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Proteolysis of Noncapsid Protein 2 of Type 3 Poliovirus at the Restrictive Temperature: Breakdown of Noncapsid Protein 2 Correlates with Loss of RNA Synthesis†

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Inhibition of RNA synthesis of the LH strain of type 3 poliovirus at the restrictive temperature occurs concurrently with the breakdown of noncapsid virus-specific peptide 2. This finding, along with other reported data (B. D. Korant, *In Proteases and Biological Control*, p. 621-644, 1975), suggests that noncapsid virus-specific peptide 2 is a component of the viral replicase.

A combination of genetic data (5) and biochemical mapping with pactamycin (11, 12) shows the gene coding for poliovirus replicase to be located at the 3′ end of the viral chromosome. Experiments performed to identify peptides that comprise the poliovirus replicase have provided varied results (3, 4, 7, 9, 14). Partially purified preparations of the viral replication complex contain capsid as well as several noncapsid virus-specific peptides (NCVP). Genes coding for capsid peptides are located at the 5′ end of the genome (10-12), and it is unlikely that capsid proteins are part of the viral enzyme. The best evidence to date indicates that NCVP4 and NCVP2 are constituents of poliovirus replicase (6, 7). Purified enzyme preparations contain predominantly NCVP4 (7). Viral replicase undergoes metabolic turnover during the late stages of infection, a time when NCVP2 is cleaved to NCVP4 and NCVP7 (6). Inhibition of cleavage with protease inhibitors partially suppresses loss of RNA synthesizing activity in vivo and concomitantly retards cleavage of NCVP2 (6). It is likely that NCVP2 and NCVP4 are subunits of viral replicase and supply different functions. The present note shows that NCVP2 of strain LH of type 3 poliovirus is degraded at the restrictive temperature concurrent with blockage of viral RNA synthesis. This finding also suggests that NCVP2 is essential for synthesis of viral RNA in vivo.

Leon (ATCC VR62) and LH (ATCC VR63) strains of type 3 poliovirus were obtained from the American Type Culture Collection. Leon virus was isolated in 1937 from the spinal cord of a patient in California. The virus was grown in primary monkey kidney cells and LL-CMK$_2$ cells prior to our passage in HeLa cells. It is a virulent prototype that causes paralysis in monkeys. LH virus was selected from Leon virus in 1951 after 24 passages in primary monkey kidney cells. The virus has been routinely maintained in mouse cord and only irregularly produces paralysis in monkeys. Both viruses were plaque purified with HeLa O (OHIO) cells obtained from Flow Laboratories, and stocks were grown in roller vessels as described (13). Virus yields over one growth cycle at 35°C were 1,500 PFU/cell and at 39.7°C (restrictive temperature) were reduced 98.5% with Leon virus and 99.9% with LH virus. Kinetic studies measuring viral protein and RNA synthesis were done as described (1, 13), and polyacrylamide gel electrophoresis was performed with 7.5% sodium dodecyl sulfate gels as described (8, 13).

The production of viral proteins and RNA at the permissive temperature and following shift-up to the restrictive temperature 2 h after infection is shown in Fig. 1. At the permissive temperature, viral proteins were produced linearly up to about 2.5 h after infection. At the restrictive temperature, the synthesis of Leon virus-specific proteins was similar to the control up to 3.5 h after infection, at which time net synthesis declined. Synthesis of LH virus-specific proteins is blocked about 30 min sooner at 39.7°C compared with Leon virus. Net synthesis of LH virus-specific RNA is completely blocked within 30 min after shift-up to the restrictive temperature, whereas Leon virus-specific RNA is synthesized for 75 min following shift-up to 39.7°C. To determine whether synthesis and degradation of virus-specific macromolecules occurred simultaneously at the restrictive temperature, the stabilities of viral proteins and RNA were also examined (Fig. 2). After incubation of infected cultures for 30 min with [^{35}S]methionine or [^{14}C]uridine, 150 mM excess NaCl (to block

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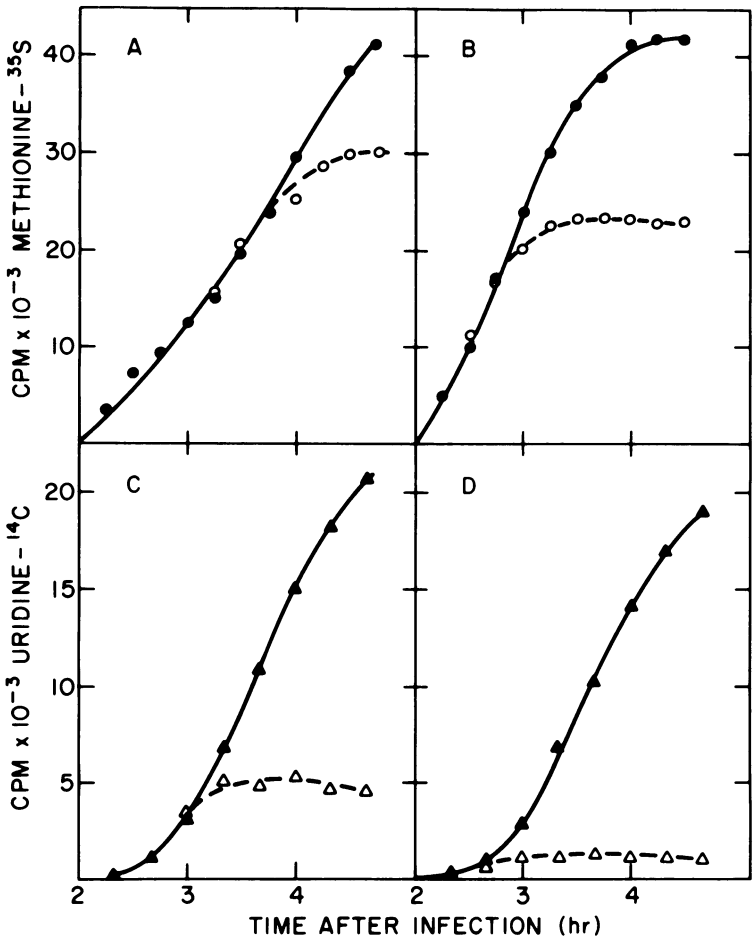

FIG. 1. *Kinetics of macromolecular synthesis. HeLa cells were infected on monolayer with 150 PFU/cell. After 1 h, cells were removed with trypsin and resuspended to 4 × 10⁶ cells/ml with labeling medium as described (1, 13). After an additional 1 h in suspension at 35°C, 4 μCi of [³⁵S]methionine per ml (327 Ci/mM; New England Nuclear) was added and half of each culture was placed at 39.7°C. At intervals 0.25-ml duplicate samples were analyzed for acid-insoluble radioactivity (1, 13). To study viral RNA synthesis, a similar experimental design was used, but 5 μg of actinomycin D per ml was added 1 h before addition of 0.3 μCi of [¹⁴C]uridine per ml (454 mCi/mM; New England Nuclear). (A) Leon protein, (B) LH protein, (C) Leon RNA, (D) LH RNA. Symbols: cultures incubated at 35°C throughout the study (●, ▲); cultures placed at 39.7°C 2 h after infection* (○, △).

further protein synthesis; 10) or 3 mM guanidine (to block further RNA synthesis; 2) was added to the infected cultures; one-half of each culture was incubated at 39.7°C. After 2 h there was only about 10% loss of protein with both viruses at 35 and 39.7°C. Because guanidine does not immediately inhibit RNA synthesis there was continued synthesis of viral RNA for about 30 min after addition of the inhibitor to Leon virus-infected cultures (Fig. 2C). After complete blockage of RNA synthesis, only about 10% of Leon virus RNA was degraded. The restrictive temperature and guanidine together immediately inhibit synthesis of LH virus RNA, but there is only a 20% breakdown of RNA over 2 h, and the final rate of breakdown appears to parallel degradation at 35°C (Fig. 2D). The limited breakdown of viral protein and RNA at the restrictive temperature cannot completely account for the rapid blockage of synthesis noted in Fig. 1. Sucrose density gradient analysis of viral RNA was also performed, and no qualitative differences were found in the distribution of single-stranded RNA, double-stranded RNA, or replicative intermediate at 35 or 39.7°C (data not shown).

From the above it appears that viral RNA synthesis is rapidly blocked at the restrictive temperature. It is possible that selective degra-

dation of one or of several viral polypeptides could be responsible for the observed loss of RNA synthesis. Such selective degradation would not be detected by the kinetic experiments if the polypeptide(s) involved comprised only a small portion of the total viral protein. Also, defective cleavage of a precursor polypeptide could be responsible for the data in Fig. 1. Electrophoretic analysis of viral peptides was undertaken to determine whether selective degradation or altered cleavage occurred at the restrictive temperature.

Viral proteins were pulse-labeled for 30 min at 35 or 39.7°C and chased for 60 min at 35 or 39.7°C. Electropherograms were obtained and plotted as percent total counts for accurate comparison. In all cases at least 75,000 total cpm were loaded per gel, and virtually all counts were recovered. Peptides of Leon virus, after a chase at 35 or 39.7°C, were identical to those found before the chase (Fig. 3). The slight mismatching of protein peaks is attributable to stretching and cutting variations of the gels. With LH virus the cleavage of NCVP1a to VP1 and VP3 was more apparent during the chase at the permissive temperature than it was with Leon virus (Fig. 4). NCVP2 appears to be very stable during the chase at 35°C, but at 39.7°C 70% of NCVP2 disappears during a 60-min chase without a corresponding increase in its normal cleavage prod-

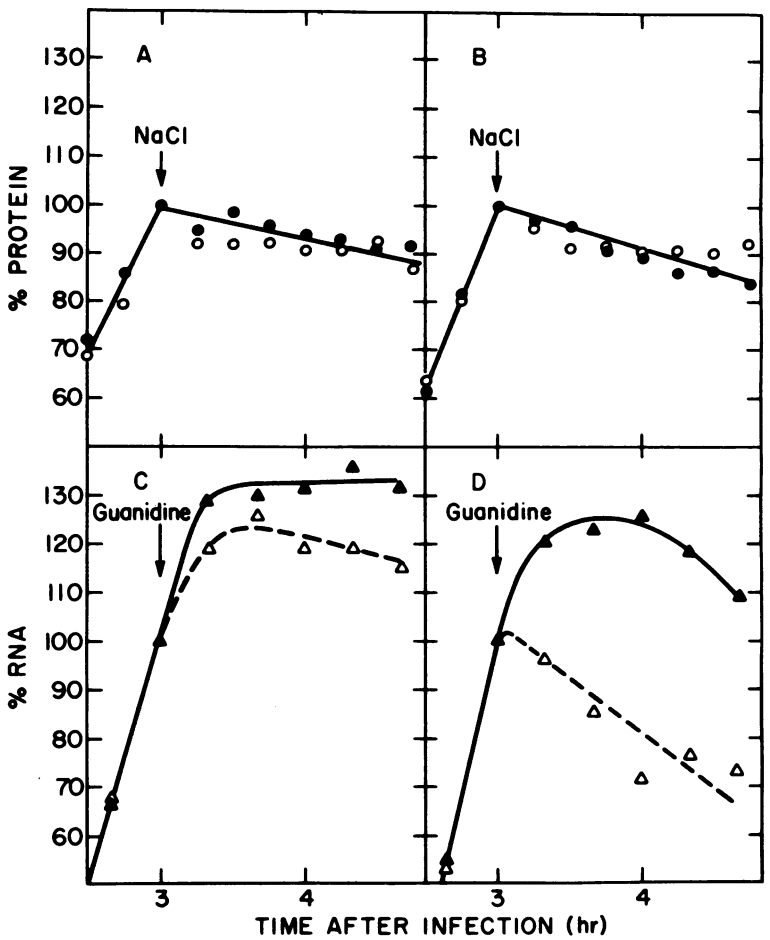

FIG. 2. *Degradation of viral macromolecules. Cultures were infected as in Fig. 1, and [^{35}S]methionine or [^{14}C]uridine was added 2.5 h after infection. At 3 h of infection, 150 mM NaCl was added to block protein synthesis in cultures incubated with [^{35}S]methionine. Guanidine (3 mM) was added to cultures exposed to [^{14}C]uridine to block further RNA sythesis. Cultures were divided equally and held at 35 or 39.7°C. Incorporated [^{35}S]methionine and [^{14}C]uridine were measured at intervals (1, 13). Counts at 3 h of infection correspond to 100%. (A) Leon protein, (B) LH protein, (C) Leon RNA, (D) LH RNA. Symbols: cultures incubated at 35°C (●, ▲) throughout and at 39.7°C (○, △) 3 h after infection.*

ucts, NCVP4 and NCVP7. The restrictive temperature appears to induce degradation of NCVP2 with LH virus.

Kinetic studies were also performed to determine whether protease inhibitors could block the turn-off of RNA synthesis at 39.7°C with LH virus. Iodoacetamide, $ZnCl_2$, phenylmethylsulfonyl-fluoride, L-1-tosylamide-2-phenylchloromethyl ketone, and *N*-α-*p*-tosyl-L-lysine chloromethyl ketone at concentrations ranging from 0.1 mM to 5 mM had no effect on reversing blockage of viral RNA synthesis (data not shown). Because results were negative, polyacrylamide gel electrophoresis studies were not undertaken.

The data indicate that RNA synthesis of Leon and LH poliovirus strains is inhibited at 39.7°C prior to overall inhibition of protein synthesis. Even though RNA synthesis of both Leon and LH viruses is severely inhibited at the restrictive temperature, only NCVP2 of LH virus is degraded. Conformational changes in NCVP2 probably occur with both viruses at the restrictive temperature but only expose the LH peptide

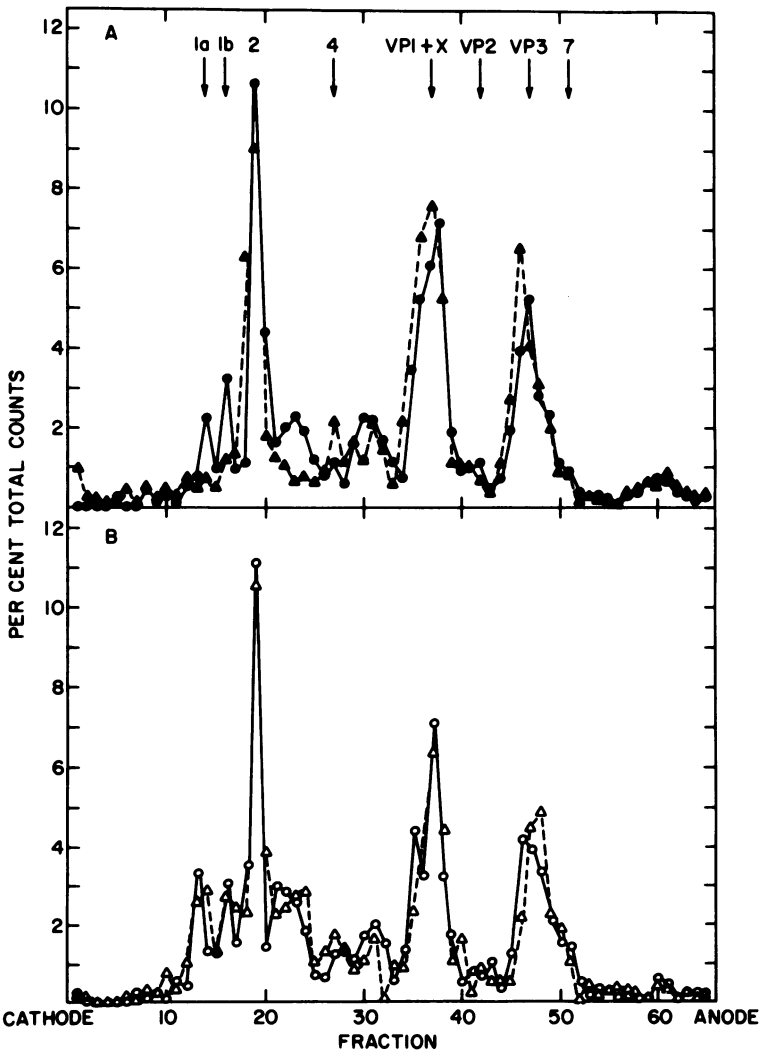

FIG. 3. *Electropherograms of intracellular peptides of Leon virus. Cells were infected as in Fig. 1 and pulse-labeled with 4 μCi of [^{35}S]methionine between 2.5 and 3 h after infection. NaCl (150 mM) was added to block further protein synthesis. Half the cultures were chased at 35°C, and the remaining portions were chased at 39.7°C for 1 h. Viral peptides were extracted and examined with 7.5% sodium dodecyl sulfate-polyacrylamide gels as described (8, 13). (A) Pulsed at 35°C (●) and chased at 35°C (▲). (B) Pulsed at 35°C (○) and chased at 39.7°C (△).*

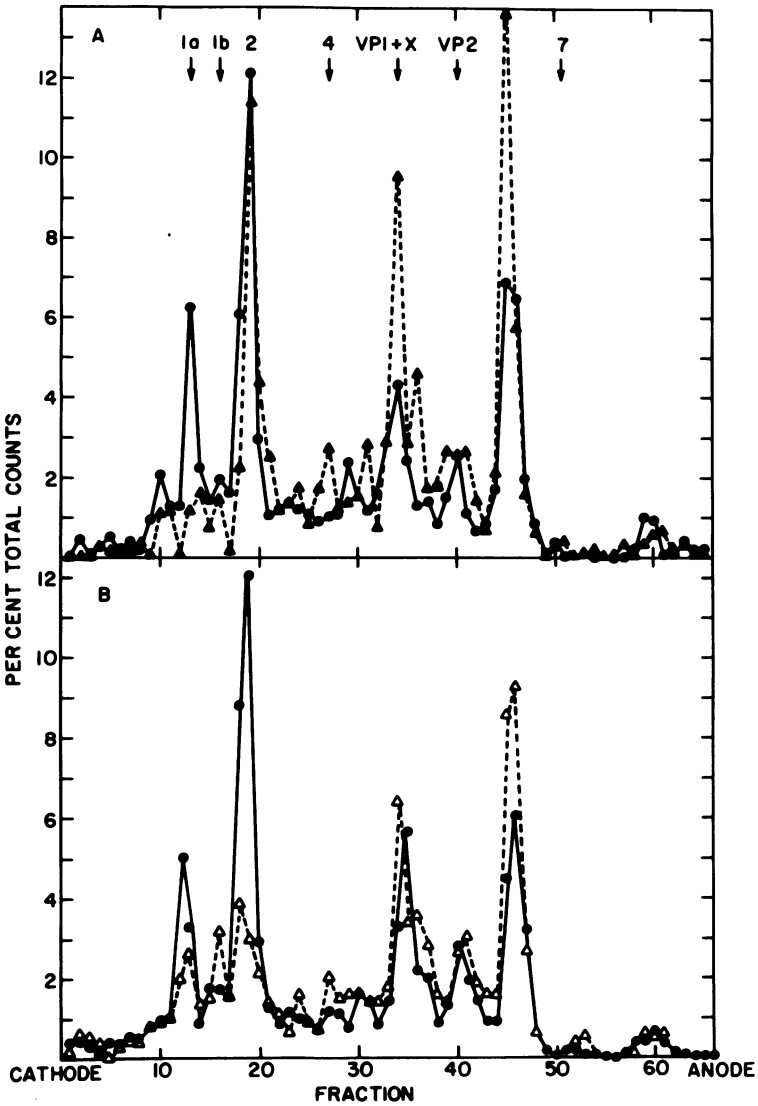

FIG. 4. *Electropherograms of intracellular peptides of LH virus. The study was performed exactly as in Fig. 3. (A) Pulsed at 35°C (●) and chased at 35°C (▲). (B) Pulsed at 35°C (●) and chased at 39.7°C (△).*

to proteases either because of degree of conformational change or release of NCVP2 from a protective structure such as a membrane. There is no generalized breakdown of viral proteins.

Taken together, the data in this report support previous studies indicating that NCVP2 is probably a component of the viral replicase. This is not necessarily contradictory to studies showing that NCVP4, a cleavage product of NCVP2, has replicase activity. If NCVP2 were degraded, NCVP4 would not be formed to replenish that lost by natural turnover of enzyme. Examination of Fig. 4 shows only an indistinct peak of radioactivity in the region of NCVP4. Possibly each polypeptide is involved in a distinct phase of RNA synthesis. The existence of two replicases, one for single-stranded RNA synthesis and one for double-stranded RNA synthesis, has been proposed (5).

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