Isolation and Preliminary Characterization of Temperature-Sensitive Mutants of Poliovirus Type 1

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We isolated six temperature-sensitive mutants of poliovirus type 1 (Mahoney) by hydroxylamine mutagenesis and replica plating at 31, 33 (permissive), and 39°C (restrictive). One of these mutants, designated *tsB9*, was chosen for more detailed examination. *tsB9* accumulated 25% of the wild-type amount of virus-specific RNA at the restrictive temperature. We found that *tsB9* was not able to synthesize mature, 35S single-stranded RNA at the restrictive temperature. In spite of the absence of significant RNA synthesis, *tsB9* retained the ability to inhibit host protein synthesis during infection at 39°C at about the same rate as wild-type virus.

The poliovirus type 1 (PV1) genome has been completely sequenced (12, 17a) and, in agreement with earlier data, appears to code for a polyprotein of 247,000 daltons. This large precursor undergoes a series of post-translational proteolytic cleavages to yield a variety of viral products. Many of these proteins have been located within the RNA sequence (12, 17a). The functions of the coat proteins (5' region of the genome) are known, and the locations of the putative replicase (p63) and VPg have been defined. However, mutants have been characterized only for the coat protein region (defective-interfering particles; 2). Cooper (4) has reported extensive genetic mapping of poliovirus (PV), using a variety of temperature-sensitive (*ts*) and other mutants, but has demonstrated an extremely low recombination frequency. Biochemical characterization of these mutants has been hampered in some cases by the relatively high levels of expression ("leakiness") at restrictive temperatures. Other mutants of PV, namely, deletion mutants (defective interfering particles; 3) and guanidine-resistant and guanidine-dependent mutants (13), have been isolated. Defective interfering particles appear to be deletions restricted to the coat protein region (5' end) of the genome (2, 15, 17). Genetic analysis of other picornaviruses has resulted in the identification of maturation mutants of encephalomyocarditis virus (18) and of RNA-negative mutants of foot and mouth disease virus (14). Thus, a clear biochemical and genetic assignment for each PV protein has not yet been made.

Replication of PV RNA involves the synthesis of both minus and plus viral molecules. These

processes have been shown to involve at least two viral proteins (p63 and probably VPg; 5, 7-9, 16) and a host protein (6). We have been interested in isolating *ts* mutants of PV1 that can be shown to be defective in some stage of viral RNA replication. We were particularly interested in mutants whose level of expression of the function at restrictive temperatures would be low enough to allow biochemical characterization. We report here the isolation of *ts* mutants of PV1 (Mahoney) that display a relatively nonleaky phenotype. One of these mutants, designated *tsB9*, has been characterized in some detail. *tsB9* displays an RNA-negative phenotype, in that only 25% of the wild-type level of RNA is synthesized at the restrictive temperature. We present preliminary evidence which suggests that this mutant may be defective in an early stage of viral RNA replication, leading to the synthesis of plus strand.

HeLa S3 cells were grown in suspension culture in Joklik modified minimal essential medium (MEM) containing 5% horse serum, as described by Baltimore et al. (1). PV1 (Mahoney) was used as the wild-type parent in these experiments and was grown in HeLa cell cultures as described elsewhere (1). Plaque assays of PV at the indicated temperatures were carried out essentially as described by Cooper (4) and modified by Cole et al. (3).

Mutagenesis of PV1 was carried out by mixing 1 volume of stock virus in MEM with 4 volumes of freshly prepared 2.0 M hydroxylamine-H₂SO₄ in 4 M NaCl-0.8 M Tris (pH 7.4) at room temperature. At various times mutagenesis was arrested by diluting a portion of the mixture 1:100 with MEM containing 10% acetone. Survival curves of PV1 were determined by plaque

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assay at 37°C. Mutagenized PV1 was plated on HeLa cells in agar overlay at 33°C. Randomly selected plaques were transferred to replica plates and incubated at 33 or 39°C. Mutants which showed no growth at 39°C were used to prepare virus stocks. The efficiency of plating (EOP) at 39 versus 33°C was determined, and mutants with an EOP of less than 10^{-2} were retained for further use.

Table 1 lists several *ts* mutants selected during three separate rounds of mutagenesis and testing. Mutants with an EOP of less than 10^{-2} occurred with a frequency of about 1 in 200 randomly selected plaques. *ts10* was retained, even though it has a somewhat higher EOP than desired. *ts10* has a different RNA-negative phenotype than *tsB9* (K. Storm and M. Hewlett, unpublished data). Although some mutant selection was carried out in agar at 31°C, preparation of stocks in suspension culture was carried out at 33°C, since our suspension cultures of HeLa S3 cells did not grow well at the lower temperature. We found that the mutant phenotype is only expressed at temperatures above 37°C, as reported for mutants selected by Cooper (4). RNA phenotypes were determined by observation of total virus-specific RNA synthesis at the two temperatures. The mutant designated *tsB9* was selected for further work.

HeLa cells were infected with either wild-type or mutant PV1 at a multiplicity of infection of 20 and treated with actinomycin D (5 µg/ml), and [³H]uridine (5 to 10 µCi/ml) was added to radiolabel virus-specific RNA. Accumulation of RNA was measured as ³H incorporation into material insoluble in cold 5% trichloroacetic acid (TCA). Infections were carried out at 33°C as the permissive temperature and 39°C as the restrictive temperature. Other experiments involved shift-up or -down between these two temperatures.

Figure 1 shows the data resulting from infection of HeLa cells with wild-type virus or *tsB9* at 33 or 39°C. Accumulation of virus-specific RNA was reduced to about 25% of the wild-type level when *tsB9* was used at 39°C. Since some viral RNA was being synthesized by this mutant at

FIG. 1. Viral RNA synthesis in wild-type- and *tsB9*-infected HeLa cells. Cells were infected with either wild-type or mutant virus at a multiplicity of infection of 20 and placed at either 33 or 39°C. The cultures were treated with actinomycin D (5 µg/ml), and [³H]uridine (5 µCi/ml) was added. Samples were taken at the indicated times, and total radioactivity was measured as TCA-insoluble material.

TABLE 1. *ts* Mutants of PV1

Virus	EOP ^a	Phenotype
Wild type	1.0	RNA +
<i>tsB9</i>	1.6×10^{-4}	RNA -
<i>ts2</i>	4.0×10^{-4}	RNA -
<i>ts8</i>	1.0×10^{-3}	RNA +
<i>ts10</i>	3.5×10^{-2}	RNA -
<i>tsD</i>	5.0×10^{-5}	ND ^b
<i>tsE</i>	2.0×10^{-3}	ND

^a PFU/ml at 39°C/PFU per ml at 33°C.

^b ND, Not determined.

the restrictive temperature, we chose to examine the nature of the species produced.

HeLa cells, harvested at various times after infection, were lysed with cold RSB (RSB = 0.1 M NaCl-0.01 M Tris, pH 7.4) containing 1% Nonidet P-40. Cytoplasmic extracts were obtained after the removal of nuclei and debris by low-speed centrifugation. These extracts were

made 1% with sodium dodecyl sulfate, 0.01 M with EDTA, and 0.4 M with NaCH_3COO . Total nucleic acid was recovered after extraction with phenol-chloroform-isoamyl alcohol (50:48:2), as described by Spector and Baltimore (19) and ethanol precipitation at -20°C overnight. Viral RNA species were separated by electrophoresis through 1% agarose gels (10). The position of radiolabeled RNA species was determined after electrophoresis by fractionation and analysis in a Packard liquid scintillation spectrometer.

When the RNA species produced during infection by either wild-type virus or *tsB9* at the permissive temperature were examined by agarose gel electrophoresis, no difference was seen. At the restrictive temperature, the mutant virus synthesized the same species in the same relative amounts as did the wild type. However, at the restrictive temperature, *tsB9* synthesized little 35S RNA relative to a slower-migrating species (Fig. 2). The small amount of 35S RNA in the case of *tsB9* was probably due to sampling technique, since we observed that when the *tsB9* infection was halted quickly by mixing a sample of the cells with an equal volume of frozen medium, essentially no 35S RNA was seen.

tsB9 virus-specific RNA migrated as an apparently homogeneous species on agarose gels (Fig. 2). Total intracellular RNA from wild-type- or *tsB9*-infected cells (at 39°C) was precipitated in 2 M LiCl to separate single-stranded from double-stranded (ds) RNA (19). The soluble and insoluble RNA was separated by gel electrophoresis. Figure 3 shows the pattern for wild-type RNA, with 35S RNA and a small amount of replicative-intermediate RNA in the insoluble fraction and ds RNA in the soluble fraction [(A) and (B), note 10-fold increase in scale in (B)]. However, *tsB9* RNA produced at the restrictive temperature consisted of ds RNA (Fig. 3C) in the soluble fraction, whereas the insoluble fraction (Fig. 3D) contained heterogeneous RNA of a size ranging from full genome length down and a relatively homogeneous peak migrating at about the position expected for fully ds RNA. Replicative-intermediate RNA from wild-type infections migrated as a heterogeneous species and was insoluble in 2 M LiCl. Thus, the *tsB9* structure had properties of both replicative-intermediate RNA and fully ds viral RNA.

Cells were infected with virus and treated with actinomycin D at 39°C , and at either 90 or 180 min, portions of the culture were transferred to 33°C . Thirty minutes after the down-shift, [^3H]uridine was added, and the infections were assayed for incorporation into TCA-insoluble material (Fig. 4). Down-shift of wild-type-infected cells resulted in essentially the same level of synthesis of viral RNA. Down-shift of *tsB9* at 90 min resulted in a significant increase in viral

FIG. 2. Agarose gel electrophoresis profile of intracellular RNAs from infections at the restrictive temperature. Infections were carried out at 39°C as described in the legend to Fig. 2, except that the infection was halted rapidly by mixing 1 volume of culture with 1 volume of crushed frozen media kept at -20°C . Viral nucleic acids were separated by electrophoresis through 1% agarose gels as described (10). Total ^3H counts per minute were determined in each 1-mm gel slice.

RNA synthesis. However, down-shift at 180 min yielded no increase in synthesis. When the converse experiment was performed and infected cells were shifted to 39°C at various times, immediate cessation of RNA synthesis in *tsB9*-infected cells was observed (data not shown). These data suggest that the defect in these cells is reversible and acts at an early time during the infectious cycle.

HeLa cells were infected with either wild-type or *ts* virus in leucine-free MEM containing dialyzed serum. At various times after infection, portions of the culture were removed and pulsed with [^3H]leucine ($10 \mu\text{Ci/ml}$) for 10 min. The cell suspension was treated with an equal volume of 0.5 N NaOH to hydrolyze amino acyl-tRNAs and then precipitated in the presence of an excess of Casamino Acids in 10% TCA. Total radioactivity incorporated into protein during the pulse was then measured by liquid scintillation. Under these conditions, we found that the rate of inhibition of host protein synthesis is the same after infection with wild-type or *tsB9* virus at 39°C . These results suggest that protein syn-

FIG. 3. Agarose gel profile of LiCl-soluble and -insoluble viral RNAs from cells infected at the restrictive temperature with either wild-type virus or *tsB9*. Infections were carried out at 39°C, as described in the legends to Fig 1 and 2. Total intracellular RNA was separated into material soluble (A) and (C) and insoluble (B) and (D) in 2 M LiCl as described by Radloff (18). Symbols: ●, wild-type-infected cells; ○ cells infected with *tsB9*.

thesis inhibition does not require continuous viral RNA synthesis or, specifically, synthesis of viral mRNA (35S RNA) within the infected cells. Although this conclusion had been reached in earlier studies (10, 11), our results were obtained without the intervention of either drugs or UV inactivation of virus.

The availability of mutants of PV for biochemical analysis should be of significant value in examining the genetics and specific functions of the picornavirus proteins. *ts* mutants which are relatively "non-leaky" were isolated, and we have presented a preliminary characterization of one of these, designated *tsB9*. The set of mutants includes others which are apparently RNA negative (e.g., *ts10*), and our present efforts focus on the characterization of this subset. Mutants were produced by hydroxylamine treatment of intact virus. This agent was chosen to avoid the possibility of double mutants. The set of mutants we produced includes only individ-

uals with EOP at 39 versus 33°C of less than 10^{-2} , except that *ts10* is somewhat higher than this.

It is possible that a defect in capsid maturation and assembly might lead to the premature degradation of mature viral RNA within the infected cells. In fact, we observed that capsid assembly is indeed defective in this mutant (unpublished data). However, the mere lack of capsid precursors cannot explain the observed RNA events in *tsB9*. When cells are infected with purified defective interfering (1) particles of PV1, no capsid protein precursors are synthesized (2). In this situation, viral 35S RNA is produced within the cell in apparently normal amounts and in relatively intact form. Therefore, the lack of normal RNA synthesis by *tsB9* at the restrictive temperature is not solely due to aberrant capsid assembly.

We propose that *tsB9* produces an RNA structure at high temperature consistent with a blocked or inhibited replicative intermediate.

FIG. 4. Effect of temperature down-shift on the synthesis of RNA in wild-type- and *tsB9*-infected HeLa cells. Cells were infected, placed at 39°C, and treated with actinomycin D (5 µg/ml). Cells were left at the restrictive temperature (circles), downshifted to 33°C at 90 min postinfection (squares), or at 180 min postinfection (triangles). Thirty minutes after the temperature shift, [³H]uridine (5 µCi/ml) was added, and samples were taken as indicated for the determination of TCA-insoluble radioactivity. The closed symbols are data from the wild-type infection, and the open symbols are data from the *tsB9* infection.

This would be supported by our finding that one structure which accumulates at the high temperature is homogeneous in size (comigrating with ds RNA) and yet retains sufficient single-stranded character to be insoluble in 2 M LiCl. The data shown in Fig. 3 indicate that this RNA species represents about 13% of the total RNA synthesized at 39°C by *tsB9*. In other experiments the amount of this species was even greater. The block is such that synthesis of RNA resumes upon lowering the temperature. In collaboration with J. Flanagan (University of Florida, Gainesville), we examined the temperature sensitivity of p63 purified from *tsB9*-infected cells (J. Flanagan and M. Hewlett, unpublished data). The viral protein p63 was able to synthesize viral RNA in vitro when provided with 35S RNA as template and oligodeoxyuridylic acid as primer (20). When p63 was prepared from *tsB9*-infected cells, it displayed only two or threefold more temperature instability than the corresponding wild-type protein. We do not consider this an overwhelming effect, and therefore it is not clear whether *tsB9* is, in fact, a p63 mutant. Other proteins are involved in PV RNA synthesis, including a host factor (6). We are currently investigating other RNA-negative *tsB9* mutants in this same manner.

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