min chase (lane A) a protein, which comigrates with protein A, is labeled but this label disappears within the next 45 min (lane B), suggesting that this protein is the immediate precursor of the ns-70.

During the 4 min pulse proteins up to 80,000 daltons should be labeled. This is illustrated in lanes C and D (Fig. 3), where capsid and p-62 proteins are heavily labeled, while only traces of E-1 are detected. After the 15 min chase (lane C) protein A and ns-70 are again labeled, but so also is ns-86. The label in protein A disappears after the 60 min chase, and that in ns-70 and ns-86 increases (lane D). This suggests that ns-70 and ns-86 are translated sequentially, and that both are derived from a short-lived precursor, presumably protein A.

After the 10 min pulse, which should allow the labeling of proteins of up to 200,000 daltons, all the structural proteins (capsid, p-62 and E-1) are labeled (lanes E and F). This confirms the result of Clegg who used the same technique (6). One more nonstructural protein, ns-78, has appeared in addition to ns-70 and ns-86. This suggests that these proteins are all synthesized, in sequence, as parts of a large polyprotein. After the 15 min chase (lane E), the B protein is detectable, but the label almost disappears during the 60 min chase (lane F). The appearance of this protein only when labeling reached ns-78 would mean that it is a precursor of this nonstructural protein.

Since the molecular weight of B is about 135,000, it is logical to assume that it contains another nonstructural protein. This fourth nonstructural protein should have a molecular weight of 135,000 minus 78,000. Since the molecular weight of ns-78 was determined in 11% polyacrylamide gels, we re-examined it using 7.5% gels. Under these conditions a value of 72,000 was obtained. This gives the fourth nonstructural protein postulated a molecular weight very similar to that of p-62.

Labeling of COOH-terminal proteins in pactamycin-treated cells

To overcome the difficulty in resolving two overlapping proteins, the dominant structural precursor protein p-62 and the fourth nonstructural protein, we used pactamycin. At low concentrations this drug is a specific inhibitor of initiation of protein synthesis (19). When initiation is inhibited but elongation continues the COOH-terminal regions of proteins, or COOH-terminal proteins are preferentially labeled (19). To achieve this, ts-1-mutant-infected cells were exposed to 1 μM pactamycin for 2, 4, and 8 min and thereafter labeled with [35S]methionine for 15 min in the presence of pactamycin. The pulse was followed by 45 min chase to enable the larger precursor proteins to undergo posttranslational modification.

The results obtained after 4 min pre-treatment with pactamycin are shown in Fig. 4. Compared to the untreated control, the proportion of both capsid (NH2-terminal in the structural polyprotein) and E1 (which is COOH-terminal) is much reduced. The small amounts of capsid synthesized during the pulse are presumably due to incomplete inhibition by the drug. There are two major peaks at the position of ns-78 and p-62. The
latter was the dominant peak when the cells were pretreated for 8 min with pactamycin (not shown). Since only small amounts of structural proteins were synthesized, the peak with apparent molecular weight of 62,000 cannot be the p-62. It is most probably the fourth nonstructural protein, which we have designated ns-60.

**DISCUSSION**

Single-stranded RNA viruses infecting prokaryotic and eukaryotic cells have been shown to have different ways of expressing their genetic information. The genome of the small bacteriophages comprises three cistrons, and their expression to yield different amounts of coat protein is controlled at the level of translation (21). On the other hand, the whole genome of picornaviruses is translated into a single polyprotein with a molecular weight of over 200,000 (22). From this the four structural proteins and nonstructural proteins, including the RNA polymerase, are cleaved (22-25).

The genome of viruses with negative stranded RNA must be transcribed into positive strands before translation can occur. The genome of influenza virus consists of at least seven different pieces of RNA with negative polarity (26, 27), which are transcribed by a virion polymerase (28). The resulting positive strands are translated into proteins, most of which are of their final size (29-31). The continuous negative-stranded genome of paramyxovirus and rabdoviruses is transcribed by the virion RNA polymerase into several short positive strands which together account for the full genetic information in the virus genome. These messenger RNAs are translated to yield the structural and nonstructural proteins in their final size (32, 33). Thus, in the cells infected by the negative-stranded viruses the genetic information is divided among small messenger RNAs, which may be easier to handle by the translational machinery of the eukaryotic cell. In this respect they apparently imitate eukaryotic genes, most of which seem to be expressed by translation of monocistronic messengers (34, 35).

The alphaviruses share features of both the picorna- and negative-strand RNA viruses. They induce a special small messenger, the 265 RNA, for the translation of the structural proteins (3). However, the structural proteins are translated as a polyprotein from which the individual proteins are cleaved (8, 36-38). The synthesis of a specific messenger for structural proteins with the same polarity as the genome is strikingly similar to the situation in some plant-virus-infected cells, in which the coat protein is translated from specific monocistronic messenger RNA (39).

The results presented in this paper suggest that in cells infected with Semliki Forest virus the nonstructural proteins are also translated as a giant polyprotein with a molecular weight of close to 300,000. Since we have not yet found the uncleaved nonstructural polyprotein, the possibility that the large proteins A and B represent different translational units cannot be excluded. The appearance of B at the same time as ns-78 but not ns-86 would mean that the whole A protein (consisting of ns-70 and ns-86) must be translated before initiation of B protein (consisting of ns-78 and ns-60) can take place. Our recent in vitro results with formyl[35S]methionyl-tRNA yield only one labeled peptide after tryptic digestion of the 42S-RNA-directed product. This peptide is different from the corresponding peptide in the 265-RNA-directed product (N. Glanville, M. Ranki, J. Morser, L. Kääriäinen and A. E. Smith, to be published). A similar result has been obtained by Cancereda et al. for the Sindbis virus 42S RNA (40). These results support the conclusion that all the nonstructural proteins of alphaviruses are translated as a polypeptide rather than having two independent initiation sites for their synthesis.

We have found that the same nonstructural proteins are synthesized in the wild-type-infected cells in the same order as in ts-1-infected cells, but in much smaller quantities. This must mean that the processing of the nonstructural proteins is not affected by the mutation, but rather that the difference between ts-1- and wild-type-infected cells is quantitative.

We thank Drs. A. Vaheri and J. Keski-Oja for the labeled and cross-linked molecular weight markers. We thank Miss Riitta Saarinen and Miss Mirja Salonen for excellent technical assistance. This work was supported by grants from the Sigrid Juselius Foundation and the Finnish Academy. B.L. is a recipient of a pre-doctoral European Molecular Biology Organization Fellowship. Pactamycin was a very generous gift from Dr. R. Taber from the Swiss Institute for Experimental Cancer Research, Lausanne, Switzerland; and actinomycin D was a gift from Merck, Sharp and Dohme.