Antinoro, Norla Marie Walser

THE BIOCHEMICAL AND PHYSIOLOGICAL CHARACTERIZATION OF POLIOVIRUS MUTANTS

The University of Arizona PH.D. 1984

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THE BIOCHEMICAL AND PHYSIOLOGICAL CHARACTERIZATION OF POLIOVIRUS MUTANTS

by

Norla Marie Walser Antinoro

A Dissertation Submitted to the Faculty of the

DEPARTMENT OF MOLECULAR AND CELLULAR BIOLOGY

In Partial Fulfillment of the Requirements
For the Degree of

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In the Graduate College

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THE UNIVERSITY OF ARIZONA
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As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Norla Marie Walser Antinoro

entitled The Biochemical and Physiological Characterization of Poliovirus Mutants

and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Philosophy.

Edward Ehrenfeld	July 13, 84
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Don P. Bourgeois	Date 7/17/84

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

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Edward Ehrenfeld	July 13, 84
Dissertation Director	Date

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SIGNED: Norla Marie Walser Contreras

For Jarrod, who waited;
For Frank, who knows where the commas go;
For my Father, who loaned me the typewriter.

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ABSTRACT

Poliovirus is a small, structurally simple virus that can serve as a powerful model system for the elucidation of the basic processes involved in the genetic control of macromolecular synthesis. The physical and biochemical characterization of temperature-sensitive and drug-resistant mutants of this virus can provide insight into the normal sequence of events during the replication and assembly of the wild-type (wt) virus.

The specific interference of the infecting virus with the major pathways of macromolecular synthesis in the host cell offers a possible inroad to the exploration of the relevant control systems.

This research is divided into two major segments: (1) a temperature-sensitive mutant of poliovirus type 1, tsB9, and a guanidine-resistant mutant, g^rH, were characterized physiologically; (2) a physiological screening procedure that quickly and efficiently reveals the phenotype of a large number of poliovirus mutants in a short period of time was developed and validated. Two guanidine-resistant and twelve temperature-sensitive mutants of poliovirus type 1, Mahoney, were generated and compared with wt, defective-interfering particles, g^rH, and tsB9 using the screening procedure herein developed.

The temperature-sensitive mutant, tsB9, appears to be a structural protein mutant bearing a related defect in ribonucleic acid synthesis at the restrictive temperature. The mutant $g^{r}H$ was found to differ from the wt virus only in the guanidine resistance of its growth and ribonucleic acid synthesis, although a detailed electrophoretic analysis of its proteins was not done.

Among the newly isolated mutants, strains were found with defects in each of the major viral functions except one. No mutant was found to be defective in the ability to inhibit host-cell protein synthesis.

The screening procedure developed met all of the criteria set for it mentioned above. It has the potential of being adapted to many virus-cell systems for the rapid determination of mutant phenotype.

INTRODUCTION

Viruses have long been known as the etiologic agents of many diseases of man and animals. Their impact is both medical and economic. The effective treatment and prevention of viral diseases depends upon a thorough understanding of these viruses in all their aspects.

Virology has evolved beyond the consideration of the clinical aspects of viruses. Viruses have proved to be effective vehicles for the study of macromolecular and cellular processes. Much of our current understanding of the structures, synthesis, and interactions of proteins and nucleic acids, and of gene expression, has been derived from studies initiated with viruses (121). Since viruses are an excellent source of single molecular species of nucleic acids and proteins, many significant advances in understanding macromolecular function and structure have developed from the study of viral components (88). Viruses offer the opportunity for the exploration of plant and animal cells with the tools of molecular biology (88).

Viruses are diverse in size, function, organization, and strategy. They affect the host cell in a variety of specific ways. As a result, viruses can be used to manipulate cellular events at a specific level and to delineate the strategies available to the cell.

The use of viral interference as a means of exploring the functions of the cell may result in the full understanding of both normal and disordered cellular processes. Such an understanding lays the foundation for the development of preventative or, at least, ameliorative treatment.

Picornaviruses are responsible for a number of human and animal diseases. Poliovirus is a typical member of the picornavirus group. Other picornaviruses are also significant pathogens. Rhinovirus is responsible for the common cold. Picorna enteroviruses attack each of the species of major mammalian meat-producing animals as does apthovirus. An effective treatment for picornavirus-induced diseases has not yet been developed.

The major health threat posed by poliovirus in this country was brought under control primarily through the use of effective vaccines. The vaccine strains of poliovirus are temperature-sensitive strains that contain multiple mutations. The mechanism by which immunity is conferred is not fully understood, nor is the mechanism by which these vaccine strains occasionally produce clinically active cases of poliomyelitis. It would be of great value to understand the way in which these viruses function. The development of effective vaccines against picornaviruses other than polio is of obvious benefit and may depend upon more complete understanding of the host-virus interaction of picornavirus mutants.

The molecular biology of all picornaviruses is similar; therefore, knowledge gained with respect to one picornavirus offers a great deal of insight into the functions of the entire group. In addition to the potential clinical benefits to be accrued from the study of poliovirus, it offers an excellent model system for the study of many viral and cellular processes. The replication of polivorius is considered to provide a good prototype of animal virus multiplication (12, 88).

As a tool for molecular biology, picornaviruses offer a number of advantages. They are small and relatively simple in structure. They are positive-strand RNA viruses that provide a source of pure messenger RNA (mRNA). Their proteins have been analyzed and catalogued so that proteins made in response to the mRNA are clearly defined. Picornaviruses encode a limited number of functions. They are relatively stable, facilitating laboratory manipulations. One of the most striking characteristics of all picornaviruses is that they inhibit host-cell macromolecular synthesis; the predominant synthetic activities in the infected cell are virus directed. Finally, the viruses are capable of replicating in physically or chemically enucleated cells, thus facilitating the separation of nuclear and cytoplasmic events.

The biochemical and physiological characterization of mutants of poliovirus can provide insight into the normal

sequence of events during the replication and assembly of the wild-type (wt) virus.

It was the purpose of the current research to characterize a temperature-sensitive mutant of poliovirus, tsB9, and the guanidine-resistant strain, g^rH, and subsequently to develop and test a biochemical-physiological screening protocol using tsB9, defective-interfering particles (DI), g^rH, and wt Mahoney type 1 poliovirus as model strains. The screening procedure was aimed at quickly characterizing the mutant strains. The developed procedure proved extremely suitable for the phenotypic analysis of large numbers of mutants with the expenditure of minimal amounts of time, money, and virus stock. The need to conserve time and minimize cost is obvious. The need to conserve virus stock is perhaps more subtle and deserves explanation.

The utilization of large amounts of virus stock for assays and experiments necessitates frequent passage of these stocks. Poliovirus is known to have a relatively high reversion rate (32, 33), and these wt revertants quickly outproduce the mutants in the stock; therefore, it is imperative to keep the number of virus passages as small as possible to minimize the opportunity for reversion. It is a serious drawback of the current techniques that by the time the general physiological character of a mutant has been determined very

little low-passage stock remains for further, more detailed studies.

The number of virus passages can best be minimized by developing experimental techniques that use very small amounts of virus stock. The multiplicity-of-infection (m.o.i.) must be maintained, however, at a level appropriate to the experimental protocol. Thus the virus cannot be conserved by simply by decreasing the volume of stock used. The entire experiment must be scaled down to as small a level as is feasible within the limits of detection.

The techniques developed here for the phenotypic characterization of poliovirus mutants meet the criteria mentioned above; however, their validity for examination of the poliovirus system had to be verified. In addition, the capacity to yield clear and meaningful results had to be assessed. For these reasons, it was decided that the techniques to be examined as potential screening tools must be validated using well-known strains of poliovirus as model strains. The virus strains chosen as model strains for the validation of techniques were Mahoney type 1 as the wt agent; DI-1 derived from Mahoney type 1 in the laboratory of Dr. D. Baltimore (Department of Biology, Massachusetts Institute of Technology, Cambridge); g^rH, a guanidine-resistant strain developed from Mahoney type 1 in the laboratory of Dr. J. Holland (Department of Biology, University of California at San Diego); and

tsB9, a temperature-sensitive mutant of Mahoney type 1 generated and purified in the laboratory of Dr. Baltimore.

The physiological characteristics of wt (2, 5-44, 49-55, 57-71, 73, 74, 77-87, 89, 95-105, 208-120, 122-124, 126-133, 136-142) and DI (27-29) have been well established in the literature. The physiological characteristics of tsB9 and g^rH were determined in preliminary investigations as part of the current research. Once a new technique was determined to be economical, and relatively simple, it was used to evaluate all four of these model virus strains. It was to be finally retained in the screening protocol if it clearly differentiated the different model strains, yielded clear and reliable results, and provided information not available by yet simpler techniques.

The current research is divided into two main segments:

1. The physiological characterization of tsB9 and g^rH.
2. The development and validation of a physiological screening procedure that quickly and efficiently reveals the physiological character of a large number of poliovirus mutants in a short period of time.

REVIEW OF THE LITERATURE

The poliovirus infection cycle occurs in the cytoplasm of the infected cell (12, 83, 88). The complete infection cycle will occur normally in anucleate cells and in nucleate cells treated with actinomycin-D, indicating that the nucleus is not required (12, 83, 88).

Soon after the initiation of poliovirus infection, the synthesis of host-cell protein and RNA are inhibited with the cellular synthetic machinery being totally subverted to the generation of viral products (116). An inhibition of host-cell DNA synthesis occurs later, beginning about 2 h post-infection (p.i.), and is probably due to the earlier inhibition of cellular protein and RNA synthesis (84).

During the virus cycle a number of cytopathic effects (CPE) is seen in the infected cells as virions and other viral products accumulate. The virions accumulate in crystalline arrays in the cytoplasm until cell lysis occurs (37, 38, 39).

Virion Properties

The virion of poliovirus is composed solely of protein 70% w/w) and RNA (30% w/w) (12, 26, 42, 83, 88, 118, 120). The virion is 27-30 nm in diameter and consists of an icosahedral protein capsid enclosing a single linear molecule of plus-sense, single-stranded RNA (12, 34, 69, 88, 116, 120).

The capsid consists of 60 structural units and is composed of approximately equimolar amounts of the four capsid proteins: VP 1 (35,000 d), VP 2 (28,000 d), VP 3 (24,000 d), and VP 4 (5,000 d) (12, 29, 34, 69, 120).

The polio virion has a sedimentation coefficient of 150 S and a buoyant density of 1.34 g/ml (12, 29, 34, 83, 116). The total molecular weight of the intact virion is 8.0-8.5 x 10^6 d (34, 120). The virions are relatively stable and resist disruption by neutral sodium dodecyl sulfate (SDS) (12, 34, 88, 89).

The virion RNA is plus-sense, single-stranded, having a molecular weight of 2.6 x 10^6 d and a sedimentation coefficient of 35 S (57, 83, 88, 116). When extracted from the virion this RNA is infectious and serves as a messenger RNA (mRNA) in cell-free translation systems (34, 124). The virion RNA of poliovirus has been completely sequenced by two separate approaches and was found to be 7,410 to 7,733 nucleotides in length, potentially coding for a protein of 242,000 to 245,000 d (73, 113, 136, 138).

A segment of variable length of polyadenylic acid (poly A) is covalently attached to the 3' end of polio virion RNA (4, 41, 88, 116, 122, 123, 124, 141). At the 5' end, polio virion RNA is covalently linked to a small protein, VPg, via a phosphodiester bond between the hydroxyl group of the tyrosine side chain and the 5' pUp of the RNA (2, 16, 50, 82, 88).

The characteristic eucaryotic methylated cap structure is not present (4, 36, 82, 99, 116), and there are no unusual or methylated bases in the molecule (99, 120).

Inhibition of Host-cell Macromolecular Synthesis

Poliovirus infection drastically depresses synthesis of cellular protein (6, 78, 79, 103) even when viral RNA synthesis is inhibited by the presence of guanidine (78, 84, 104, 128). If protein synthesis is measured as the amount of radioactive amino acids incorporated into acid insoluble material within 60 min p.i., a decrease in the rate of synthesis is seen (84, 96). By 90 min p.i., protein synthesis has declined to 20%-25% of the unifected control levels (96, 104, 130, 134). The low point in total protein synthesis occurs at approximately 180 min p.i. The rate of total protein synthesis then increases until about 300 min p.i., and falls again (84). The rate of viral protein synthesis in poliovirus-infected cells never rises as high as that of host protein seen in the uninfected cells (85, 86).

Concomitant with the decrease in cellular protein synthesis, there is a dissociation of host-cell polysomes and the formation of viral polysomes (37, 62, 85, 103, 119, 130). Viral polysomes are larger than host polysomes (37, 84, 103, 119). The bulk of viral protein synthesis occurs in membrane-bound polysomes (37).

Poliovirus inhibits cellular protein synthesis at the level of initiation (8, 45, 56, 59, 71, 96). It has been shown that poliovirus RNA outcompetes cellular mRNA by interaction with host initiation factors, specifically, the cap-binding protein of the multimeric eIF3 (8, 56, 60, 71, 84, 107, 114).

Infection with poliovirus further leads to inhibition of host RNA synthesis and DNA synthesis (5, 6, 8, 14, 63, 84).

Cytopathic Effects of Poliovirus Infection

Normal HeLa cells possess large, spherical or oval, centrally placed nuclei, numerous mitochondria distributed throughout the cytoplasm, a relatively well-developed endoplasmic reticulum, and a typical Golgi apparatus (37). After infection with poliovirus, vacuols become prominent (1, 37, 38), numerous smooth membranous cisternae appear (16, 37, 38), and the nucleus migrates to one pole of the cell and eventually disintegrates (16, 38). For these degenerative changes to occur, active replication of the virus is required (6, 7).

Intracellular smooth membranes of the cisternae proliferate after poliovirus infection, increasing 8-fold in protein content and 13-fold in phospholipid content between 150 and 360 min p.i. (25). The newly added membranes are assembled partly from nascent macromolecules and partly from pre-existing phospholipids in the cell (1). Some of the newly formed membranes may contain viral products and the assembly of the membranes may be virus directed as the formation of

these new membranes occurs at a time when cellular protein, RNA and DNA synthesis have been suppressed.

It is possible that the inhibitory effect of guanidine on viral RNA synthesis is mediated by the membranes (102, 104). It is consistent with data currently available in the literature to suggest that viral protein synthesis evokes the proliferation of membranous cisternae which, in turn, harbor viral RNA synthesis (16, 109). There is also evidence for the possible functional involvement of poliovirus morphogenesis with intracellular virus-modified membranes (108, 109).

Synthesis of Viral RNA

Five species of viral RNA are found in the cytoplasm of poliovirus-infected cells: 35 S virion RNA, 35 S polysomal RNA, 20 S replicative form (RF) RNA, 35 S minus-sense RNA, and replicative intermediate (RI) RNA (9, 11, 12, 83, 88, 105). The 35 S virion RNA is of messenger-(plus) sense and has a 3' poly(A) tract and is covalently linked to VPg at the 5' end. Polysomal 35 S RNA is of plus-sense and has a 5' protein attached to both the plus and the minus strands, a poly(A) tract at the 3' end of the plus strand, and a poly(U) tract at the 3' end of the minus-strand (9, 11, 12, 88, 105). The plus-strands are all infectious. All full-length 35 S plus-strands have the same base composition and ribonuclease T1 oligonucleotide map (12, 48, 86, 99, 105, 118, 120, 126).

In addition to the above RNA species, an RNA of unknown function with a sedimentation coefficient between 5 S and 10 S has been found in poliovirus-infected cells, but not in uninfected cells (9; personal observation).

If actinomycin-D-treated poliovirus-infected cells are labeled at mid-cycle with radioactive uridine for 3 min or less, the RI RNA is preferentially labeled (14,64, 117). A labeling period of 10 min or more labels the 35 S RNA predominantly (22, 49, 66, 117). This 35 S RNA is 95% sensitive to single-strand specific ribonucleases (9, 22, 105).

Although the viral RNA in poliovirus-infected cells is predominantly 35 S, that species of RNA is not found in free-form in the cell (66). The 35 S RNA released from polysomes by EDTA or released from the replication complex (RC) is found in association with a ribonuclear protein complex (RNP) with a sedimentation coefficient of 80 S (66). Exogenous virion RNA added to a cytoplasmic extract of poliovirus-infected cells is also recovered as an 80 S RNA. Both of these 80 S RNPs have the same buoyant density of 1.4 g/cm^3 (66).

Replicative form RNA is a virus-specific, fully double-stranded RNA synthesized at a constant rate throughout the virus cycle (62, 105). This species accumulates so that it represents 10%-20% of the viral RNA in the cell when the estimated numbers of molecules are compared (12, 14, 62, 105). Replicative form has a molecular weight of 5.2×10^6 d, twice

that of 35 S single-stranded RNA (105). It is 80% resistant to single-strand specific ribonucleases under high salt conditions (9, 14, 83, 88). Its function is not known.

Poliovirus RF is infectious only when the synthesis of host-cell RNA and protein have not been inhibited (40, 76, 105 106).

Replicative intermediate RNA is the site of synthesis of plus-strand poliovirus RNA and is found in the RC together with the replicase proteins, tightly associated with smooth membranes (9, 11, 19, 23, 44, 51, 52, 55, 88, 89, 105, 117). It is not known whether the RC is enclosed in a membranous vesicle or if its components are integral to the membrane structure (16). The RI is synthesized at its maximum rate early in infection (14, 105, 117). The RI consists of one complete strand of minus-sense genome-length RNA and several (1-7) nascent strands of plus-sense (88). When examined by electron microscopy, the RI molecules are seen as branched structures, apparently the result of the single-stranded portions of the nascent plus-strand RNA extending from the double-stranded portions. The RI exhibits a variable resistance to ribonuclease A under high salt conditions, ranging from 10% to 60% (11, 19, 22, 46, 105, 117). The sedimentation behavior of RI is variable and heterogeneous and cannot be duplicated from one experiment to the next. It also varies with the method of extraction (10, 11, 14, 17, 105,

117) and the conditions of storage (18). The sedimentation values of RI range from 10 S to 70 S (11, 105, 117), with the more rapidly sedimenting molecules having more single-stranded branches (19).

The complementary minus-strand RNA cannot be isolated from the cytoplasm in the free state. It is only isolated as a component of RF or RI (115). The minus strand, once purified, is not infectious (115).

Viral Proteins

Poliovirus proteins are synthesized de novo from the free amino-acid pool of the cell (39). Poliovirus mRNA is translated on virus specific polysomes which contain a plus strand of viral RNA (12, 65, 68, 80, 124, 129, 132, 133). It is generally accepted that ribosomes begin translation of the poliovirus mRNA at the 5' end and proceed to the 3' end, yielding the large precursor protein NCVP 00, which may represent the entire coding capacity of the genome (8, 12, 20, 31, 77, 80). There is some evidence to suggest that a second initiation site exists that produces a polypeptide which contains sequences common to VPg, NCVP 4 (replicase), and the putative protease (20, 26, 67, 70, 74, 100, 134).

As each ribosome leaves the region of the 95,000 d structural coding region, a primary cleavage of the nascent chain occurs (80). A second nascent cleavage seems to occur at the end of the NCVP 3b coding region and the ribosome

then completes the protease-replicate precursor (58, 73, 80). Secondary cleavages require several minutes and lead to extensive refolding of the structural precursor (20, 77, 80).

Primary cleavage of NCVP 00, which is apparently membrane-bound, is due to host enzyme (77, 100). Secondary and later cleavages are carried out by viral proteases, presumably NCVP 7c and NCVP X (78, 80, 81, 101) or NCVP 6a (58).

The rate of protein synthesis in poliovirus-infected cells is considerably decreased late in the cycle, although the same spectrum of viral proteins is maintained (131).

Morphogenesis of Poliovirus

The morphogenesis of poliovirus particles involves a number of events that can be separated for analysis but which normally occur in an integrated fashion (94). Figure 1 illustrates a proposed scheme of poliovirus morphogenesis (88, 112, 116, 120). Five NCVP 1a molecules assemble to form an immature procapsid precursor that sediments at 13 S (109).

Immature 13 S particles undergo cleavage and rearrangement to yield mature 14 S procapsid precursors consisting of $(VP\ 0\text{-}VP\ 1\text{-}VP\ 3)_5$ (110). Twelve of the 14 particles assemble to form the procapsid with the sedimentation values of 80 S (18). Virion RNA and the procapsid interact in such a way that VP 0 is cleaved to VP 2 and VP 4, and the RNA is encapsidated to form the mature virion which sediments at 150 S (28, 67, 69, 94).

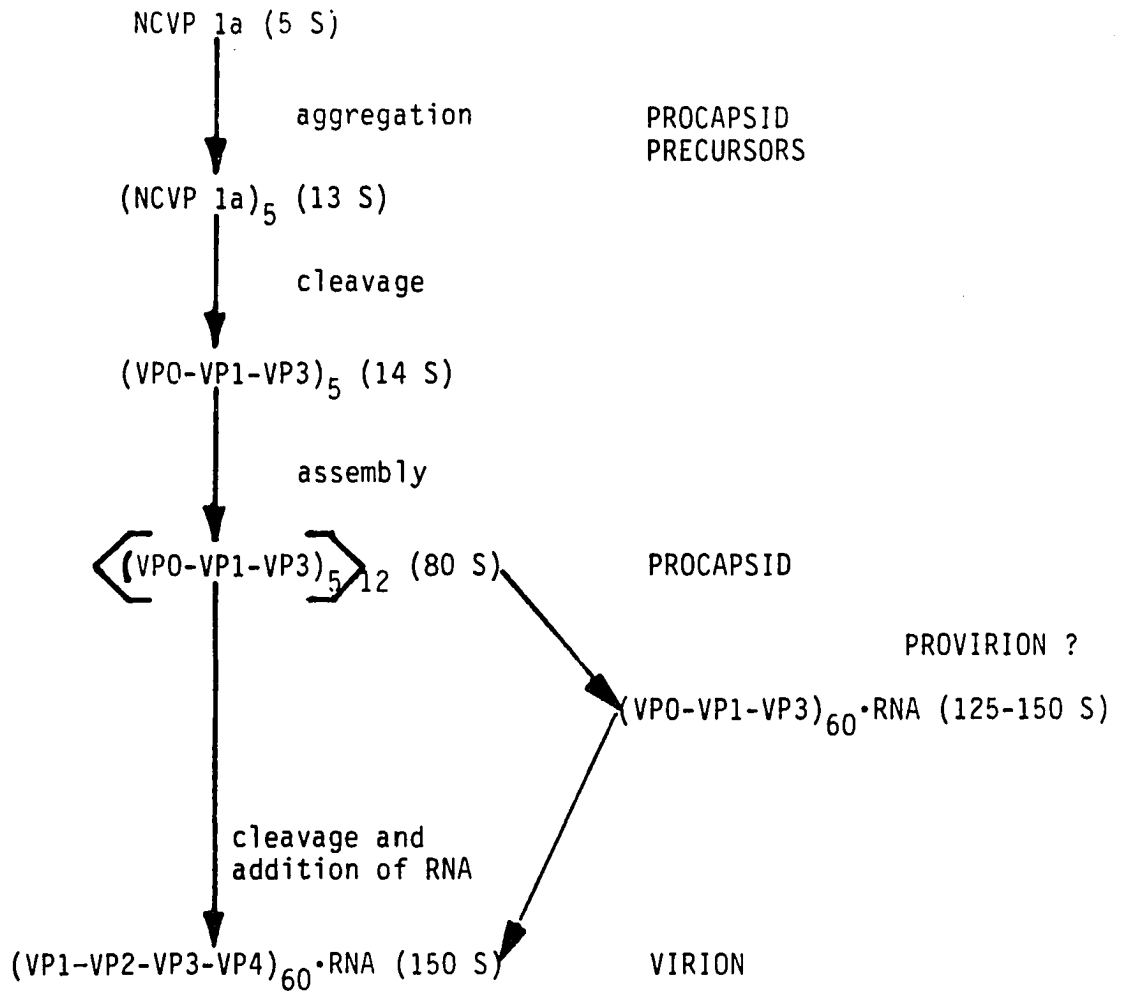


Figure 1. Proposed scheme of poliovirus virion morphogenesis.--The existence of the provirion is questionable (88, 111, 116, 120).

The presence of virus-modified membranes appears to be essential for the formation of infectious virion (25). Phillips has suggested that virus-modified membranes concentrate the 14 S particles to the threshold required for their self-assembly (108, 109), and that the membranes contain an "assembly factor" which fixes 14 S particles into a metastable configuration that acts as a nucleation site for procapsid assembly (112).

Effects of Guanidine

Guanidine is a specific inhibitor of poliovirus replication at concentrations that have no effect on cellular functions (1-5 mM) (105). A 100- to 1,000-fold decrease in the titer of guanidine-sensitive strains is seen in the presence of guanidine (6). It should be emphasized that guanidine is not 100% effective in blocking virus production. Stocks of poliovirus typically have titers of 10^7 to 10^{10} plaque-forming-units (pfu)/ml. In the presence of guanidine, the titers decrease to 10^4 to 10^7 pfu/ml, leaving a substantial residual infectivity.

Guanidine at a concentration of 1-5 mM inhibits: (1) the formation of virions (24, 66, 78); (2) synthesis of viral replicase (6, 79); (3) RNA synthesis (79); and (4) the movement of newly synthesized RNA into virions (69). It also causes an accumulation of procapsids, diminishes the virus-induced incorporation of choline into membranes,

delays the onset of cytopathic effects (25, 79), delays host macromolecular synthesis inhibition (6), and prevents the association of capsid proteins with the smooth membrane fraction (79, 139, 140).

Guanidine-resistant (g^r) and guanidine-dependent (g^d) mutants of poliovirus can be isolated after repeated passage of poliovirus in the presence of guanidine (24, 105). These mutants differ from their parent strain in the structure of one or more capsid proteins and the procapsid precursor NCVP 1a (79).

These findings, together with the results of genetic mapping, have prompted Korant (79) and Cooper et al. (36) to suggest that the guanidine locus is in the capsid protein coding region. It has also been argued that the guanidine locus is not in the capsid region (135). This is on the basis of the finding that defective-interfering (DI) particles replicate by a guanidine-sensitive mechanism while they do not make full-length NCVP 1a. An alterantive interpretation of that finding is that the guanidine locus is in the region coding for the 69,000 d fragment of NCVP 1a that DI does synthesize (79).

A number of simple compounds have been shown to inhibit guanidine effects on poliovirus: certain amino acids (methionine, leucine, and isoleucine) and methylated or ethylated amino alcohols or amines (95, 96). The lipid

precursors which antagonize guanidine act synergestically with the amino-acid guanidine antagonists (95). It is possible that both guanidine and its antagonists act via the virus-induced membranes.

Defective-interfering Particles

One of the best characterized sets of poliovirus mutants is a group of deletion mutants designated as "defective-interfering particles." These mutants interfere with wild-type (wt) virus during co-infection so that cells co-infected with DI and wt produce fewer progeny than cells infected with only wt (28).

The DI strains are deletion mutants which lack about 40% of the RNA encoding the capsid precursor NCVP 1a (27, 28, 87, 111, 137). This represents approximately 15% of the total genome RNA (29, 62, 87, 111). Wild-type and DI can be resolved by several techniques including agarose gel or composite agarose-acrylamide gel electrophoresis (62, 111). The DI RNA migrates faster than wt RNA and sediments more slowly in sucrose density gradients (29). The corresponding two virions can be separated by CsCl equilibrium centrifugation (29, 134).

Cells infected with DI do not produce 5 S, 13 S, or 14 S procapsid presursor, procapsid, or virion (110).

The Genetic Map of Poliovirus

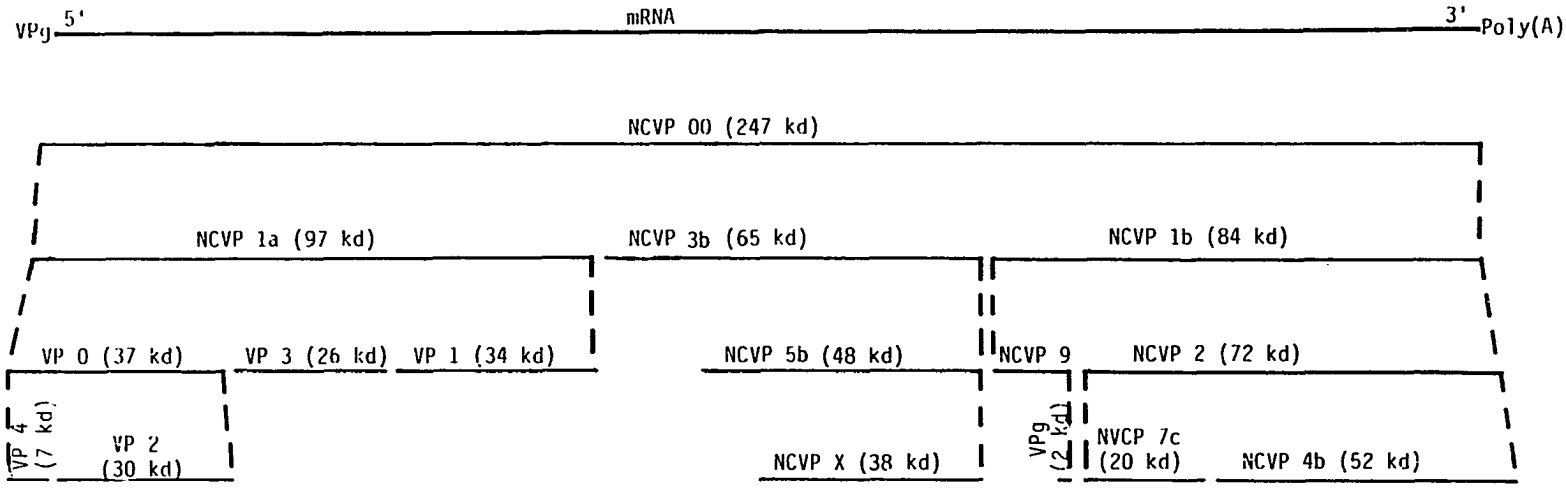
The study of poliovirus genetics is neither as simple nor as straightforward as had once been hoped in view of its size and apparent structural simplicity. Poliovirus RNA translation is initiated at one or two sites, generating large proteins that must be processed into functional proteins by proteolysis. In this system, therefore, the concept of "gene" or cistron is not truly useful. Instead, the term "coding region" will be used. The coding region contains the genetic information coding for a protein created by post-translational cleavage. Proximate coding regions may overlap, may be totally contained in other coding regions, or may not directly meet at all. Initial and terminal sequences may not be uniquely defined. Signals for initiation and punctuation of translation are not included in all coding regions. The boundaries of the coding regions are probably determined by certain amino-acid sequences, which in turn determines the overall conformation of the primary translation product and, thus, in reaction with the proteolytic specificities of its environment, delineate the final product proteins (33).

It is conceivable that the cleavage of the primary translation product(s) of poliovirus results in molecules with differing, overlapping, or antagonistic activities. These possibilities exist at each consecutive cleavage.

The genetic map of poliovirus has been examined in a number of different ways. A map has been generated using the recombination frequencies of mutants of an LsC revertant (33) and of Mahoney type 1 poliovirus (135). These two maps are similar to each other and to maps generated from apthovirus, cardioviruses, and rhinoviruses (33, 135). Another type of map has been generated by the labeling of polypeptides of poliovirus under restrictive conditions such as high osmolarity, the presence of emitine, or the presence of pactamycin. These maps are all consistent with each other if the assumption is made that poliovirus RNA is translated from one unique initiation site near the 5' end.

Capsid proteins and their precursor NCVP 1a map at one end of the genome by all mapping techniques, while the replicase activity maps at the opposite end. Biochemical mapping techniques have placed the capsid proteins at the 5' end and this information is used to orient the translational and recombination maps relative to each other (126). The gene order arrived at by these techniques is (5' to 3') NCVP 1a-NCVP 3b-NCVP 1b (9, 52, 58, 129).

Figure 2 depicts the composite map arrived at by combining the data available to date. The primary cleavage products of the protein precursor NCVP 00 are (5' to 3') NCVP 1a, NCVP 3b, andNCVP 1b. The polypeptide NCVP1a is the precursor of the structural proteins, NVBP 1b is the precursor



This figure represents a composite genetic map of poliovirus type 1 arrived at by combining the information available to date, including biochemical and genetic data. The proteins coded by the RNA (5' to 3') are shown, along with their post-translational cleavage patterns (8, 12, 13, 20, 26, 33, 52, 58, 129, 135).

Figure 2. The genetic map of poliovirus type 1

of the protease and replicase proteins, while the functions of NCVP 3b have not been fully elucidated. Secondary, tertiary, and maturation cleavages of NCVP 1a yield the capsid proteins (5' to 3') VP 4-VP 2-VP 3-VP 1 (58, 129). Secondary cleavage of NCVP 3b yields NCVP 5b which is then cleaved to yield NCVP X which is stable (58, 129). The secondary cleavage of NCVP 1b yields NCVP 2 and NCVP 9. Tertiary cleavage of NCVP 9 yields VPg, while tertiary cleavage of NCVP 2 yields NCVP 7c and NCVP 4b. The apparent function of NCVP 4b is replication of RNA (9) while NCVP 7c is a viral protease (58).

As can be seen from the above, only three functions of poliovirus have been mapped with any confidence: capsid proteins, proteases, and replicases. The other activities taking place during poliovirus infection have not yet been clearly explained or related to the genetic map. Conceptual and methodological difficulties contribute to this lack. For example, co-reversion from temperature sensitivity to wt of two characteristics is commonly taken to indicate that both characteristics are in the same gene or coding region (125, 134). Often the assumption is made that co-reversion indicates a functional relationship between the characters. However, since poliovirus proteins are generated by post-translational cleavage, it is conceivable that two characters are functionally independent, but cleaved

from the same precursor or that the coding regions of the two characters are overlapping or closely adjacent.

In addition, since the poliovirus proteins are all cleavage products, the conformation of the precursor molecule (proteolytic substrate) can affect the products yielded. Therefore, a mutation may exist that affects the conformation of the precursor, resulting in altered cleavage patterns that could affect all or most of the functions associated with cleavage products of that precursor. In such a case, the mutation could be in a region unrelated to the functions that are ultimately affected. Co-reversion of characters in such a mutant could lead to erroneous conclusions.

Another possible type of mutation that could lead to ambiguous co-reversion data is a temperature-sensitive mutation in a protease such that cleavage is modified but not inactivated at the restrictive temperature. The above discussion relating to altered substrate conformation holds true here also.

A number of poliovirus effects that have not been related to a particular protein remain. These include inhibition of host macromolecular synthesis, proliferation of cytoplasmic membranes, induction of cytopathic effects, assembly of virions from procapsids and RNA, asymmetry of viral plus- and minus-strand RNA synthesis, lysis of the host cell, penetration and uncoating, and selection of

nascent plus strands among their three functions (translation, encapsidation, and acting as a template for synthesis of minus strands).

METHODS AND MATERIALS

Culture Procedure

Cell Culture

HeLa S-3 cells were grown in suspension culture in Joklik's modified Eagle's medium (Gibco, Grand Island, NY) supplemented with 5% horse serum (Gibco), (3). Gentamycin sulfate was added to prewarmed (37°C) growth medium immediately before use. The cell cultures were maintained at 37°C in a Thelco (Precision Scientific, Los Angeles, CA) Model 6M forced-air incubator.

Preparation of Virus Stock

The type 1 Mahoney poliovirus used as the wild-type (wt) strain throughout these studies was a gift from Dr. M. J. Hewlett (Department of Molecular and Cellular Biology, The University of Arizona, Tucson). The defective-interfering strain (DI) was isolated from type 1 Mahoney poliovirus in the laboratory of Dr. D. Baltimore. It was purified and supplied as a gift by Dr. M. J. Hewlett. The guanidine-resistant (g^r) strain, g^rH, was a kind gift from Dr. J. Holland. The temperature-sensitive (ts) mutant, tsB9, was selected by replicative plating of hydroxylamine mutated type 1 Mahoney poliovirus at 31°C and 39°C in the laboratory

of Dr. D. Baltimore (47). All other mutants were generated and selected as described below.

Stocks of all virus strains, except DI, were grown at 33°C. The only DI used was supplied in purified form by Dr. Hewlett's laboratory and had been grown at 37°C. All other material designated "virus stock" was prepared by the following procedures. HeLa S-3 cells grown to a density of 4×10^5 cells/ml were pelleted by centrifugation (800 x g; 10'; room temperature (RT)) and washed once with isotonic saline (Hank's Balanced Salt Solution; Gibco). Virus stock was added at a multiplicity-of-infection (m.o.i) of 5 plaque-forming-units (pfu) per cell to HeLa S-3 cells at a density of 4×10^7 cells/ml and allowed to adsorb to the cells at RT for 30 min in the absence of serum. The infected cells were then diluted to a density of 4×10^6 cells/ml with JMEM plus 5% horse serum and stirred at 33°C.

After 8 h the infected cells were harvested by centrifugation (800 x g; 4°C), washed once in isotonic saline, and frozen as a pellet at -20°C. The frozen cells were thawed in Earle's saline, resuspended at a density of 4×10^7 cells/ml, and subjected to three cycles of freezing and thawing. Care was taken during the thawing procedure to keep calls at or below the permissive temperature of the temperature-sensitive strains, 33°C. The nuclei were removed by centrifugation (2,000 x g; 10'; 4°C) and the resulting

supernatant was frozen in 2.0-ml aliquots at -20°C and designated as virus stock.

Plaque Assays and Efficiency-of-plating Quotients

The titers of the virus stocks were determined by a modification of the Cooper (31) plaque assay at 33°C and 39°C. A nutrient layer of 0.9% nutrient agar (Difco, Detroit, MI) in JMEM plus 10% horse serum was pipetted into 60-mm plastic Petri dishes (Falcon Plastics, Oxnard, CA). HeLa S-3 cells (4×10^6 per dish) were suspended in 0.6 ml of JMEM. A 0.2-ml inoculum of diluted virus stock was added to each tube of cells and mixed by vortexing. The cell-virus suspension was combined with 0.8 ml of 0.9% bacto agar in JMEM, vortexed, and plated over the nutrient layer. After the top layers solidified, the plates were incubated under 5%-10% CO_2 at 33°C or 39.5°C.

Plaques were counted at 48 h (39.5°C) and 72 h (33°C) after inoculation.

The efficiency-of-plating quotient (e.o.p. 33/39.5°C) was determined by dividing the titer of the virus stock at 33°C by the titer at 39.5°C.

The titer determined at 33°C was used to determine the m.o.i. for all infections.

Standard Infection Procedure

Unless otherwise indicated for specific experiments, all infections were carried out by the following procedure. HeLa S-3 cells grown to a density of 4 x 10^5 cells/ml were pelleted (800 x g; 10'; RT) and washed in isotonic saline. Virus stock was added at a m.o.i. of 10 pfu/cell and the cells were suspended at a density of 4 x 10^7 cells/ml in the absence of serum. The virus was allowed to adsorb to the cells for 30 min at RT. The infected cells were diluted to 4 x 10^6 cells/ml with JMEM plus 5% horse serum and put to stir at the temperature appropriate to the experiment or procedure. This is defined as zero-time post-infection.

At 60 min post-infection (p.i.), actinomycin-D was added to a final concentration of 5 µg/ml. Tritiated uridine (10 µCi/ml of culture) was added at 90 min p.i.

Harvesting Infected Cells

To harvest the infected cells, the cultures were quickly chilled in ice. The cells were pelleted (800 x g; 5'; 4°C) and frozen at -20°C. Lysis was carried out by adding 2.0 ml of ice-cold RSB buffer (0.01 M NaCl; 0.01 M Tris; pH 7.5) plus 1% Nonidet P 40 (NP-40) per 10.0 ml of infection volume to the frozen cell pellet and gently vortexing. Nuclei were removed from the extract by centrifugation (2,000 x g; 10'; 4°C). The extracts were made 1% in sodium dodecyl

sulfate (SDS) and then subjected to various analytical techniques appropriate to the individual experiment.

Generation and Selection of Guanidine-resistant Mutants

The method of Korant (78) was used to select guanidine-resistant mutants. Wild-type poliovirus was plaque-purified by selecting single, well-defined plaques from a plate prepared as for plaque assay. Using a standard bacteriological inoculating needle, material from these plaques was plated at 33°C and 39.5°C in the presence and absence of 5 mM guanidine hydrochloride (Sigma, St. Louis, MO). Plaques that were not temperature sensitive and grew only in the absence of guanidine were cored from the plates using sterile Pasteur pipets. The cored plaques were pipeted into 1.0 ml of Earle's saline, subjected to five cycles of rapid freezing and thawing, and used to infect five 100-mm plates of HeLa cells in soft agar prepared as for plaque assay (10^7 cells/plate). The plates were incubated at 37°C under 5%-10% CO_2 until the plaques were just touching but not overlapping. The top agar from all five plates was pooled into 5 ml of Earle's saline and subjected to five cycles of freezing and thawing. Agar was cleared from the virus suspension by centrifugation (1,000 x g; 10'; 4°C). The supernatant was used to infect HeLa S-3 cells in suspension in the presence of 5 mM guanidine HCl.

A virus stock was prepared from these cultures as described above for virus stock preparation. After three consecutive undiluted passages in the presence of 5 mM guanidine, the stock was plated in HeLa S-3 cells in soft agar as described for the plaque assays with 5 mM guandine HCl present in both the top agar and nutrient bottom agar. Using a standard bacteriological inoculating needle, plaques grown in the presence of guanidine were plated in replicate in the presence and absence of guanidine at 37°C. All plaques that grew in both the presence and absence of guanidine were cored and designated g^r. No variants unable to grow in the absence of guanidine (i.e., guanidine-dependent (g^d)) were selected, as the g^d strains are presumed to be double mutants (Holland, personal communication, 1978 (64)).

All g^r mutants thus selected were used to grow stocks as previously described for virus stock preparation except that these stocks were grown in the presence of 5 mM guanidine. These mutant stocks were then plaque assayed in the presence and absence of 5 mM guanidine at 37°C. Variants that exhibited a significant difference between their titers in the presence and absence of guanidine (2-fold or more) were discarded. Variants that exhibited a difference between the two titers of less than 2-fold were divided into 2.0-ml aliquots and stored frozen at -20°C.

Generation and Selection of
Temperature-sensitive Mutants

Plaque-purified wt virus was prepared as described above for g^r mutant generation. A 1.0-ml aliquot of this purified stock was treated for 30 min at RT with 4.0 ml of hydroxylamine sulfate (2 M hydroxylamine sulfate; 2 M sodium acetate; pH 6.0). At this pH, hydroxylamine modifies uridine residues primarily, allowing them to pair with guanidine residues rather than adenine during replication, resulting in point mutation(s) in which cytosine has replaced uridine (75).

The mutagenesis was halted by diluting hydroxylamine-treated stock 100-fold with JMEM plus 10% acetone. The pH of the diluted stock was adjusted to pH 7.4 with 0.1 N NaOH. This stock was used to infect HeLa S-3 cells in soft agar prepared as for plaque assay. Twenty plates of mutant-infected HeLa S-3 cells were incubated at 33°C (5% CO_2) for 72 h.

Using standard bacteriological inoculating needles, plaques were selected and plated in replicate at 33°C and 39.5°C (5% CO_2) in lawns of HeLa S-3 cells in soft agar. Plates were observed at 48 and 72 h. All variants that formed a plaque at 33°C and none at 39.5°C ($33^+/39.5^-$) were cored from the agar plates at 33°C using sterile Pasteur pipets and were used to prepare virus stocks as described for g^r mutants with the exception that no guanidine was present and all stocks were grown at 33°C.

Plaque assays were performed at 33°C and 39.5°C as previously described. It was decided on the basis of previous literature (32, 33, 34, 135) that ts mutants with a titer greater than 10^4 pfu/ml at 39.5°C or an e.o.p. 33/39.5 of less than 10^2 would be discarded. All selected strains were stored frozen at -20°C in 2.0-ml aliquots.

Characterization of tsB9 and g^rH

Efficiency-of-plating Quotient

In order to determine the e.o.p. 33/39.5 of tsB9, the virus stock was subjected to the standard plaque assay at 33°C and 39.5°C. The e.o.p. 33/39.5 of a given stock was defined as the titer of that stock at 33°C divided by the titer of that stock when assayed at 39.5°C. Any stock having an e.o.p. 33/39.5 of less than 10^2 or a titer at 39.5°C of greater than 10^4 was discarded.

The e.o.p. was also determined in the presence and absence of guanidine and the e.o.p. g^-/g^+ calculated. In the guanidine + assay, all media and agar contained 3 mM guanidine HCl. The e.o.p. g^-/g^+ was defined as the titer of the virus in the absence of guanidine divided by the titer in the presence of guanidine. This was used as the measure of the guanidine sensitivity of virus replication. These assays were carried out at 33°C.

Generation of Cytopathic Effects

To determine the stimulation of cytopathic effects (CPE) by tsB9 and g^rH, the uptake of trypan blue by infected cells was observed. Cultures of tsB9, g^rH, and wt were established at 39.5°C using the standard infection procedures except that no actinomycin-D nor radioactive label were added. At 0 min p.i., a 0.20-ml sample of infected cells was diluted 10-fold with isotonic saline and made 0.4% in trypan blue. The number of viable cells, those not stained by the dye, was determined for each sample using a hemacytometer. Samples were taken every 2 h for a period of 24 h.

Electron Microscope Observations

In order to determine whether virion lattices were formed in tsB9-infected cells at the restrictive temperature, infected cells were examined by electron microscopy. HeLa S-3 cells were infected with wt or tsB9 virus using the standard infection technique without the addition of actinomycin-D or radioactive precursors. Cultures of g^rH were not examined since it was not a ts strain. Cultures of wt and tsB9 were incubated at 33°C and 39.5°C. Mock-infected cells were incubated at 39.5°C.

At 8 h p.i. (39.5°C) and 10 h p.i. (33°C), the cells were pelleted (800 x g; 10'; 4°C) and immediately fixed in glutaraldehyde. The fixed cells were embedded in epon and thin sections were prepared and examined in the Phillips 200

electron microscope. Sections of each culture were scanned for the presence of crystalline lattices of virions. General morphological observations were recorded. No micrographs were taken.

Effects of Temperature Shift-down on tsB9 Replication

The effects of shifting from 39.5°C to 33°C during the virus cycle on the final titer of the virus stock was determined for tsB9 and wt. Virus stocks were prepared as previously described. A stock of each strain was established at 39.5°C and 33°C. A portion of each stock was shifted down (39.5°C to 33°C) at 90 and at 240 min p.i. and incubated until 8 h p.i. The titer of each stock was determined by plaque assay at 33°C and 39.5°C.

Sensitivity of Virus Infectivity to Low pH (6.8)

In order to determine the sensitivity of the virus stock to low pH (6.8), the procedure of Cooper et al. (35) was followed. Aliquots of 0.5 ml of virus stock were adjusted to pH 6.8 by addition of 0.1 N HCl. After 15, 30, and 45 min at RT, the stocks were adjusted to pH 7.4 by the addition of 0.1 N NaOH and immediately frozen at -20°C. The remaining infectivity was determined by plaque assay at 33°C. Stocks of tsB9, $g^{r}H$, and wt were examined in this manner.

Sensitivity of Virus Infectivity to Heat (45°C)

In order to determine the sensitivity of virus infectivity to heat, 0.5-ml aliquots of tsB9, wt, and g^rH stock were exposed to 45°C in a water bath for 15, 30, and 60 min. The samples were then chilled in ice and frozen at -20°C. The remaining infectivity was determined by plaque assay at 33°C.

Complementation between tsB9 and Defective-interfering Strains

Two tests for complementation between tsB9 and DI were carried out. Uninfected HeLa S-3 cells were plated as described for plaque assay in soft agar except that 35-mm Petri dishes were used. The DI stock was purified and generously supplied by Dr. M. J. Hewlett. Stocks of wt, DI, and tsB9 were made equal in their infectivity by appropriate dilutions. Equal volumes (hence equal pfu) were combined to produce mixed stocks. With a standard bacteriological inoculating needle, samples of the following stocks were inoculated into duplicate established cell lawns: wt, DI, tsB9, tsB9/wt, wt/DI, and tsB9/DI. The plates were then incubated at 39.5°C under 10% CO_2 and observed daily for a period of 4 days for any signs of clearing in the cell lawn around the point of inoculation which would indicate cell death.

These same mixed and titer-equalized pure stocks were used to generate a one-step growth curve at 39.5°C. HeLa S-3 cells were infected with a total of 10 pfu/cell. Samples of the cultures were taken at six time points and the pfu/cell were determined by plaque assay at 33°C.

Determination of the Presence of Virions

In order to determine whether tsB9 produced detectable amounts of virion at the restrictive temperature, wt and tsB9 cultures were compared using the procedure of Cole and Baltimore (27). HeLa S-3 cells were infected with wt or tsB9 using the standard infection procedures. To label virion RNA, either 20 μCi of 5,6-^{3}H-uridine or 1.0 mCi of carrier-free $^{32}PO_4$ were added per ml of culture. Whenever $^{32}PO_4$ was used, the infection was carried out in phosphate-free JMEM + 5% dialyzed horse serum. In experiments involving the labeling of virion proteins, either ^{14}C- or ^{3}H-amino acids or ^{35}S-methionine were added. In such cases, the JMEM was formulated to contain none of the specific amino acid used as label or to contain 1/10 of the normal concentration of essential amino acids. The concentration of glutamine was always maintained at the normal level as this is an essential nutrient for poliovirus replication (43). The growth medium was supplemented with 5% (v/v) dialyzed horse serum.

Cultures of g^rH were also examined for the presence and characteristics of virions. The g^rH virion RNA was labeled by the uptake of ^{14}C-uracil and g^rH virion proteins were labeled by the uptake of ^{3}H-amino acids. The g^rH cultures were incubated at 33°C except when otherwise indicated.

Both actinomycin-D and radioactive precursors were omitted when unlabeled virions were produced.

Infected cells were harvested at 360 min p.i. (39.5°C) or 480 min p.i. (33°C). To determine the presence of virions in the samples, the extracts were subjected to ultracentrifugation through linear gradients of 5%-30% sucrose in RSB + 1.5 mM $MgCl_2$ (SW 27; 3 h; 27,000 rpm; 4°C).

Virions were purified from infected cell cytoplasmic extracts prepared as described above. The cytoplasmic extracts were adjusted to 1% SDS and diluted to 10.0 ml with TNE buffer (0.1 M NaCl; 0.01 M Tris; 0.001 M EDTA; pH 7.5) plus 0.5% SDS, and the virions were pelleted in a Spinco (Irvine, CA) type 40 rotor (35,000 rpm; 90 min; 21°C). The pelleted virions were resuspended in 0.5 ml of TNE + 0.5% SDS by stirring overnight at room temperature. The resuspended virions were layered on 37 ml of a 15%-30% (w/w) linear sucrose gradient (sucrose in TNE + 0.5% SDS) and centrifuged in a Spinco SW 27 rotor (27,000 rpm; 2.5 h; 21°C). One-milliliter fractions were collected and the presence of virion was established by measuring the radioactivity of a

small aliquot of each fraction in a Beckman liquid scintillation spectrophotometer. Fractions containing the virion peaks were pooled and stored at -20°C for use as purified virion.

Determination of the Presence of Procapsid

To detect the presence of procapsid, standard infection procedures were carried out as above using ^{35}S-methionine, ^{3}H-amino acids, or ^{14}C-amino acids to label capsid proteins. The cultures were harvested and lysed as described above. The extracts were layered onto linear gradients of 5%-30% (w/w) sucrose in RSB + 1.5 mM $MgCl_2$. The gradients were centrifuged (SW 27; 3 h; 27,000 rpm; 4°C) and divided into 1.0-ml fractions. The radioactivity was determined as previously described. Both virions and procapsids were detected on the same gradients by this technique.

Complementation of Particle Formation between tsB9 and Defective-interfering Strains

In order to determine whether tsB9 and DI were able to complement each other with respect to particle formation at 39.5°C, mixed and pure cultures were prepared as described above for virion and procapsid labeling. The total m.o.i. was 10 pfu/cell of the following stocks: wt, DI, tsB9, wt/DI, and tsB9/DI. The viral proteins were radioactively

labeled with 50 μCi/ml of ^{35}S-methionine using the standard infection procedure. The cultures were harvested at 300 min p.i., extracted and analyzed as described above for their content of procapsid and virion.

Comparison of the Buoyant Density

Purified wt virions labeled with ^{3}H-uridine were mixed with either purified tsB9 virions labeled with $^{32}PO_4$ at 33°C or with g^{r}H virions labeled with ^{14}C-uracil and the mixed virions were subjected to equilibrium centrifugation as follows. To 0.3 ml of mixed virion suspension was added 4.72 ml of NEB (0.01 M Tris, 0.01 M NaCl, 0.02 M EDTA; pH 7.4), 0.8 ml of 10% Brij-58, and 4.18 ml of CsCl stock (ρ = 1.79) to a total volume of 10.0 ml and average ρ of 1.33. The gradients were centrifuged for 20 h in a Spinco type 40 rotor at 35,000 rpm at 4°C and 0.25-ml fractions were collected. The radioactivity of each fraction was measured in Bray's mixture using a Beckman liquid scintillation spectrophotometer; the channels and gain were set for simultaneous counting of ^{3}H and ^{32}P, or ^{3}H and ^{14}C.

Comparison of Sedimentation of Virions in Sucrose Density Gradients

Mixed, differentially labeled virion species were prepared as above and layered onto 15%-30% (w/w) sucrose gradients (in TNE + 0.5% SDS). The gradients were subjected

to ultracentrifugation (SW 27; 2.5 h; 25,000 rpm; 21°C) and 1.0-ml fractions were collected. Radioactivity was determined as described above.

Comparison of Sedimentation of Virion RNA in Sucrose Density Gradients

Mixed, differentially labeled virion suspensions were prepared as described above. The virions were pelleted and resuspended in TNE + 0.5% SDS adjusted to 0.1 M sodium acetate (pH 3.5) and immediately layered onto sucrose density gradients (TNE + 0.5% SDS) (SW 27; 16.5 h; 21,000 rpm; 21°C). Fractions of 1.0 ml were collected and the radioactivity in each fraction was determined.

Preparation of Radioactively Labeled Virus-specific Cytoplasmic RNA

For the preparation of radioactively labeled total cytoplasmic RNA, the standard infection and harvesting procedures were carried out as previously described. The RNA was labeled by the uptake of radioactive precursors such as ^{3}H-uridine, $^{32}PO_4$, or ^{14}C-uracil. Cultures were harvested at 240 min p.i. unless specifically indicated otherwise.

In order to label replicative-intermediate (RI) RNA, the standard infection procedure was modified. A m.o.i. of 150 pfu/cell was used to maximize the number of replication foci (11, 12, 55). For preparative purposes, ^{3}H-uridine

(20 μCi/ml) or $^{32}PO_4$ (1 mCi/ml of culture) was added at 90 min p.i. and the infected cells were harvested at 180 min p.i. For analytical purposes, ^{3}H-uridine (70 μCi/ml) was added at 180 min p.i. and the cells were harvested at 2.5 min later. It is known that the predominant species labeled in such conditions of pulse-labeling is RI RNA (11, 12, 55).

Determination of the Uptake of Radioactive Precursors to Monitor RNA Synthesis

As a measure of RNA synthesis, RNA accumulation was monitored following the standard infection procedure. Samples of 0.2 ml were withdrawn from the cultures at 90 min p.i. and every 30 min thereafter and analyzed for the presence of acid-precipitable radioactively labeled material. Experiments done at 39.5°C were usually terminated at 360 min p.i., those at 33°C at 480 min p.i. The 0.2-ml samples were pipetted into 0.5 ml of ice-cold 10% trichloroacetic acid (TCA) and chilled in ice for at least 10 min. The acid-insoluble material was collected by vacuum filtration onto Whatman GF/C glass fiber filters and its radioactivity was measured.

In experiments designed to determine the rate of RNA synthesis, the standard infection procedure was slightly modified. No radioactive precursors were added to the whole cultures. Instead, 0.2-ml samples were withdrawn and placed in 1.5-ml mocrocentrifuge tubes in the same water bath as the

parent culture. Two microcuries of ^{3}H-uridine were added and the samples were incubated at the appropriate temperature for 10 min. The microfuge tubes were then quickly chilled in ice and 0.5 ml of 10% TCA was added to each sample. The samples were kept on ice for another 10 min. The acid-insoluble material was collected as described above and the radioactivity was measured.

Determination of the Presence of VPg on tsB9 RNA

The procedure of Flanegan et al. (53) was used to determine whether VPg was present on tsB9 RNA. Purified $^{32}PO_4$-labeled virion RNA or cytoplasmic RNA was digested to completion in low salt with a mixture of ribonucleases T1, T2, U, and A (200, 5, 0.1, and 50 units/ml, respectively). Digested samples were spotted on Whatman 3M paper and subjected to high-voltage electrophoresis for about 2.5 h at 5,500 V at room temperature. The protein-pUp spot migrates in the opposite direction from the nucleotides and free phosphate and can thus be easily located (53). Wild-type RNA of the corresponding type was run in parallel as a control and to provide a marker for the migration of VPg-pUp. Unlabeled 5' nucleotides were mixed with the digested samples and spotted as ultraviolet (UV) markers for the migration of the nucleotides.

The 3M paper was dried and autoradiographed to determine the location of the radioactive material. The nucleotide and VPg-pUp spots were cut out, submerged in Bray's scintillation mixture and the radioactivity was determined. A UV lamp was used to verify the location of the nucleotides.

In addition to virion RNA produced at 33°C, cytoplasmic viral RNA labeled with $^{32}PO_4$ was produced at 33°C and 39.5°C. After harvesting the cells, the RNA was extracted three times with a mixture of phenol-chloroform-isoamyl alcohol (50:48:2), three times with chloroform-isoamyl alcohol (96:4), and ethanol precipitated. The RNA was pelleted from the ethanol suspension (Brinkman microcentrifuge; 12,000 x g; 4°C; 10'), dried under vacuum, and resuspended in TNE + 0.5% SDS. The resuspended samples were analyzed on sucrose density gradients (SW 27; 21,000 rpm; 21°C; 15%-30% sucrose in TNE + 0.5% SDS). The radioactivity of a 0.1-ml aliquot of each fraction of the gradient was measured. The 35 S and 20 S peaks, located by the presence of purified wt markers run in a parallel gradient, were pooled and the RNA was ethanol-precipitated. This RNA was analyzed for the presence of VPg as described above.

Agarose Gel Filtration of RNA

Samples of RNA were phenol-extracted as previously described. The ethanol-precipitated, vacuum-dried RNA was resuspended in TNE + 0.5% SDS and layered onto a Sepharose

2B (Pharmacia, Piscataway, NJ) column previously equilibrated with TNE + 0.5% SDS. Purified radioactively labeled wt form (RF) (20 S) and virion (35 S) RNA, obtained from Dr. M. J. Hewlett, were mixed and filtered through the column as control markers.

Agarose Gel Electrophoresis of RNA

A modification of the procedure of Hewlett et al. (62) was used for agarose gel electrophoresis of RNA. Agarose gels (1% w/w) were prepared by melting agarose (Sigma) in E buffer (40 mM Tris, 10 mM sodium acetate, 1 mM EDTA, pH 7.3) containing 10% (v/v) glycerol. The gels were formed in acid-cleaned glass tubes and pre-run in E buffer at 10 V/cm for 45 min at room temperature.

Vacuum-dried RNA was suspended in 10 µl of TNE + 0.5% SDS and 5 µl of 60% sucrose-0.2% bromophenol blue in TNE. The samples were layered onto the pre-run gels under the buffer. Electrophoresis was carried out at 10 V/cm (no more than 8 mA/gel) for approximately 2 h, at which time the tracking dye had migrated 10 cm from the origin.

The gels were cut into 1-mm slices with a manual gel slicer (Hoefer Scientific, San Francisco, CA) and placed in open liquid scintillation vials to air dry. The gel slices were then soaked in an aqueous scintillation cocktail, such as Bray's solution, overnight and the radioactivity was measured.

Determination of the Ribonuclease Sensitivity of Viral RNA

In order to determine the ribonuclease-A sensitivity of RNA samples, the dried, ethanol-precipitated RNA was suspended in 4.0 ml of 2X SSC buffer (0.15 M NaCl, 0.015 M sodium citrate). Treated gradient fractions were diluted to a total volume of 4.0 ml with 2X SSC buffer, mixed thoroughly, and divided into two equal aliquots. One-half of each gradient fraction was treated with 0.1-mg ribonuclease-A (Worthington, Freeland, NJ) per ml for 15 min at 37°C (10). The TCA insoluble radioactivity was determined as previously described for nuclease-treated and untreated samples. Ribonuclease sensitivity was defined as the percentage of TCA insoluble radioactivity in the untreated fraction found remaining in the nuclease-treated sample.

Lithium Chloride Precipitation of RNA

The cytoplasmic extract prepared as described for harvesting of virus-infected cells was adjusted to 2 M LiCl and maintained at -20°C overnight (10). The samples were centrifuged in a Brinkman microcentrifuge (12,000 x g) for 20 min at room temperature. The supernatant and pellet were individually ethanol precipitated. Each was then treated with 2 M LiCl again. The RNA thus purified was stored at -20°C as an ethanol suspension (70% ethanol, 0.2 M sodium acetate, pH 7.0). Purified wt 35 S virion and

20 S RNA were mixed and subjected to the entire LiCl treatment as a monitor of the efficiency of separation of single- and double-stranded RNA.

Comparison of Virus-specific Cytoplasmic RNA

Virus-specific cytoplasmic RNA from tsB9 and wt cultures incubated at 33°C and 39.5°C, and from g^rH cultures incubated at 39.5°C were labeled with ^3H-uridine (wt), $^{32}PO_4$ (tsB9), or ^{14}C-uracil (g^rH). The RNA was prepared from cytoplasmic extracts generated as described in the cell-harvesting procedure. The RNA was phenol-chloroform extracted and ethanol precipitated as described in the procedure for the determination of the presence of VPg on cytoplasmic RNA. The RNAs extracted from the differentially labeled virus cultures were mixed and analyzed by agarose gel electrophoresis (tsB9 + wt only) and by sucrose density gradient centrifugation (tsB9 + wt and g^rH + wt). In some cases, the wt and tsB9 or g^rH RNA were displayed on separate gradients or gels in parallel and the same radioactive label was used for both viruses.

Determination of the Effects of Temperature Variation Near the Restrictive Temperature on tsB9 RNA

In order to determine the effects of temperature variation near the restrictive temperature on RNA synthesis in

tsB9, the standard infection procedure was carried out with wt and tsB9 cultures incubated at 38°C, 38.5°C, 39°C, 39.5°C, 40°C, 40.5°C, and 41°C. The accumulation of TCA insoluble material labeled by the uptake of ^{3}H-uridine was monitored, as previously described, as a measure of RNA synthesis. The RNA samples were harvested, phenol-chloroform extracted, ethanol precipitated, and analyzed on linear gradients of 10%-22% sucrose in TNE (SW 50; 39,000 rpm; 5.5 h; 4°C). Two-drop fractions were collected and the radioactivity was determined.

Uptake of Radioactive Precursors to Monitor Protein Synthesis

To monitor protein synthesis in infected cells, the standard infection procedure was carried out in JMEM containing 1/10 the normal concentration of essential amino acids and supplemented with 5% dialyzed horse serum. The labeling and sampling procedure was the same as that used to determine the rate of RNA synthesis, but with some minor exceptions. The samples were labeled with 5 μCi of ^{3}H-amino acids (Amersham, Arlington Heights, IL) per sample. After incubating for 10 min, the samples were made 0.5 N in NaOH and incubated 15 min at the same temperature as growth to remove any amino-acyl label from tRNA (84). The samples were then TCA-precipitated as previously described.

Complementation of Protein Synthesis between tsB9 and Defective-interfering Strains

In order to determine whether tsB9 and DI were able to complement with respect to protein synthesis at the restrictive temperature, the rate of protein synthesis was determined as described above for the following virus stocks: wt, DI, tsB9, wt/DI, and tsB9/DI.

Inhibition of Host-cell Protein Synthesis

In order to demonstrate the poliovirus-triggered inhibition of host-cell protein synthesis, the procedure described above for monitoring protein synthesis was used, except that no actinomycin-D was added to the cultures. The samples were taken at 0 min p.i. and every 30 min thereafter. The cultures were labeled with ^{3}H-leucine.

SDS-Polyacrylamide Electrophoresis of Cytoplasmic Proteins

Cultures of tsB9 and wt were established at 33°C and at 39.5°C using the standard infection procedure. Proteins in the wt cultures were labeled with ^{35}S-methionine, while the tsB9 proteins were labeled with a mixture of ^{3}H-tyrosine, ^{3}H-leucine, and ^{3}H-lysine. Cultures were harvested at 240 min p.i. at 39.5°C and 360 min p.i. at 33°C for cytoplasmic proteins. A portion of the wt culture at 39.5°C

was incubated until 360 min p.i. for the preparation of labeled virions.

Virions were purified as previously described from the wt culture at 39.5°C harvested at 360 min p.i. Cytoplasmic extracts were prepared as previously described from all other cultures.

Small aliquots of each cytoplasmic extract and the purified wt virion suspension were mixed with an equal volume of electrophoresis sample buffer (0.625 M-Tris, pH 6.8, 5% mercaptoethanol, 10% glycerol; 2% SDS) and placed in a boiling water bath for 2 min. The samples were electrophoresed at room temperature in cylindrical gels of 9% acrylamide (30:0.8, acrylamide:bisacrylamide) (0.375 M Tris, 0.1% SDS, 10% glycerol) at 20 mA until the bromophenol blue tracking dye migrated out of the gel (about 3 h). They were then fixed in 50% TCA for 30 min, cut into 1-mm slices with a manual gel slicer (Hoefer Scientific), and soaked in aqueous scintillation cocktail overnight. The radioactivity was determined in a Beckman liquid scintillation spectrophotometer.

Synthesis of Procapsid Precursors by tsB9

In order to monitor the presence of procapsid precursor proteins in tsB9-infected cells, the procedure of Phillips (109) was used. The standard infection procedure

was slightly modified. The cells were infected at a m.o.i. of 150 pfu/cell and incubated at 39.5°C. Guanidine HCl was added to each culture at 150 min p.i. to a final concentration of 3 mM. At 210 min p.i., ^{3}H-amino acids were added to each culture (10 µCi/ml) and the cultures were harvested at 360 min p.i. Pelleted infected cells were resuspended in RSB at 4°C for 5 min. A cytoplasmic extract was prepared by 50 strokes of a Dounce homogenizer and the nuclei were removed by centrifugation (2,000 x g; 5'; 4°C). The extract was layered on a linear gradient of 5%-30% sucrose in RSB + 1.5 mM $MgCl_2$ and subjected to ultracentrifugation (SW 27; 25,000 rpm; 46 h; 4°C). A control gradient was run in parallel with 0.25-mg catalase (11 S) and 0.25-mg urease (16 S) (92). The control gradient was fractionated using a Gilford flow-through spectrophotometer at 280 nm and the positions of the marker proteins were recorded.

Effects of Temperature Shift on tsB9 RNA

Cultures of tsB9 and wt were established using the standard infection procedure. Control cultures of wt and tsB9 were maintained at 33°C and at 39.5°C. At 90 min p.i., sample cultures of both wt and tsB9 were shifted from 33°C to 39.5°C (shift-up) and from 39.5°C to 33°C (shift-down). After allowing 5 min for temperature equilibration, 10 µCi/ml of ^{3}H-uridine was added to the shifted cultures which

were labeled for 1 h and harvested. At 240 min the shift procedure was repeated. One set of control cultures was labeled for 1 h from 95 to 155 min p.i., while a second set of control cultures was labeled from 245 to 305 min p.i.

All cultures were harvested, phenol-chloroform extracted, and the RNA was ethanol precipitated as previously described. The extracted RNA was analyzed by electrophoresis in 1% agarose gels.

Effects of Temperature Shift on Virions and Procapsids of tsB9

The effect of temperature shift-down on virion and procapsid production in tsB9-infected cells was investigated. The standard infection procedure was followed to establish cultures of wt and tsB9 at 39.5°C and 33°C. The cultures were incubated in JMEM lacking tyrosine, leucine, and lysine, and supplemented with 5% dialyzed horse serum. At 90 min p.i., 20 µCi each of ^{3}H tyrosine, ^{3}H-lysine, and ^{3}H-leucine per ml of culture was added to one set of cultures at 39.5°C. These cultures were down-shifted at 180 min p.i. Cycloheximide (0.1 mg/ml) was added to one-half of each down-shifted culture. After 1 h at 33°C, the cultures were harvested. At 300 min p.i., the labeling and shift procedures were repeated. Control cultures were labeled from 240 to 360 min p.i. in the absence of cycloheximide.

The infected cells were harvested and analyzed by sucrose density gradient centrifugation as described earlier for the examination of virion and procapsid (SW 41; 30,000 rpm; 90 min; 4°C; 10%-22% sucrose in TNE).

Development of a Phenotype Screening Procedure

Synthesis of Actinomycin-D-resistant RNA

The procedure for determining the extent of accumulation of TCA insoluble RNA was described earlier. In the screening procedure, a m.o.i. of 10 pfu/cell was always used. In order to establish the criteria for scoring the mutants, cultures of wt, DI, g^rH, and tsB9 were analyzed at the same temperature as the newly generated mutants. Mutants which stimulated RNA accumulation equal to wt ± 10% at 39.5°C were scored as "+". Mutants which stimulated RNA accumulation less than or equal to 50% of wt levels at 39.5°C, as does tsB9, were scored as "-". Mutants which produced 51%-80% of the wt amount of 39.5°C were scored as "+/-".

Guanidine Sensitivity of RNA Synthesis

In order to determine whether the RNA synthesis of a given strain was sensitive to guanidine, the rate of RNA synthesis and the accumulation of TCA insoluble RNA at 33°C were determined as previously described. Guanidine HCl (5 mM) was added to each g+ culture at 150 min p.i. Replicate

cultures were maintained in the absence of guanidine (g-). Each virus strain was scored as g^s or g^r on the basis of the difference between the acid-insoluble material seen in the presence and in the absence of guanidine. If the acid-insoluble radioactivity found in the g+ culture was significantly less than that of the g- culture of the same strain, that strain was designated g^s. If no significant difference was seen, the strain was designated g^r.

Protein Synthesis at the Restrictive Temperature

The procedure for monitoring protein synthesis was described earlier. For the screening procedure, JMEM containing 1/10 the normal concentration of essential amino acids and supplemented with 5% dialyzed horse serum was used. The newly synthesized cytoplasmic proteins were labeled with ^{3}H-amino acids (Amersham).

Cultures of wt and DI were used to define the phenotype of wt (+) protein synthesis at 39.5°C. Cultures of tsB9 were used to define the ts mutant (-) phenotype. All mutants which reached the rate of protein synthesis of the wt at 39.5°C were scored "+". Those equal to or less than tsB9 were scored "-". Any mutants whose rate of protein synthesis at 39.5°C fell between that of the tsB9 and wt were scored "+/-".

Choline Incorporation into Membranes

The procedure for determining uptake of radioactive choline into acid-insoluble material was the same as that used to measure the accumulation of acid-insoluble RNA with a few exceptions. The newly assembled membranes were labeled with ^{3}H-methyl-choline (20 μCi/ml).

The wt cultures were used to define the "+" phenotype for membrane synthesis at 39.5°C. Cultures of tsB9 were used to define the mutant "-" phenotype at 39.5°C. All mutants that were equal to wt ± 20% in incorporation of choline at 39.5°C were scored as "+"; those that were equal to or less than 50% of wt were scored as "-", and those that were 51%-80% of wt were scored as "+/-".

The pH of the cultures was maintained by careful gassing with CO_2 generated by the sublimation of dry ice. This was crucial, as pH increases result in the uptake of choline in both infected and uninfected cells (102).

Presence of 35 S and 20 S RNA

A modification of the standard infection procedure was used to determine the presence of 35 S and 20 S RNA in infected cells. HeLa S-3 cells grown to a density of 4 x 10^5 cells/ml were pelleted (800 x g; 10'; RT) and washed with isotonic saline. A sample of 6 x 10^6 cells were infected at a m.o.i. of 10 pfu/cell and allowed to adsorb

for 30 min at room temperature. The infected cells were diluted to 6×10^6 cells/ml with JMEM + 5% horse serum. The cultures were then placed in a water bath maintained at 39.5°C ± 0.2°C. At 60 min p.i., actinomycin-D was added to each culture (7 µg/ml culture). At 90 min p.i., 60 µCi/ml of ^{3}H-uridine was added to each culture. The cultures were harvested at 240 min p.i. The pelleted cells were lysed in 0.225 ml of RSB + 1% Nonidet P-40 + 1% sodium deoxycholate (DOC). The nuclei were removed by centrifugation (2,000 x g; 10'; 4°C). The cytoplasmic extract was analyzed on linear gradients of 14% to 30% (w/w) sucrose in TNE (SW 50; 39,000 rpm; 5 h; 4°C). Two-drop fractions were collected directly into liquid scintillation minivials (West Chem, San Diego, CA) by bottom puncture and the radioactivity was measured.

Mutants producing amounts of 35 S RNA equal to that of wt at 39.5°C were scored as ss^+, using the nomenclature of Cooper (32, 33) and Cooper et al. (34). Those producing amounts of 20 S RNA equal to wt were scored ds^+. Mutants producing little or no 35 S were scored as ss^-, and those producing little or no 20 S were scored ds^-. The +/- designation was used for mutants with intermediate amounts of particular RNA.

Detection of 80 S RNP
by "In Situ Lysis"

In order to investigate the production of 80 S RNP produced by the various mutant strains during infection, cells were infected and labeled as described above for the determination of the levels of 35 S and 20 S RNA. The infected cells were pelleted (800 x g; 5'; 4°C) and resuspended in 0.25-ml RSB. The resuspended cells were layered onto linear gradients of 14% to 30% (w/w) sucrose in TNE overlaid with 0.25 ml of 5% sucrose in RSB + 1% Nonidet P-40 + 1% DOC. These "in situ lysis" gradients were subjected to ultracentrifugation in a Spinco SW 50 rotor. The gradients were centrifuged at 1,000 rpm for 2 min to move the cells into the lysis layer containing the detergents. The speed was then increased to 39,000 rpm and maintained for 3.5 h. All manipulations using the in situ lysis procedure were carried out at 4°C unless specifically indicated otherwise. Two-drop fractions were collected directly into minivials and the radioactivity was determined. The area under the 80 S RNP curve was determined for each strain and the wt value was arbitrarily set equal to 1.0.

Detection of 150 S Virion
by In Situ Lysis

The infection, harvesting, and analysis procedures were as described above for 80 S RNP with the following exceptions. The cultures were harvested at 360 min p.i.

(39.5°C) or 480 min p.i. (33°C). The in situ lysis gradients were centrifuged for 90 min after increasing the speed. Radioactivity was determined as described below.

Detection of 80 S Procapsid by In Situ Lysis

To detect the presence of 80 S procapsid, the cells were infected as described for 80 S RNP except that a m.o.i. of 150 pfu/cell was used. At 150 min p.i., guanidine HCl was added to a final concentration of 5 mM. At 210 min p.i., 60 μCi of ^{3}H-amino acids was added to each culture. The cultures were harvested at 270 min p.i. (39.5°C) and analyzed by the in situ lysis procedure described above. The RSB used for procapsid analysis contained 1.5 mM $MgCl_2$. The gradients were centrifuged for 3.5 h after increasing to full speed. All g^s strains were compared to wt by this technique. All g^r strains were analyzed as described for virion (not using guanidine) except that buffers and centrifugation times were as just described for procapsid. The g^r strains were compared by this technique with g^rH.

Inhibition of Host-cell Protein Synthesis

The procedure for monitoring the inhibition of host-cell protein synthesis was described previously. All virus strains were evaluated for the ability to inhibit host-cell protein synthesis at 39.5°C.

Analysis of Plaque Size

Plaque size and appearance vary among mutants of a given picornavirus (32). The significance of this feature is unknown. Wild-type plaques observed with the Cooper-type plaque assay previously described are 5 to 7 mm in diameter and clearly defined (personal observation). The plaques formed by the mutant strains at 33°C and 39.5°C were scored as "+" (5 to 7 mm), "-" (3 to 4 mm), minute (1 to 3 mm), and large (7 to 10 mm).

RESULTS

Characterization of the tsB9 and g^rH Virus Strains

Growth Characteristics

Plaque Assays and Efficiency-of-plating Quotients. Standard plaque assays were performed under several conditions in order to demonstrate the typical growth characteristics of the virus strains. The plaque-forming ability of tsB9, g^rH, and wild-type (wt) strains were examined at two temperatures and in the presence and absence of guanidine to establish the temperature sensitivity and guanidine sensitivity of these virus strains.

The titer of wt at 33°C was determined to be 1.0002×10^{10} plaque-forming-units (pfu)/ml while at 39.5°C the titer was found to be 1.1×10^{9} pfu/ml. This is an efficiency-of-plating (e.o.p.) quotient 33/39.5 of 0.91×10^{1} (Table 1).

The titer of g^rH at 33°C was found to be 8.5×10^{9} pfu/ml in the absence of guanidine while at 39.5°C it was 7.96×10^{8} pfu/ml in the absence of guanidine. This is an e.o.p. 33/39.5 of 1.07×10^{1}, approximately the same as wt. It can be concluded, therefore, that g^rH is not a temperature-sensitive (ts) mutant with respect to growth.

Table 1. Titers of virus stocks at the permissive (33°C) and restrictive (39.5°C) temperatures.--No guanidine was present during assay.

Virus Strain	Average pfu/ml		e.o.p. 33/39.5°C
	33°C	39.5°C	
Wilt-type	1.00×10^{10}	1.10×10^{9}	0.91×10^{1}
tsB9	5.95×10^{9}	3.20×10^{4}	1.86×10^{5}
$g^{r}H$	8.51×10^{9}	7.96×10^{8}	1.07×10^{1}

It was found that the average titer of tsB9 at 33°C was 5.95×10^{9} pfu/ml, while at 39.5°C it was 3.2×10^{4} pfu/ml. This yields an average e.o.p. 33/39.5 of 1.86×10^{5}; thus tsB9 can be designated as temperature-sensitive.

Effects of Temperature on the Infectivity of tsB9. Once it was determined that tsB9 was temperature-sensitive with respect to plaque formation, it was decided to determine at what temperature this defect was expressed. Standard plaque assays were carried out on tsB9 at temperatures ranging from 33°C to 41°C.

The plaque-forming ability of tsB9 was essentially the same at temperatures ranging from 33°C to 36°C (Table 2). At 37°C a drop in titer of approximately 10-fold occurred. Another 10-fold drop in titer was observed at 38°C. At 39°C the titer decreased to 3.3×10^4 pfu/ml, a decrease factor of 1.7×10^5 from the titer at the fully permissive temperature. At 40°C no plaques were seen at any dilution or with undiluted tsB9 stock, although the cell lawns were well developed. No cell lawns were established at 41°C, at which temperature the cells clumped and died within 10 h.

Table 2. Temperature curve of infectivity of tsB9 in HeLa cells in soft agar

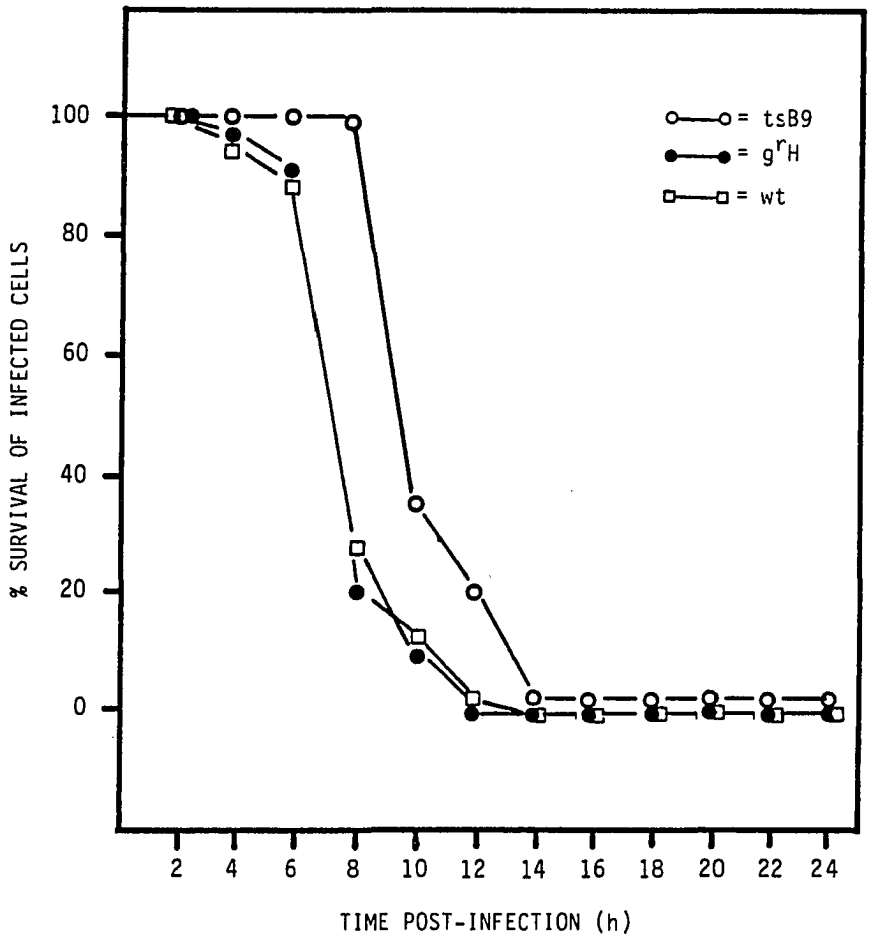
Temperature (°C)	Titer (pfu/ml)
33	5.6×10^9[a]
34	5.2×10^9
35	5.4×10^9
36	5.0×10^9
37	4.8×10^8
38	4.3×10^7
39	3.3×10^4
40	0
41	---

a. These figures reflect the titer of a signle virus stock.

Generation of Cytopathic Effects in tsB9 and g^rH Cultures. The generation of cytopathic effects (CPE) is one of the characteristic actions of poliovirus infection on the host cell. Garwes, Wright, and Cooper (54) isolated a number of ts mutants of poliovirus that were significantly depressed in the ability to generate CPE at the restrictive temperature. Garwes et al. evaluated the viability of infected cells from their ability to exclude trypan blue. Cells that excluded trypan blue were defined as viable and not exhibiting CPE.

The ability of tsB9, g^rH, and wt to generate CPE was measured by the method of Garwes et al. (54). Cultures were established at 39.5°C. The viability of the infected cells was measured at 0 h post-infection (p.i.) and every 2 h for a period of 24 h. From 0 to 4 h p.i. no decrease in viable cell count occurred in tsB9, g^rH, or wt cultures. The first morphological changes occurred at 2 h p.i. in wt and g^rH cultures, and at 4 h p.i. in tsB9 cultures. These changes included the appearance of the usually centered HeLa cell nucleus off to one side of the cell and an alteration of the cell membrane's appearance from smooth to rough and irregular. Appearance of these changes preceded the uptake of trypan blue by the cells.

Figure 3 shows the decrease in the percentage of viable cells as the time p.i. increased with the viable



Trypan blue exclusion was used to determine the viable cell count at various times post-infection (p.i.). HeLa S-3 cells grown to a density of 4 x 10^5 cells/ml were pelleted and washed in isotonic saline (750 x g, 10 min at room temperature (RT)). The cells were infected at a multiplicity-of-infection of 10 plaque-forming-units/cell with wild-type (wt) or g^rH and allowed to adsorb for 30 min at RT at a cell density of 4 x 10^7 cells/ml. The infected cells were then diluted to a density of 4 x 10^6 cells/ml with Joklik's modified Eagle's medium + 5% horse serum and stirred at 39.5°C. This point is defined as 0 min p.i. At 0 min p.i., a 0.2-ml sample of infected cells was diluted 10-fold with isotonic saline and made 0.4% in trypan blue. The number of viable cells, those not stained, was determined for each sample using a hemacytometer and a hand tally counter. Samples were taken from each culture every 2 h and 24 h p.i.

Figure 3. Cytopathic effects of poliovirus strains at 39.5°C

cell count at "0" h defined as 100%. By 14 h p.i., no remaining viable cells were apparent in the wt or g^rH cultures. Upon examining Figure 3, the 1,000 cells/ml found in tsB9 cultures do not appear dramatically different when considered as a percentage of the original viable cell count, but it seems significant that the tsB9 culture contained 1,000 or more viable cells/ml up to 24 h p.i. when the experiment was terminated. While it is apparent that tsB9 is not as effective as wt in producing CPE, it is not defective to the same extent as the mutants found to be defective by Cooper (32, 33).

Electron Microscopic Observations. The effects of virus infection on the host cells were also examined at the ultrastructural level (data not shown). Cultures of wt and tsB9 incubated at the restrictive and at the permissive temperatures were examined by electron microscopy. The g^rH-infected cells were not examined since their infectivity was approximately equal to wt at both temperatures.

The wt-infected cells incubated at 33°C and at 39.5°C exhibited extensive disarrangement of the cytoplasm. No intact mitochondria were seen. Furthermore, there was extensive vacuolization of the cytoplasm and there appeared to be many cytoplasmic membranous cisternae. The tsB9-infected cells incubated at 33°C were indistinguishable from the wt-infected cells.

In contrast, the tsB9-infected cells incubated at 39.5°C did not exhibit any gross disorganization. The mitochondria were intact and distinct. There were no signs of vacuolization. The tsB9-infected cells incubated at 39.5°C were essentially the same in appearance as mock-infected cells.

Guanidine Sensitivity of Infectivity. Guanidine has been shown to specifically inhibit the growth of poliovirus at low concentrations which have no cytotoxic effects on the host cells (3-5 mM) (24, 25, 36). Mutants of poliovirus have been found which differ from the wt strain in their response to the presence of guanidine (24, 25, 36). Some mutants are resistant to the growth-suppressing effects of guanidine. Their titers in the presence and absence of guanidine are approximately the same. Other mutants have been found to be dependent upon the presence of guanidine for their growth; their titer in the presence of guanidine is greater than that in the absence of guanidine.

Since guanidine sensitivity is such a well-established characteristic of poliovirus, the guanidine sensitivity of wt, g^rH, and tsB9 was examined.

The presence of guanidine (5 mM) reduced the apparent titer of both wt and tsB9 virus at 33°C but had no effect on the titer of g^rH. The replication of tsB9 is only half as sensitive to guanidine as wt. The replication of g^rH

is essentially insensitive to guanidine, thus confirming its guanidine-resistant (g^r) character (Table 3).

Although guanidine can be seen to exert a significant inhibitory effect on the replication of wt and tsB9, the effective remaining titer in its presence is still considerable. This must be kept in mind when interpreting the results of experiments in which guanidine is used.

Temperature Shift-down Effects of Infectivity of tsB9. It was decided to determine whether the effects of exposure to the restrictive temperature on the growth of tsB9 were reversible by shifting down to the permissive temperature early and late in infection. The effects of temperature shift-down from 39.5°C to 33°C were examined for tsB9 and wt. Since g^rH is not temperature-sensitive, it was not

Table 3. Titer of tsB9, g^rH, and wild-type at 33°C ± guanidine HCl (5 mM)

Virus Strain	Titer - guanidine	Titer + guanidine	e.o.p. -g/+g
Wild-type	1.00×10^{10}	6.50×10^{6}	1.54×10^{3}
tsB9	5.80×10^{9}	7.11×10^{6}	8.16×10^{2}
g^rH	8.51×10^{9}	8.04×19^{9}	1.06

examined. Virus stocks were prepared as described in Methods and Materials, with the only variation being in the temperature of incubation. Control stocks were maintained continuously at 33°C and 39.5°C. Sample cultures were shifted down at 90 min p.i. and again at 240 min p.i.

Table 4 shows the titer of all stocks. No significant effects of temperature shift-down were exhibited by the wt culture. The tsB9 culture, on the other hand, showed a marked effect of temperature shift-down on the titer of the virus stock prepared from the shifted culture. The tsB9 cultures shifted down at 90 min p.i. yielded stocks with approximately the same titer as the control culture of tsB9 maintained at 33°C. Shift-down later in infection resulted in a titer 5.98×10^3 times greater than that at 39.5°C but still 3.67 times less than the 33°C control (27%).

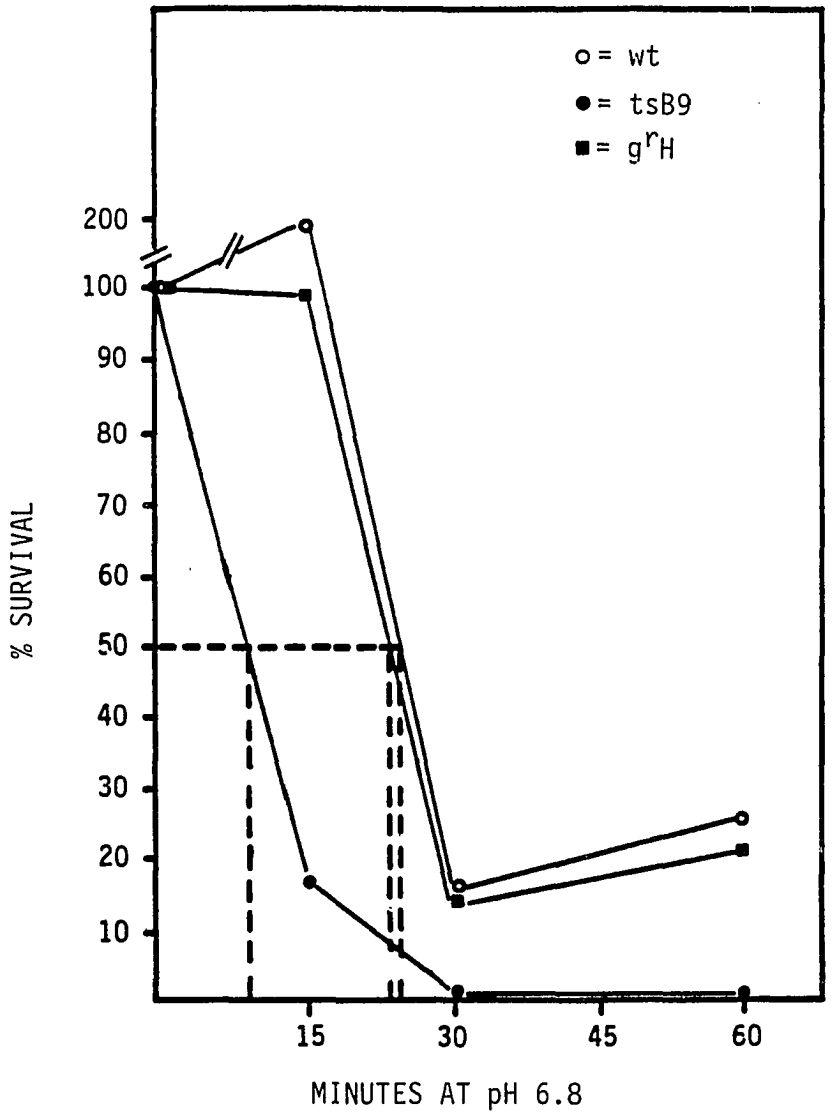
Sensitivity of the Virus Infectivity to pH 6.8.

Mutants of poliovirus with defects in the structural proteins have been found to have less stability than the parent wt strain to exposure to acid pH (90). The protocol of Cooper et al. (35) was used to determine the stability of tsB9 and wt virus to a pH of 6.8. Stocks of tsB9, g^rH, and wt were exposed to pH 6.8 for 15, 30, or 60 min as described in Methods and Materials. The remaining infectivity, as determined by plaque assay, is shown in Figure 4. The infectivity of all three virus strains decreases after exposure to

Table 4. Temperature shift plaque assays.--Virus stocks were prepared from cultures incubated at 39.5°C, 33°C, and from cultures shifted down from 39.5°C to 33°C during the infectious cycle. Results are expressed as titer (pfu/ml) determined at 33°C by standard plaque assay as described in Methods and Materials.

Virus Strain	Starting Temperature (°C)	Final Temperature (°C)	Time of Shift (min p.i.)	Titer (at 33°C)
wild-type	33	no shift	---	7.8×10^9
wild-type	39.5	no shift	---	7.5×10^8
wild-type	39.5	33	90	7.5×10^9
wild-type	39.5	33	240	5.7×10^9
tsB9	33	no shift	---	5.8×10^9
tsB9	39.5	no shift	---	2.6×10^{5}[a]
tsB9	39.5	33	90	5.43×10^9
tsB9	39.5	33	240	1.6×10^9

a. The stock of tsB9 grown continuously at 39.5°C was still temperature sensitive. Its titer at 33°C was 2.6×10^5, while no plaques were seen at 39.5°C.



The sensitivity of virus infectivity to acid pH (6.8) was determined. Aliquots of 0.5 ml of virus stocks were adjusted to pH 6.8 by the addition of 0.1 N HCl. After 15, 30, and 45 min at room temperature, the stocks were adjusted to pH 7.4 by the addition of 0.1 N NaOH and immediately frozen at -20°C. The remaining infectivity was determined by the standard plaque assay at 33°C as described in Methods and Materials. The dotted lines identify the $T_{\frac{1}{2}}$ of each strain. $T_{\frac{1}{2}}$ (wild-type (wt)) = 24.3 min; $T_{\frac{1}{2}}$ (tsB9) = 8.7 min; $T_{\frac{1}{2}}$ (g^rH) = 23.9 min.

Figure 4. Acid sensitivity of virus infectivity at the permissive temperature

pH 6.8. The g^rH stock was approximately equal to the wt stock in its acid stability. The tsB9 stock was found to be less stable than wt to acid exposure. The $T_{½}$ of wt infectivity at pH 6.8 was 24.3 min, that of g^rH was 23.9 min, and that of tsB9 was 8.7 min.

Sensitivity of Virus Infectivity to Heat. Structural protein mutants of poliovirus are also often less stable to heat exposure than the wt strain (90). It was decided to determine whether tsB9 or g^rH were significantly different from wt in their stability to heat exposure.

Stocks of tsB9, g^rH, and wt were exposed to 45°C for 15, 30, or 45 min as described in Methods and Materials. The remaining infectivity was assessed by the standard plaque assay. The infectivity of tsB9 and g^rH was slightly less heat-stable than wt but the difference was not appreciable. The $T_{½}$ of wt at 45°C was 9.2 min, of g^rH it was 8.7 min, and that of tsB9 was 8.0 min.

Complementation of tsB9 and Defective-interfering Strains

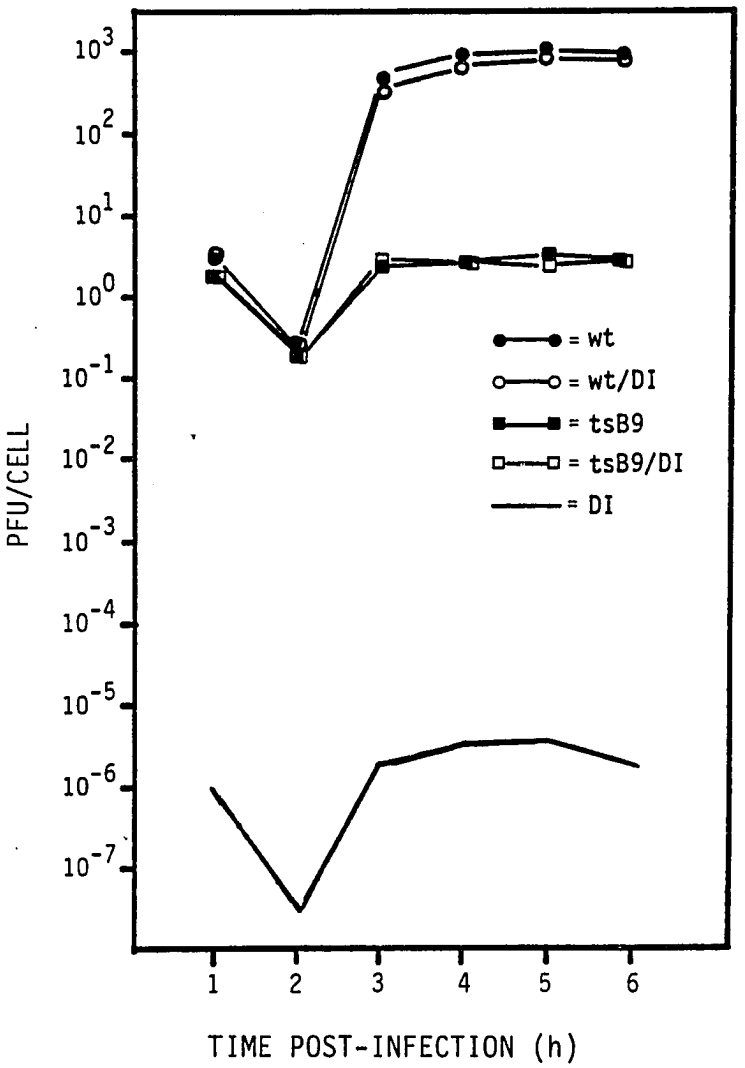
Simple Clearing of the Cell Lawn. If two poliovirus strains complement significantly under restrictive conditions, they should cause cell death indicative of a productive infection. Both pure and mixed virus stocks were inoculated into replicate cell lawns of HeLa cells in soft agar as described in Methods and Materials (data not shown). The

pure stocks of tsB9 and defective-interfering (DI) produced no clearing of the cell lawn at 39.5°C as the wt inoculation did. The mixed tsB9/DI stock also failed to produce clearing in the cell lawn in any sample. All inocula containing wt virus produced definite clearing in the cell lawn around the point of inoculation. No complementation between tsB9 and DI is indicated by these data.

One-step Growth Curve. The possible complementation of tsB9 and DI at the restrictive temperature was also examined by generation of a one-step growth curve, as described in Methods and Materials. Both pure and mixed stocks were used to infect cells which were then incubated at 39.5°C. At 1-h intervals, samples were taken and virus stocks were prepared. The pfu/cell were determined by the standard plaque assay at 33°C (Figure 5).

As expected from the literature, the wt pure culture produced slightly more pfu/cell than did the mixed culture of wt/DI. The DI culture produced few plaques, as expected from the nature of its defect. The tsB9 culture produced a maximum of 3.52 pfu/cell. The tsB9/DI mixed infection produced a maximum of 3.45 pfu/cell at the same time point. Neither complementation nor interference is indicated by these data.

The failure of tsB9 and DI to complement at the restrictive temperature agrees well with the results of Manos and Hewlett (personal communication, 1978 (91)).



HeLa cells were infected at a total multiplicity-of-infection of 10 plaque-forming-units (pfu)/cell with several poliovirus pure and mixed strains and incubated at 39.5°C. All stocks were diluted in such a way that their titers were equal. To generate mixed stocks, equal volumes of these normalized stocks were mixed. Samples were taken from each culture at 6 times post-infection, and the pfu/cell yielded was determined by standard plaque assay at 33°C.

Figure 5. One-step growth curve

Reversion to Wild-type

A common method of determining whether two temperature-sensitive characters of a virus mutant are genetically related is the measurement of the co-reversion frequency. If both characters revert to wt concomitantly, then the likelihood of the characters being the result of two unrelated mutations is considered low.

Stock of tsB9 was plaque-purified, selecting only plaques confirmed to be temperature sensitive. Four consecutive passages were made at a multiplicity-of-infection (m.o.i.) of 10 pfu/ml. At each passage, stocks were prepared and plated at 39.5°C. Plaques formed at 39.5°C were cored and tested for temperature sensitivity by replicate plating at 33°C and 39.5°C. Plaques which scored as 33 + 39.5 + were designated as wt revertants, cored and titered by the standard plaque assay. Since these were derived from plaque-purified tsB9 determined to have maintained its temperature sensitivity, it is not likely that a significant degree of wt contamination was present.

Revertants were used to infect suspension cultures using the standard infection procedure as described in Methods and Materials. Their RNA synthesis at 39.5°C was measured as the accumulation of acid-insoluble material labeled with ^{3}H-uridine. The synthesis of virion at 39.5°C was determined as total radioactivity in the virion fractions

of sucrose density gradients of cytoplasmic extracts. Results were expressed as the percentage of the wt control (Table 5).

The number of revertants was low for the first three passages. At the fourth passage, an approximate 10-fold increase in the number of revertants was seen. Subsequent experiments were done with stocks passaged only twice in my hands to avoid the complications of high passage revertants. These stocks were prepared at different times from a single passage stock maintained at -70°C in small aliquots.

In every revertant, the loss of temperature sensitivity of growth was accompanied by reversion to wt RNA synthesis and virion production. It is suggested on this basis that tsB9 is not a double mutant. It is further suggested that the RNA(-) phenotype is related to the virion(-) phenotype genetically, functionally, or by conformation of precursor proteins.

Virion and Procapsid Characteristics

Determination of the Presence of Procapsid and Virion. One of the primary characteristics of a virus is the production of virions and this is one of the functions which may be defective in a virus mutant. In order to determine whether tsB9- and g^{r}H-infected cells produced virions and procapsids at the wt level, standard infections were carried out under varying conditions. The virions and

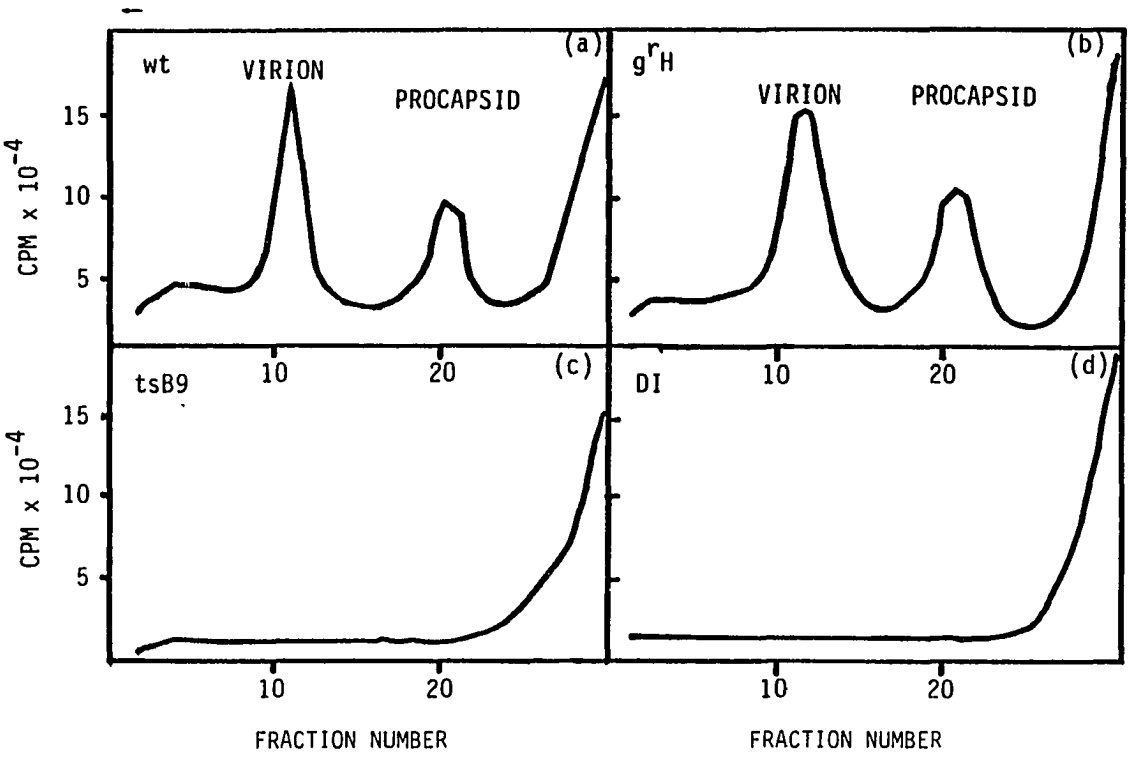
Table 5. Phenotype of tsB9 revertants.--Revertants to a non-temperature sensitive phenotype were selected at 39.5°C and used to infect HeLa spinner cultures. The RNA synthesis was determined by ^{3}H-uridine uptake into acid-precipitable material. The amount of virion was determined by uptake of ^{3}H-uridine into the virion fraction visualized on sucrose density gradients. Numbers in parentheses refer to the number of revertant plaques.

	RNA Synthesis (% of wt)	Virion (% of wt)
Passage 2		
Stock 1 (2)	96.8	98.9
Stock 2 (1)	96.3	93.6
Stock 3 (3)	98.2	98.6
Passage 3		
Stock 1 (5)	101.2	100.3
Stock 2 (1)	101.1	99.5
Stock 3 (2)	98.4	99.1
Stock 4 (1)	99.9	98.5
Stock 5 (4)	99.0	99.3
Passage 4		
Stock 1 (40)	100.2	99.1
Stock 2 (29)	96.8	97.7
Stock 3 (36)	101.7	99.9
Stock 4 (28)	101.6	99.6
Stock 5 (41)	100.5	98.5

procapsids were radioactively labeled by incubation of the cultures with 10 μCi/ml of ^{35}S-methionine (tsB9 or wt) or ^{3}H-amino acids (DI or g^{r}H) from 180 to 360 min p.i. (39.5°C) or from 180 to 480 min p.i. (33°C). The infected cells were harvested and analyzed by sucrose density gradient centrifugation as described in Methods and Materials.

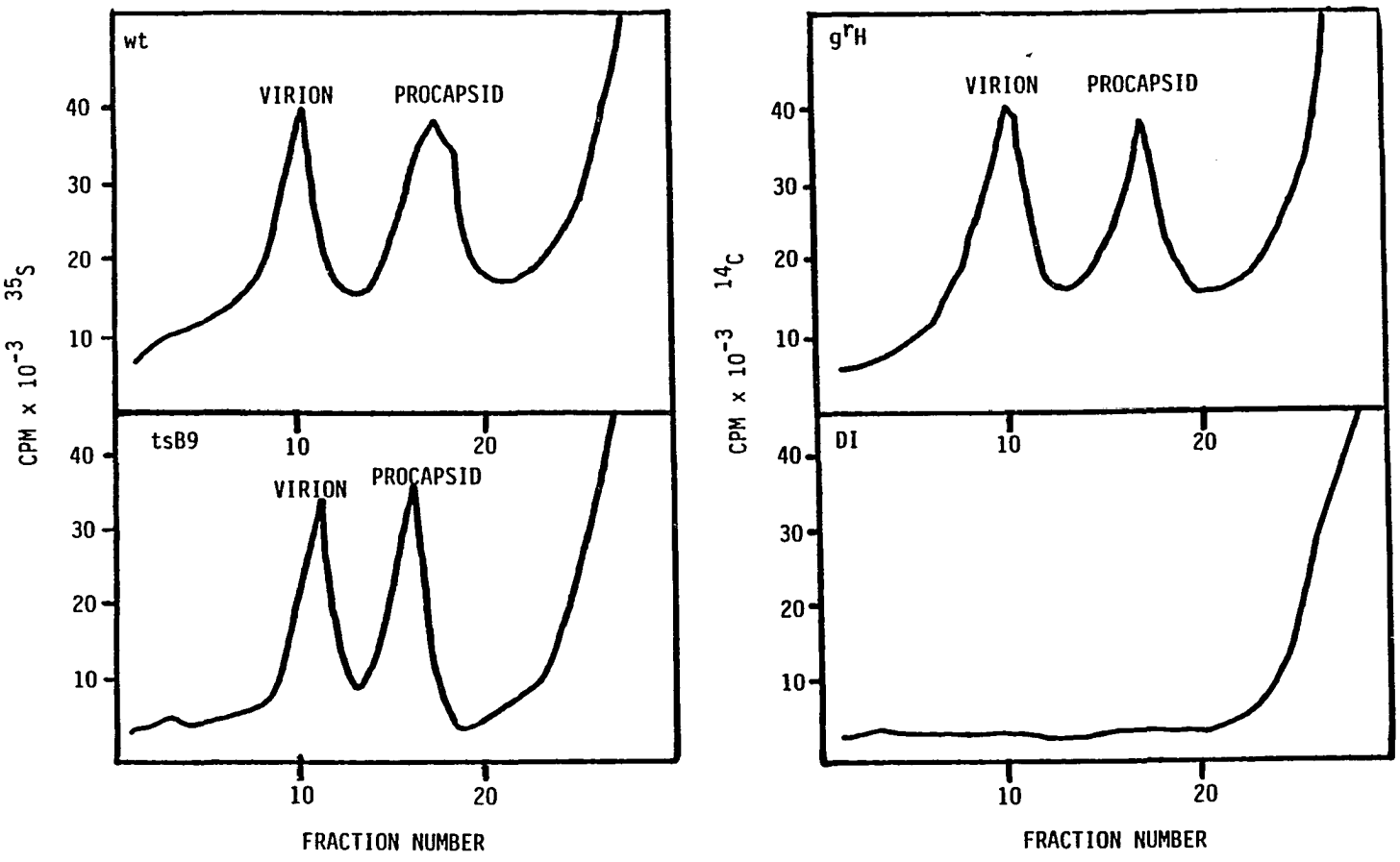
When the cultures were incubated at 39.5°C, wt produced labeled virions and procapsids, while tsB9 produced neither (Figure 6). Stocks of DI and g^{r}H were examined at 39.5°C even though they are not temperature-sensitive. When the cultures were incubated at 33°C, tsB9- and g^{r}H-infected cells produced approximately wt levels of both virions and procapsids, while DI-infected cells produced neither (Figure 7).

Complementation of tsB9 and DI with respect to particle formation at 39.5°C was examined. The above-described experiments were repeated with pure and mixed infections of wt, tsB9, DI, wt/DI, and tsB9/DI. No virions or procapsids were produced by the tsB9, DI, or tsB9/DI cultures (Figure 8). The wt and wt/DI cultures produced normal levels of both virions and procapsids. The virion peak in the wt/DI culture had a slower sedimenting shoulder corresponding to the expected lighter DI particles. The location of wt virion and procapsid peaks in the parallel gradient are indicated by arrows.



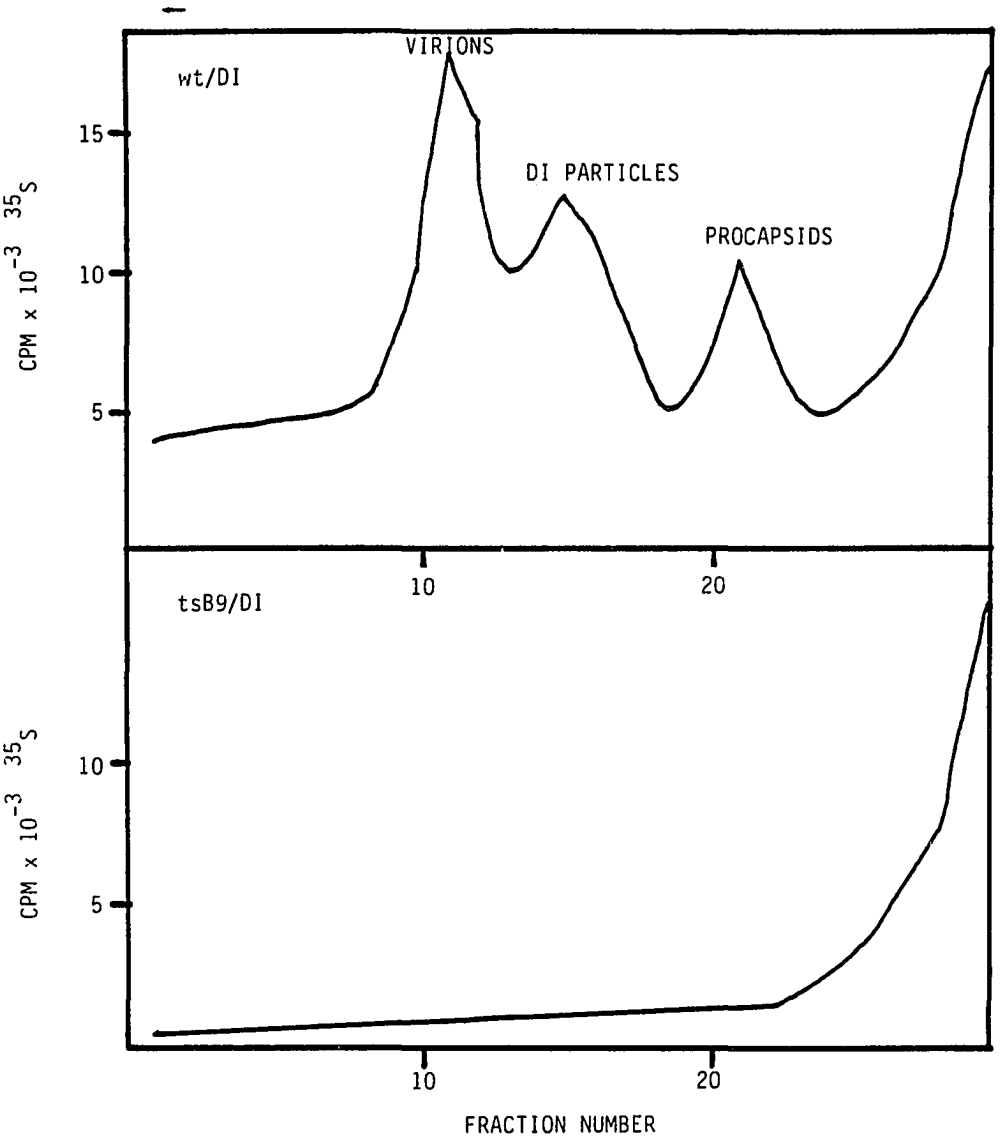
HeLa cells were infected at a multiplicity-of-infection of 10 plaque-forming-units/cell in the presence of 5 µg of actinomycin-D per ml of culture according to the standard infection procedure. The cultures were labeled with 10 µCi/ml of ^{35}S-methionine or ^{14}C-amino acids from 180 to 360 min post-infection and harvested as described in Methods and Materials. Cytoplasmic extracts were prepared in ice-cold RSB + 1% NP-40 and cleared of nuclei by centrifugation. The extracts were subjected to ultracentrifugation through linear gradients of 5% to 30% sucrose in RSB + 1.5 mM $MgCl_2$ (SW 27; 3 h; 27,000 rpm; 4°C). (a) ^{35}S-methionine; (b) ^{14}C-amino acids; (c) ^{35}S-methionine; (d) ^{14}C-amino acids.

Figure 6. Production of virions and procapsids at 39.5°C



Infection and analysis were carried out as described for Figure 6 except that the cultures were harvested at 420 min post-infection. The wild-type (wt) and tsB9 cultures were labeled with 10 μCi/ml of ^{35}S-methionine while the g^{r}H and defective-interfering (DI) cultures were labeled with 10 μCi/ml of ^{14}C-amino acid mixture.

Figure 7. Production of virions and procapsids at 33°C

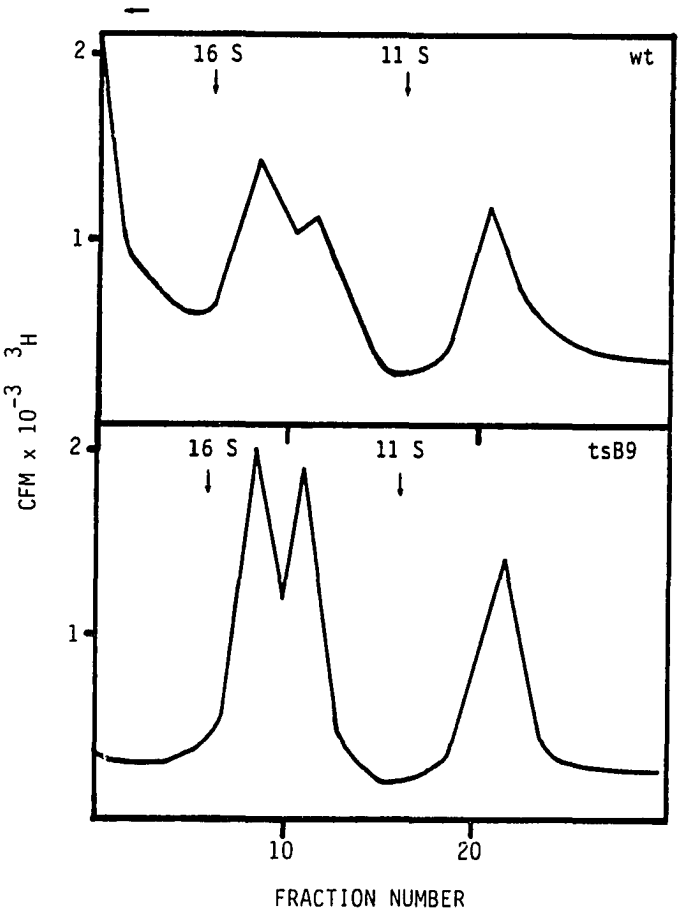


HeLa S-3 cells were infected as previously described with tsB9/DI or wt/DI mixed virus stocks and incubated at 39.5°C. Cultures were labeled with 10 µCi/ml of ^{35}S-methionine from 3 to 6 h post-infection. The infected cells were harvested and analyzed by sucrose density gradient centrifugation as described in Methods and Materials.

Figure 8. Complementation of tsB9 and DI at 39.5°C with respect to particle formation

Determination of the Presence of Procapsid Precursors. Production of procapsid precursors by tsB9-infected cells at the restrictive temperature was investigated. The tsB9-infected cells did not produce either virions or procapsids at the restrictive temperature, and it was of interest to note whether they were producing some form of the precursors to these particles. The $g^{r}H$-infected cells produced wt amounts of virions and procapsids and, therefore, they were not examined by this technique.

The presence of the 5 S and 14 S procapsid precursors in wt and tsB9 cultures at 39.5°C was examined by the procedure of Phillips, as described in Methods and Materials. The wt culture exhibited peaks at about 5 S and 14 S with a lighter shoulder which may be the 13 S immature particles found by Phillips and Fennell (110) and Phillips and Wiemart (112) (Figure 9). There is material at the bottom of the wt gradient, probably corresponding to virion and procapsid. The tsB9 culture also exhibited a peak at about 5 S and a split peak at about 13-14 S. A larger portion of the split peak was found in the slower fraction in tsB9 than in wt. It is suggested that tsB9 may produce more of the 13 S immature particles or particles which are defective in conformation at 39.5°C and cannot assemble. No material is seen at the bottom of the tsB9 gradient.



HeLa cells were infected at a multiplicity-of-infection of 150 plaque-forming-units/cell with wild-type (wt) or tsB9 virus stock and incubated at 39.5°C. Guanidine HCl was added to each culture at 150 min post-infection (p.i.) to a final concentration of 3 mM. At 210 min p.i., ^{3}H-amino acid mixture was added to each culture (10 µCi/ml) and the cultures were harvested at 360 min p.i. A cytoplasmic extract was prepared in a Dounce homogenizer and cleared of nuclei by centrifugation (2,000 x g; 5 min; 4°C). The extract was layered onto linear 5% to 30% sucrose in RSB + 1.5 mM $MgCl_2$ and subjected to ultracentrifugation (SW 27; 25,000 rpm; 46 h; 4°C). A marker gradient was run in parallel with 0.25 mg of catalase (11 S) and 0.25 mg of urease (16 S) and their position determined by absorbance at 280 nm. The positions comparable to catalase and urease are indicated by arrows.

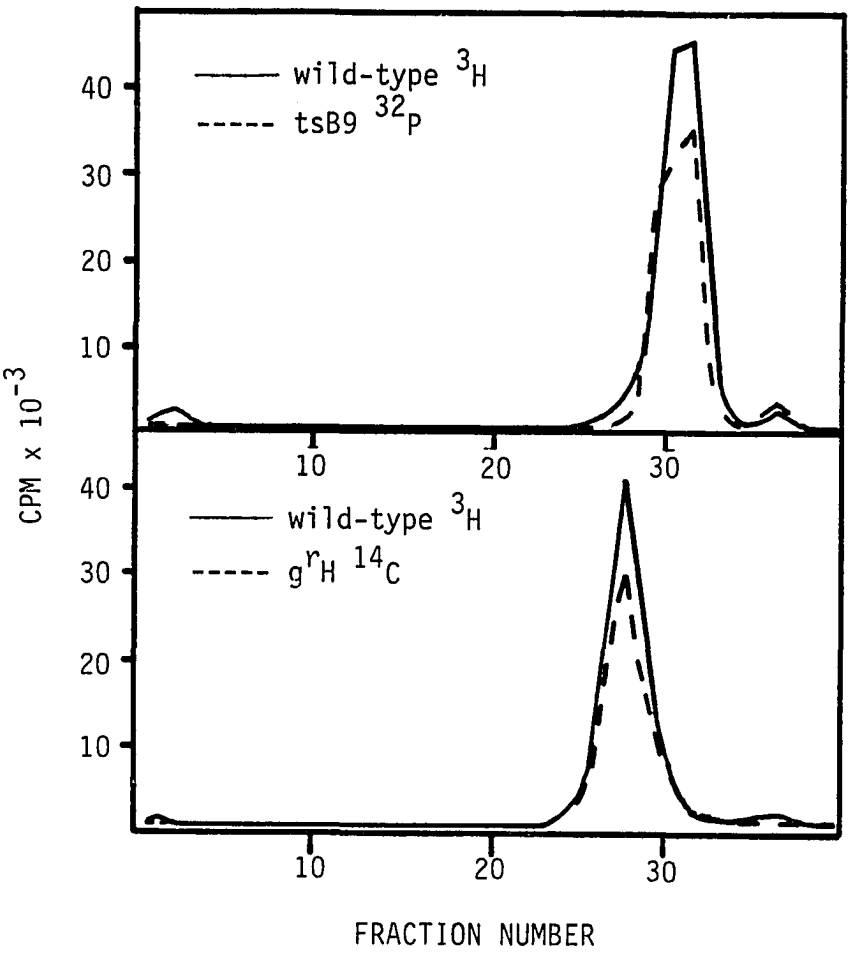
Figure 9. Synthesis of procapsid precursors by tsB9 at 39.5°C

Buoyant Density of Virions. One of the characteristic properties of a virion is its buoyant density. Mutants which differ significantly in their RNA and protein content from wt may have markedly different buoyant density than the wt virion, as is true for DI (27, 29).

To determine whether the defects in tsB9 or g^rH produced such aberrant particles at 33°C, the buoyant densities of wt, g^rH, and tsB9 virions were compared. Purified wt virions containing RNA labeled with ^3H-uridine were mixed with purified tsB9 virions containing RNA labeled with $^{32}PO_4$ and analyzed on CsCl gradients as described in Methods and Materials. Purified g^rH virions containing RNA labeled with ^{14}C-uracil were similarly mixed with wt virions containing RNA labeled with ^3H-uridine and analyzed on CsCl gradients.

No differences were seen in the banding patterns of the wt, tsB9, and g^rH virions (Figure 10). A 15% difference in RNA content is sufficient to permit resolution of the virions in this system (27, 29). It appears that neither tsB9 nor g^rH contain a large deletion of RNA such as that found in DI.

Sedimentation of Virions in Sucrose Density Gradients. The mixed virions prepared as above were also analyzed by sucrose gradient centrifugation as described in Methods and Materials. No differences were seen among the



Virions of wild-type (wt), tsB9, and $g^{r}H$ were labeled at 33°C with radioactive RNA precursors and purified through sucrose density gradients as described in Methods and Materials. The virion fractions were pooled and pelleted and resuspended in NEB buffer. The wt virions had been labeled by the uptake of ^{3}H-uridine, the tsB9 by the uptake of $^{32}PO_4$, and the $g^{r}H$ by the uptake of ^{14}C-uracil. Resuspended wt virions were mixed with tsB9 or $g^{r}H$ virions and subjected to equilibrium centrifugation in CsCl as described in Methods and Materials (20 h; Type 40; 35,000 rpm; 4°C).

Figure 10. Buoyant density of tsB9 and $g^{r}H$ virions compared to that of wild-type virions

virions produced by tsB9-, g^rH-, and wt-infected cells with respect to their sedimentation behavior (data not shown).

Sedimentation of Virion RNA in Sucrose Density Gradients and Migration in Agarose Gel Electrophoresis. Virion RNA from the cultures described above were purified separately, ethanol precipitated, and the ethanol suspensions were mixed as described in Methods and Materials (wt/tsB9 and wt/g^rH). The mixed virion RNA samples were analyzed by sedimentation through sucrose density gradients and by electrophoresis in 1% agarose gels as described in Methods and Materials. No apparent size differences exist among these three virion RNAs as determined by these two methods which consistently resolve RNAs with a 15% difference in size (12).

Determination of the Presence of VPg on Virion RNA

Picornavirus virion RNA is covalently linked at the 5' end to the small peptide, VPg, which appears to be involved in virus replication (96). Whether tsB9 virion RNA also possessed VPg was investigated. Virions radioactively labeled with $^{32}PO_4$ were prepared and the virion RNA purified as described in Methods and Materials. The virion RNA was digested to completion with a mixture of ribonucleases, and the digested samples were subjected to high-voltage ionophoresis and autoradiography.

Virion RNA from wt was analyzed in parallel as a control and to provide a marker for the migration of VPg-pUp. The autoradiograph was used to locate the nucleotide and VPg-pUp spots which were then cut out of the paper and soaked in liquid scintillation cocktail.

The VPg-pUp spot should contain approximately 2/7500 of the phosphate label of the RNA if uniform labeling is assumed. Therefore, the radioactivity found in each nucleotide spot and the VPg-pUp spot was added and the percentage of the total radioactivity in each was calculated (Table 6).

The genome-linked protein is apparently present in normal amounts on the virion RNA of tsB9. Its presence was confirmed by its ability to become labeled by Bolten-Hunter (Amersham, Arlington Heights, IL) reagent which iodinates protein but not RNA (Hewlett, personal communication, 1979 (61)). The g^rH virion RNA was not examined for the presence of VPg.

RNA Characteristics

Rate of Actinomycin-D-resistant RNA Synthesis. The synthesis of RNA is crucial to the replication of an RNA virus such as poliovirus. A number of ts mutants have been found which are defective in their synthesis of RNA at the restrictive temperature. It was, therefore, considered

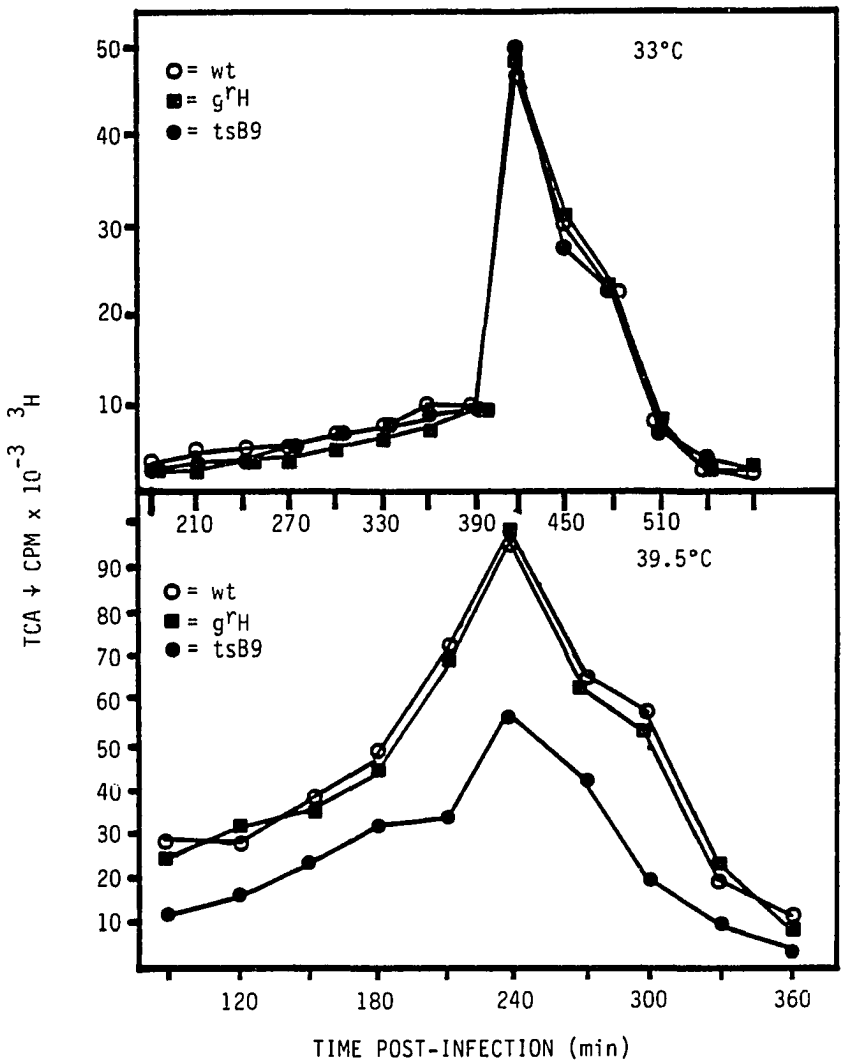
Table 6. Proportion of ^{32}P label found in nucleotides and VPg of tsB9 and wild-type (wt) virion RNA.--A greater amount of virion RNA was prepared for the tsB9 sample since the purpose of the wt was to serve as control and marker.

Virus Strain	Counts per Minute (% of total label)				
	VPg	C	A	G	U
wt	115 (0.028%)	109,628 (26.7%)	115,568 (28.1%)	92,008 (22.4%)	93,593 (22.8%)
tsB9	430 (0.027%)	408,924 (25.6%)	503,863 (31.6%)	386,206 (24.2%)	296,127 (18.6%)

essential to determine the RNA synthesis rate of tsB9-infected cells and compare it to that of wt-infected cells.

At 33°C, the peak of actinomycin-D-resistant RNA synthesis occurred at approximately 410 min p.i. for wt-, g^rH-, and tsB9-infected cells (Figure 11). The time course of RNA synthesis and the rate of synthesis by wt, g^rH, and tsB9 are similar at 33°C.

On the other hand, at 39.5°C the rate of RNA synthesis in the tsB9 cultures was considerably less than that of the wt cultures. When the average differences were examined, it was found that the rate of RNA synthesis in tsB9



The standard infection procedure was followed with the exception that the infected cells were pulse labeled instead of continuously labeled. At 90 min post-infection and every 30 min thereafter, 0.1 ml of infected cells were removed from each culture and placed in a microcentrifuge tube and incubated for 10 min in the presence of 5 μCi of ^{3}H-uridine at 39.5°C. The acid-insoluble material was collected by filtration. The vertical scale in the two panels is different for purposes of clarity.

Figure 11. Rate of actinomycin-D-resistant RNA synthesis

cultures at 39.5°C was 55% of that of wt-infected cells, while that of g^rH-infected cells was essentially equal to wt.

It is clear that tsB9 is defective in the synthesis of viral RNA at 39.5°C.

Accumulation of Acid-insoluble RNA. The accumulation of acid-insoluble material labeled with ^3H-uridine was also measured as discussed in Methods and Materials. At 33°C there were no significant differences from wt in the accumulation of RNA in tsB9- or g^rH-infected cells. As before, a difference was seen at 39.5°C between the RNA accumulation in tsB9 cultures and wt cultures. On the average, tsB9 cultures accumulated only 28% as much RNA as wt. Since the rate of RNA synthesis in tsB9 cultures at 39.5°C was 55% of that in wt cultures, it appears that a depressed rate of synthesis may not be solely responsible for the lower accumulation of RNA.

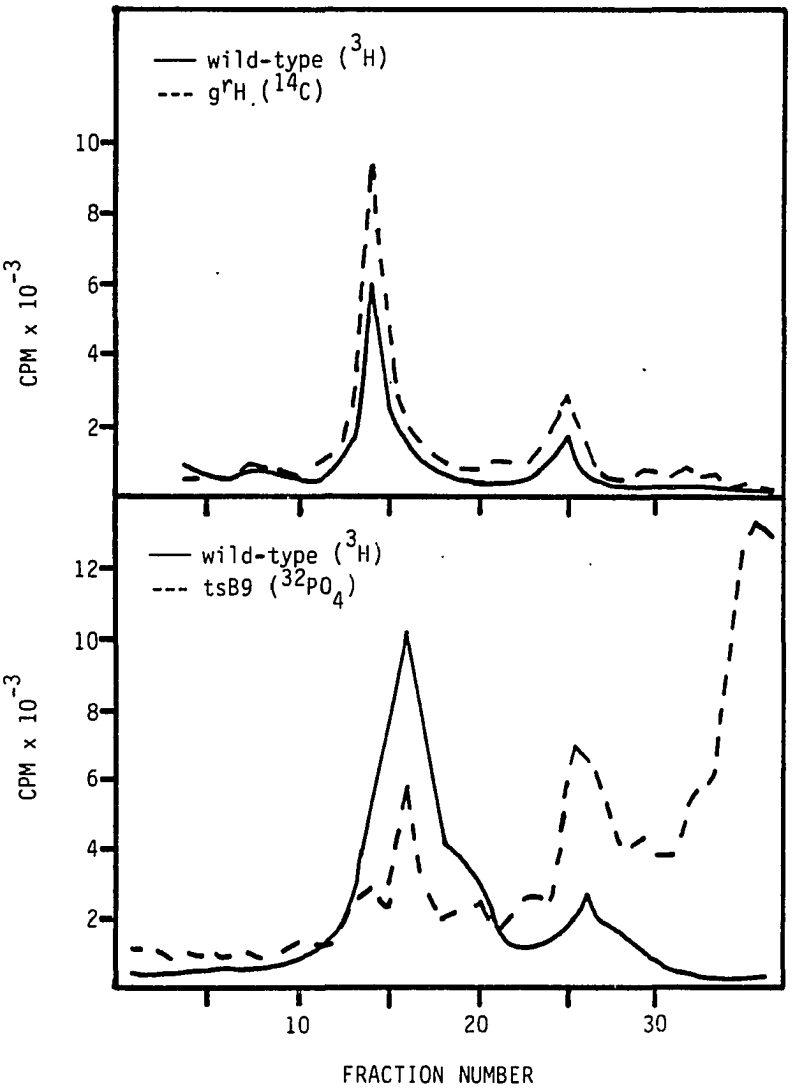
Examination of Cytoplasmic RNA. Once it was found tsB9 was defective in RNA synthesis at 39.5°C, it became necessary to determine the nature of the RNA that was synthesized by these cultures. The cytoplasmic RNA from tsB9, g^rH, and wt cultures incubated at 33°C and 39.5°C were labeled with $^{32}PO_4$, ^{14}C-uracil, and ^3H-uridine, respectively, using the standard infection procedure. The RNAs were extracted and purified as described in Methods and Materials.

The ^{3}H-labeled RNA from wt cultures was mixed with either tsB9 or g^{r}H RNA and analyzed on sucrose density gradients (SW 27; 15%-30% sucrose in TNE + sodium dodecyl sulfate (SDS); 21,000 rpm; 16.5 h; 21°C). The acid-insoluble radioactivity in each fraction was determined as previously described.

At 33°C, tsB9, g^{r}H, and wt profiles were essentially the same. A major peak representing 35 S single-stranded RNA and a minor peak representing 20 S double-stranded RNA were both present in normal proportions.

At 39.5°C, the tsB9 cultures produced less of the genome-length 35 S RNA than wt and apparently the same amount of a species that approximately co-sedimented with poliovirus wt replicative-form (RF) RNA (20 S). The g^{r}H cultures produced an essentially wt pattern at 39.5°C. Figure 12 illustrates the typical cytoplasmic RNA patterns of wt and g^{r}H or wt and tsB9 under differential labeling conditions. The actual amounts cannot be directly compared in this figure due to differences in specific activities of the radioisotope.

The RNA prepared as above was also examined by agarose gel electrophoresis. The results were the same as described above. In agarose gels, the 20 S RNA migrates as a homogeneous peak. The tsB9 RNA migrating with the 35 S RNA exactly co-migrated with the wt species although the



Cytoplasmic virus specific RNA was labeled with radioactive RNA precursors during incubation of infected cells at 39.5°C using the standard infection procedure as descirbed in Methods and Materials. The wild-type (wt) RNA was labeled with ^{3}H-uridine, g^rH by the uptake of ^{14}C-uracil, and tsB9 RNA by the uptake of $^{32}PO_4$. The RNA was phenol-chloroform extracted and ethanol precipitated as previously described. The ethanol suspension of wt RNA was mixed with an equal volume of tsB9 RNA or g^rH RNA and pelleted in a Brinkman centrifugate (12,000 x g; 10 min; 4°C). The mixed samples were then subjected to centrifugation through sucrose density gradients (SW 27; 15%-30% sucrose in TNE + 0.5% SDS, 21°C; 16.5 h; 21,000 rpm). The acid-insoluble radioactivity in each fraction was determined as described in Methods and Materials.

Figure 12. Co-sedimentation of differentially labeled cytoplasmic viral RNA synthesized at 39.5°C

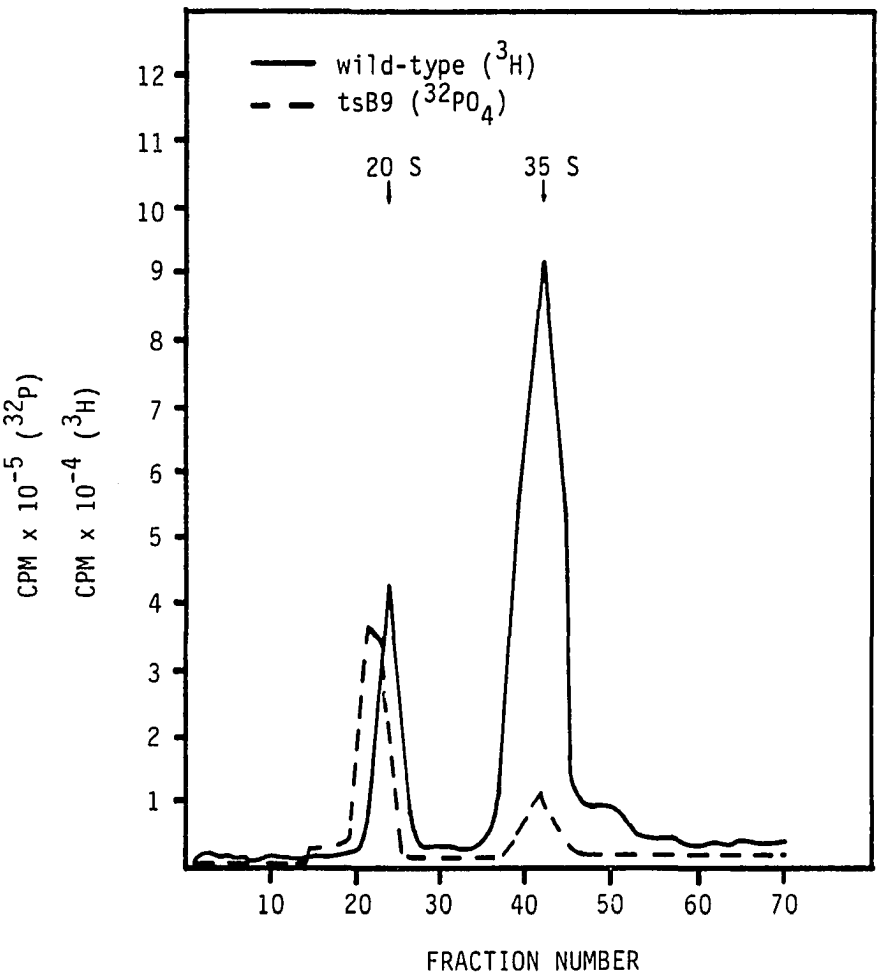
proportion was significantly reduced. The tsB9 RNA migrating with the wt RF RNA (20 S) migrated slightly slower than the wt species (Figure 13). The g^rH RNA was not analyzed by electrophoresis.

The mutant tsB9 appears to be defective in both total RNA synthesis and production of genome-length, single-stranded RNA at 39.5°C. The RNA migrating in the region of the wt RF RNA is apparently not identical with the authentic RF RNA from wt, as they do not exactly co-migrate. Repetition of the differentially labeled gradients and gels three times produced the same results.

Comparison of wt and tsB9 RNA, produced at 39.5°C, in parallel gels and gradients allowed the quantitative comparisons of 35 S and 20 S RNA (data not shown).

The mutant strain g^rH appeared not to be defective or temperature sensitive with respect to RNA synthesis.

Effects of Elevated Temperature on Unencapsidated RNA. It was considered possible that the decreased amount of genome-length, single-stranded RNA in tsB9 cultures at the restrictive temperature might be due to the unencapsidated single-strand RNA being exposed to the elevated temperature, possibly exposing it to cellular nucleases to a greater extent than the wt single-stranded RNA which is quickly encapsidated. If such a failure of RNA to be encapsidated were responsible for the observed decrease in



Cytoplasmic RNA from wild-type (wt) and tsB9 cultures incubated at 39.5°C was prepared as described in the legend to Figure 12, wt RNA being labeled by the uptake of ^{3}H uridine and tsB9 RNA by the uptake of $^{32}PO_4$. The RNA samples were purified and mixed exactly as in Figure 12 and subjected to electrophoresis in 1% agarose gel. Positions of nonradioactive purified 35 S virion and 20 S replicative-form RNA are indicated by arrows. The position of the markers was determined by ethidium bromide staining. Migration is from left to right.

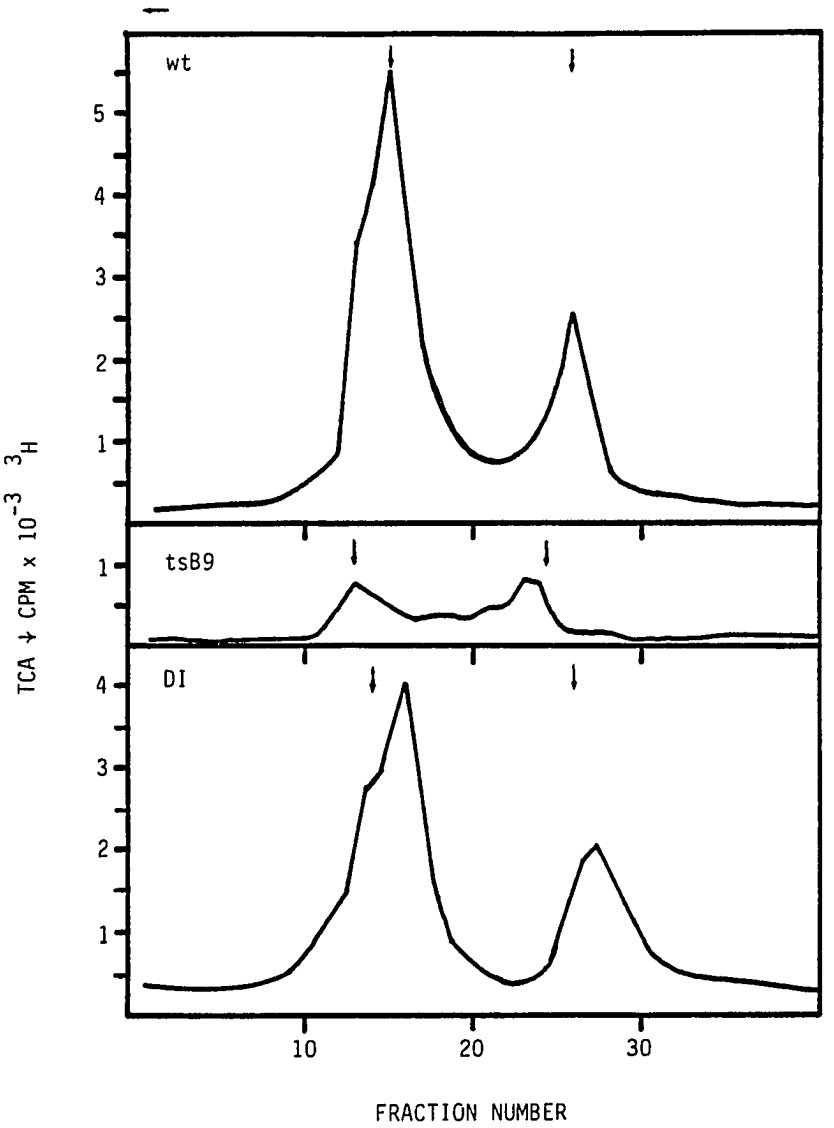
Figure 13. Agarose gel electrophoresis of cytoplasmic viral RNA produced by wild-type and tsB9 cultures at 39.5°C

35 S RNA in tsB9 at 39.5°C, a similar occurrence should be observed in DI cultures elevated to 39.5°C. To determine whether this were the case, cultures of tsB9 were compared to cultures of DI and wt at 33°C and 39.5°C.

At 33°C, the accumulation of acid-insoluble material labeled with ^{3}H-uridine was similar for all three strains. At 39.5°C, the tsB9 culture accumulated 38% of the level of RNA accumulated by wt, while the DI culture accumulated 86% as much RNA as wt.

Although the DI culture accumulated less acid-insoluble RNA than wt at 39.5°C, it did not show the dramatic difference that tsB9 did. Since DI RNA is completely unencapsidated in the absence of co-infecting virus, it is apparent that exposure of unencapsidated RNA to the cellular milieu at the elevated temperature is not alone responsible for the observed decrease in accumulation of RNA seen in tsB9 cultures at the restrictive temperature.

The cytoplasmic RNA from the above cultures was purified as described in Methods and Materials and examined by sucrose density gradient centrifugation. There were no signs of RNA degradation in any of the samples taken at 165 or 240 min p.i. The 35 S peak is larger than the 20 S peak in both wt and DI samples (Figure 14). In the tsB9 sample, the areas under the 35 S and 20 S peaks appear approximately the same, both being less than those in the wt and DI



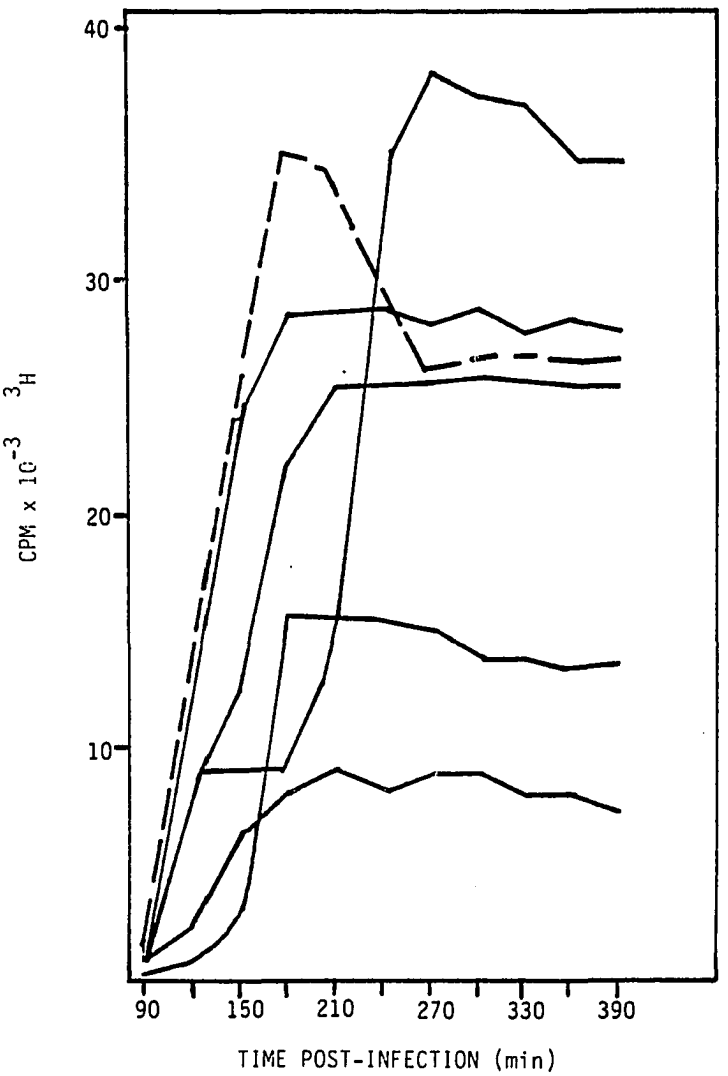
Cytoplasmic RNA was extracted from wild-type (wt), defective-interfering (DI), and tsB9 cultures incubated at 39.5°C. The RNA was ethanol precipitated and sedimented through sucrose density gradients (SW 27; 15%-30% w/v sucrose in TNE + 0.5% SDS; 21,000 rpm; 16.5 h; 21°C). The acid-insoluble radioactivity in each fraction was determined as previously described. The arrows indicate the position of ^{32}P-35 and ^{32}P-20 S replicative-form RNA internal markers.

Figure 14. Sucrose density gradient analysis of cytoplasmic RNA from 39.5°C and 240 min post-infection

samples. The 35 S and 20 S RNA in the DI samples sediment somewhat slower than the wt markers, as would be expected in view of their shorter length. The tsB9 35 S RNA co-sediments with the wt marker 35 S RNA. The 20 S RNA from tsB9, however, sediments slightly faster than the wt RF marker.

Effects of tsB9 on RNA Defective-interfering Cultures in Mixed Infections at 39.5°C. The possibility that tsB9 cultures incubated at 39.5°C contained a factor causing RNA degradation was considered. To examine that possibility, the RNA in a number of pure and mixed infections incubated at 39.5°C was examined. The following cultures were established: wt, DI, tsB9, wt/DI, tsB9/DI, and tsB9/wt. The accumulation of acid-precipitable RNA and the species of RNA produced were both examined.

It can be seen (Figure 15) that wt/DI, DI, wt, and tsB9/wt cultures accumulated comparable amounts of acid-precipitable RNA at 39.5°C. The tsB9 and tsB9/DI cultures accumulated reduced amounts of RNA, that of tsB9/DI falling between the levels seen in tsB9 and DI pure cultures. It is apparent that tsB9 does not interfere with wt total RNA synthesis, since the tsB9/wt culture accumulated somewhat more RNA than the wt culture. It should be noted, however, that the accumulation of RNA is faster in pure wt and DI cultures than in tsB9 or mixed cultures and some interaction of the virus strains in RNA synthesis in mixed cultures can be



HeLa S-3 cells were infected at a total multiplicity-of-infection of 10 plaque-forming-units (pfu)/cell or 10 pfe/cell with wild-type (wt), tsB9, defective-interfering (DI), or a mixture of wt and DI, tsB9 and wt, or tsB9 and DI. At 60 min post-infection (p.i.), actinomycin-D was added to each culture to a final concentration of 5 µg/ml of culture. At 90 min p.i., 10 µCi/ml ^{3}H-uridine was added. At 90 min p.i. and every 30 min thereafter, a 0.1-ml sample was withdrawn from each culture and precipitated in 1.0-ml ice-cold 10% TCA. The acid-insoluble material was collected on Millipore membrane filters and the radioactivity was determined.

Figure 15. Accumulation of acid-insoluble RNA in mixed and pure cultures incubated at 39.5°C

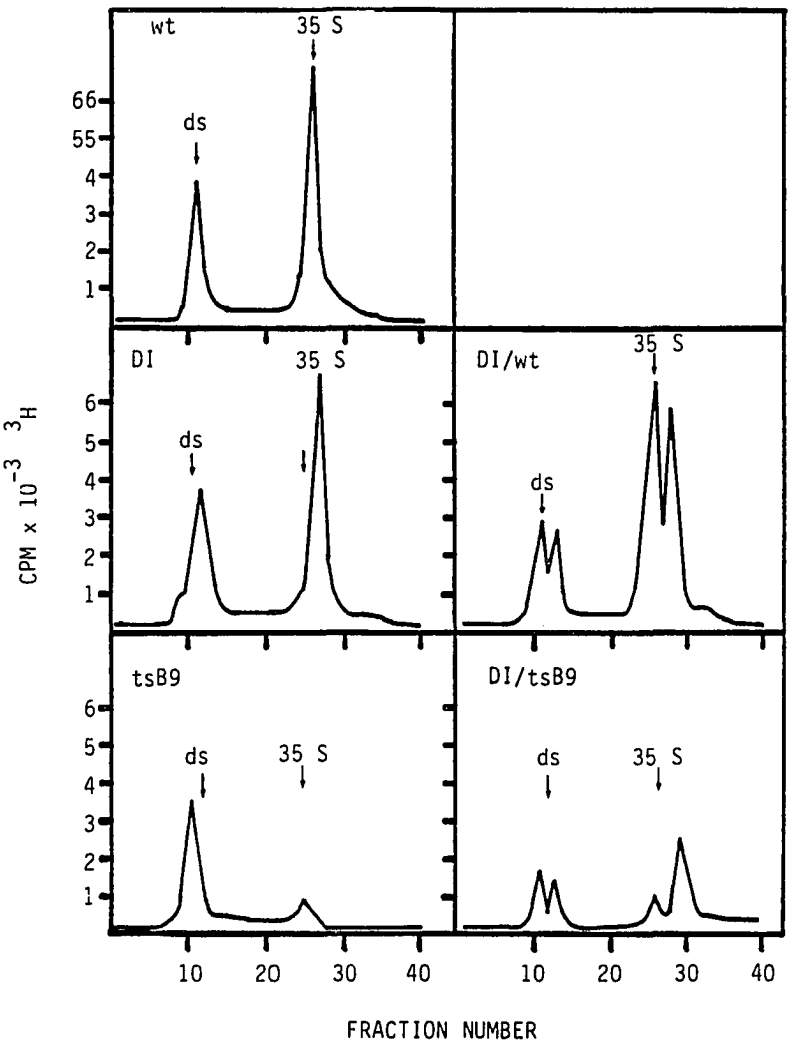
postulated. The presence of tsB9 may interfere with DI RNA synthesis in some way, as the tsB9/DI level is less than that of DI.

Purified RNAs from the above cultures were analyzed by electrophoresis in 1% agarose gels in the presence of ^{32}P-labeled authentic wt 35 S and 20 S RF RNA (Figure 16). The RNA patterns produced by wt and DI were typical, showing both 35 S and 20 S RNA as expected. Both wt and DI RNA were apparently produced in the wt/DI culture, resulting in the split peaks observed. The tsB9 culture produced its typical pattern as described earlier; a small 35 S peak and a peak migrating slightly slower than authentic RF RNA.

The tsB9/DI culture appeared to produce both tsB9 and DI RNA. The profile is one that could be obtained by superimposing the tsB9 and DI profiles with the exception that the total amount of 35 S RNA was significantly less than that produced by the pure DI culture.

The above data do not exclude the possibility that tsB9 cultures incubated at 39.5°C contain some factor that results in degradation of RNA or interferes with its synthesis.

Temperature Effects on RNA Synthesis. Due to variations observed in the amount of 35 S RNA produced by tsB9 cultures incubated in an open water bath set at 39.5°C, it became necessary to evaluate the effects of variations in



Cytoplasmic RNA from the cultures shown in Figure 15 were phenol-chloroform extracted and ethanol precipitated. The purified RNA was layered onto cylindrical gels of 1% agarose and subjected to electrophoresis at 10V/cm (≤ 8 mA/gel) for about 2 h or until the bromophenol blue-marker dye had migrated 10 cm from the origin. The gels were cut into approximate 1-mm slices and the radioactivity in the slices was measured.

Authentic 35 S and 20 S replicative-form RNA from wild-type (wt), labeled with $^{32}PO_4$ were run as internal markers in each gel. The location of the markers is indicated by arrows. Migration is from left to right.

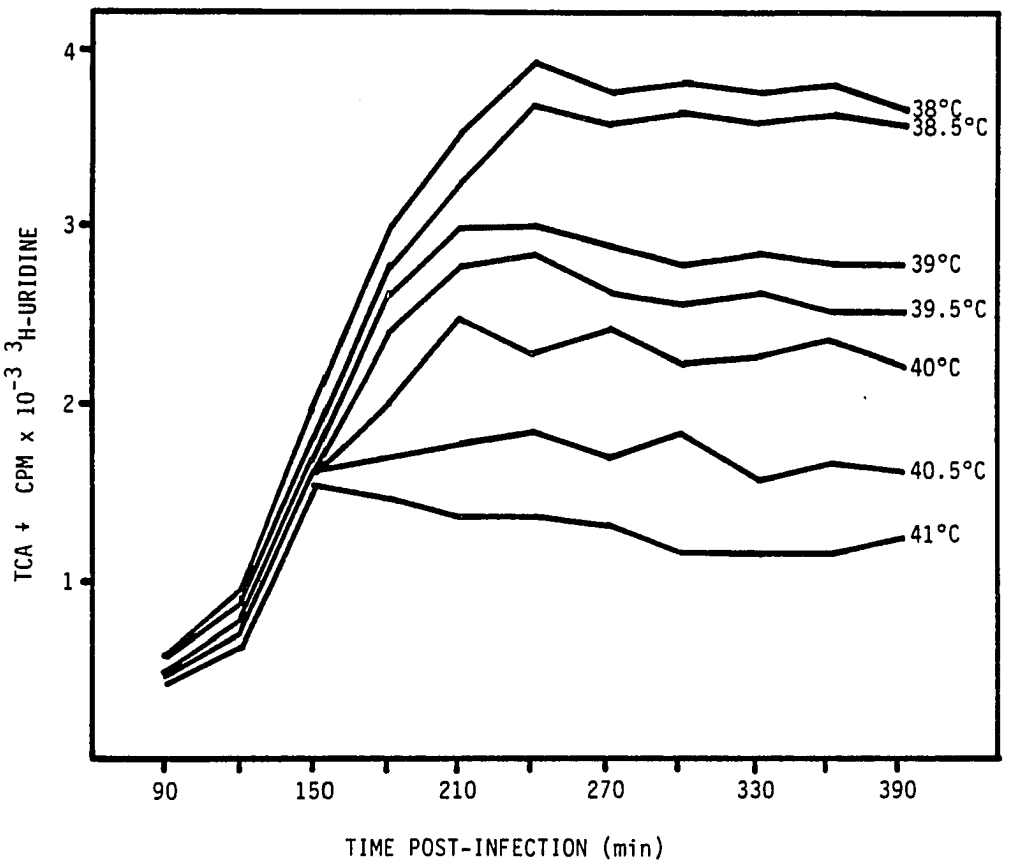
Figure 16. Agarose gel electrophoresis of cytoplasmic RNA extracted from pure and mixed cultures at 39.5°C

temperature near the restrictive temperature. Cultures of tsB9 were incubated at 38.0, 38.5, 39.0, 40.0, 40.5, and 41.0 degrees centigrade. The accumulation of acid-precipitable RNA and the sedimentation properties of purified RNA were examined as described in Methods and Materials.

The accumulation of acid-insoluble RNA decreased as the temperature increased (Figure 17). The RNA profile on sucrose density gradients also varied with the temperature (Figure 18). At all temperatures, an acid-insoluble peak occurred at about 5 S. The proportion of 35 S and 20 S RNA progressed from approximately wt at 38.0°C to the clearly defective pattern typical of tsB9 at 39.5°C. At 40.0°C to 41.0°C, little RNA larger than 5 S was seen.

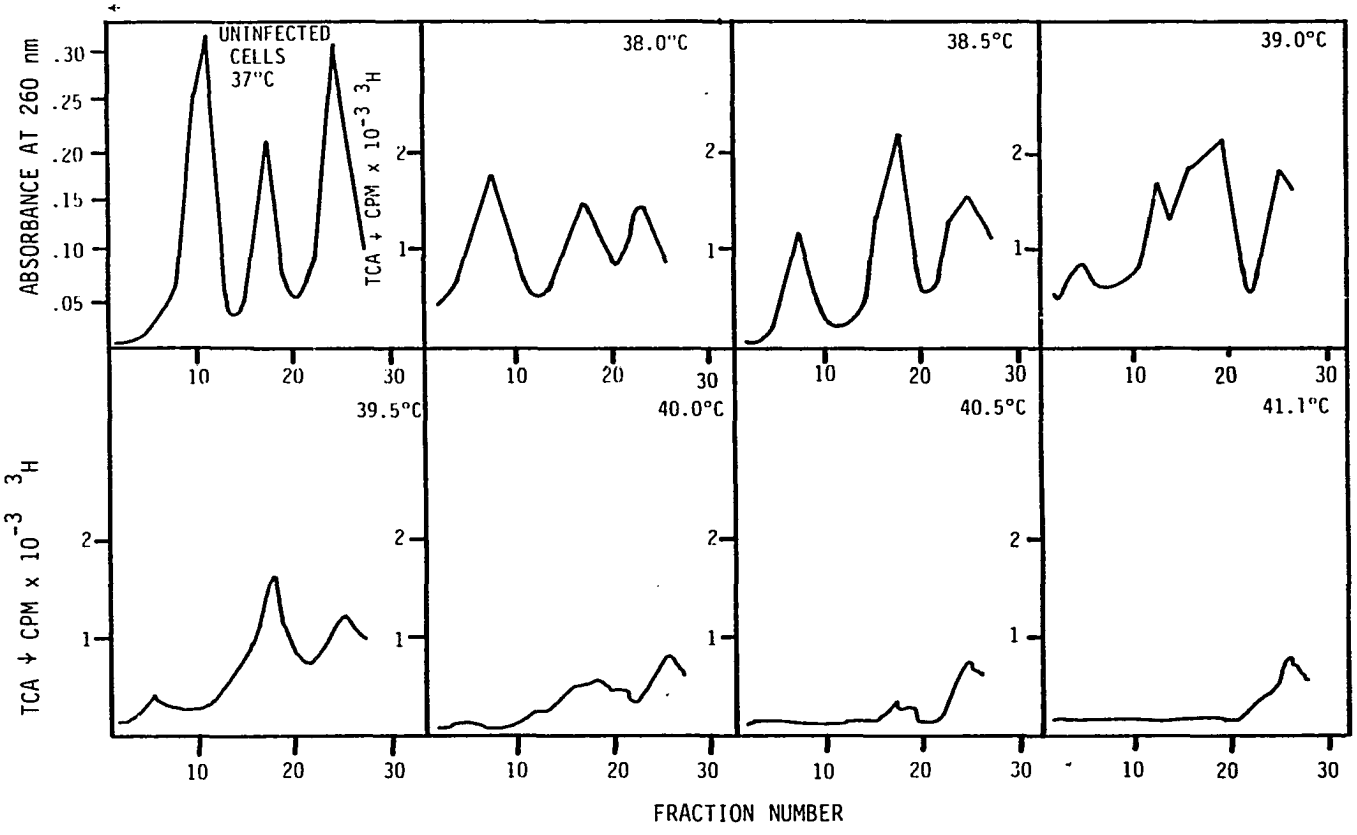
Since a 0.5°C variation in temperature resulted in an alteration in the RNA profile, care was taken to maintain the temperature of incubation in the restrictive region to 39.5°C ± 0.2°C. This was made possible by the use of a Haake (Saddlebrook, NJ) circulator and an insulated, covered water bath.

Determination of the Presence of VPg on Cytoplasmic RNA. It has been demonstrated that VPg is covalently attached to the 5' end of all poliovirus RNA except for RNA purified from the polysomes. Previously it was shown that VPg was also present on the tsB9 virion RNA. The cytoplasmic RNA species produced in tsB9 cultures at 39.5°C were also



HeLa S-3 cells were infected at a multiplicity-of-infection of 10 plaque-forming-units/cell in the presence of 5 µg of actinomycin-D per ml of culture as previously described. The cultures were incubated at 38°C, 38.5°C, 39°C, 39.5°C, 40°C, 40.5°C, or 41°C. At 90 min post-infection (p.i.), 10 µCi/ml ^{3}H uridine was added. At 90 min p.i. and every 30 min thereafter, a 0.1-ml sample was withdrawn from each culture and precipitated in ice-cold 10% TCA. The acid-insoluble material was collected on Millipore membrane filters and the radioactivity was determined.

Figure 17. Accumulation of acid-insoluble RNA in tsB9 cultures at temperatures near the restrictive temperature



Cytoplasmic RNA from the cultures shown in Figure 17 was phenol-chloroform extracted and ethanol precipitated. The purified RNA was subjected to centrifugation through linear gradietns of 14%-22% sucrose in TNE (SW 50; 39,000 rpm; 4°C). The gradients were divided into approximate 0.5-ml fractions and the acid-insoluble radioactivity in each fraction was determined.

Figure 18. Sucrose gradient analysis of tsB9 RNA at temperatures in the restrictive range

examined for the presence of VPg. Cytoplasmic RNA was labeled with $^{32}PO_4$ at 39.5°C. The RNA was purified and sedimented through sucrose density gradients as previously described. The 35 S and 20 S RNA peaks were individually pooled, ethanol precipitated, and digested to completion with a mixture of ribonucleases as described in Methods and Materials. The analysis by high-voltage ionophoresis and autoradiography was as described earlier.

The percentage of the phosphate label found in the VPg-pUp spot was approximately the same in 35 S and 20 S RNA samples taken from tsB9 cultures incubated at 33°C and at 39.5°C (Table 7). The tsB9 35 S RNA produced at both temperatures exhibited the same approximate nucleotide composition as the wt 35 S (see Table 6). The 20 S RNA produced by tsB9 at 33°C exhibited the A = U, C = G composition expected of double-stranded RNA.

The 20 S RNA produced by tsB9 cultures at 39.5°C did not exhibit the A = U, C = G composition expected of double-stranded RNA, indicating that it is probably not fully double-stranded in nature. It appears that some double-stranded tracts are to be found in this anomalous 20 S RNA since attempts to digest the RNA in high salt conditions resulted in most of the label remaining at the origin during the ionophoresis. When the ribonuclease digestion was carried out as described in Methods and Materials, but in the

Table 7. Determination of the presence of VPg on cytoplasmic RNA of tsB9.--Cytoplasmic RNA was labeled with $^{32}PO_4$ and digested to completion with a mixture of ribonucleases then run on high-voltage electrophoresis as described previously for virion RNA.

		Counts per Minute (% total radioactivity)				
		VPg	C	A	G	U
33°C						
tsB9	35 S	182 (0.030%)	172.354 (25.6%)	212,750 (31.6%)	162,929 (24.2%)	125,227 (18.6%)
tsB9	20 S	126 (0.030%)	104,623 (24.9%)	106,303 (25.3%)	104,202 (24.8%)	105,043 (25.0%)
39.5°C						
tsB9	35 S	175 (0.030%)	145,158 (25.0%)	190,447 (32.6%)	138,771 (23.8%)	109,739 (18.8%)
tsB9	20 S	250 (0.027%)	309,317 (33.8%)	314,656 (34.4%)	146,969 (16.1%)	143,135 (15.7%)

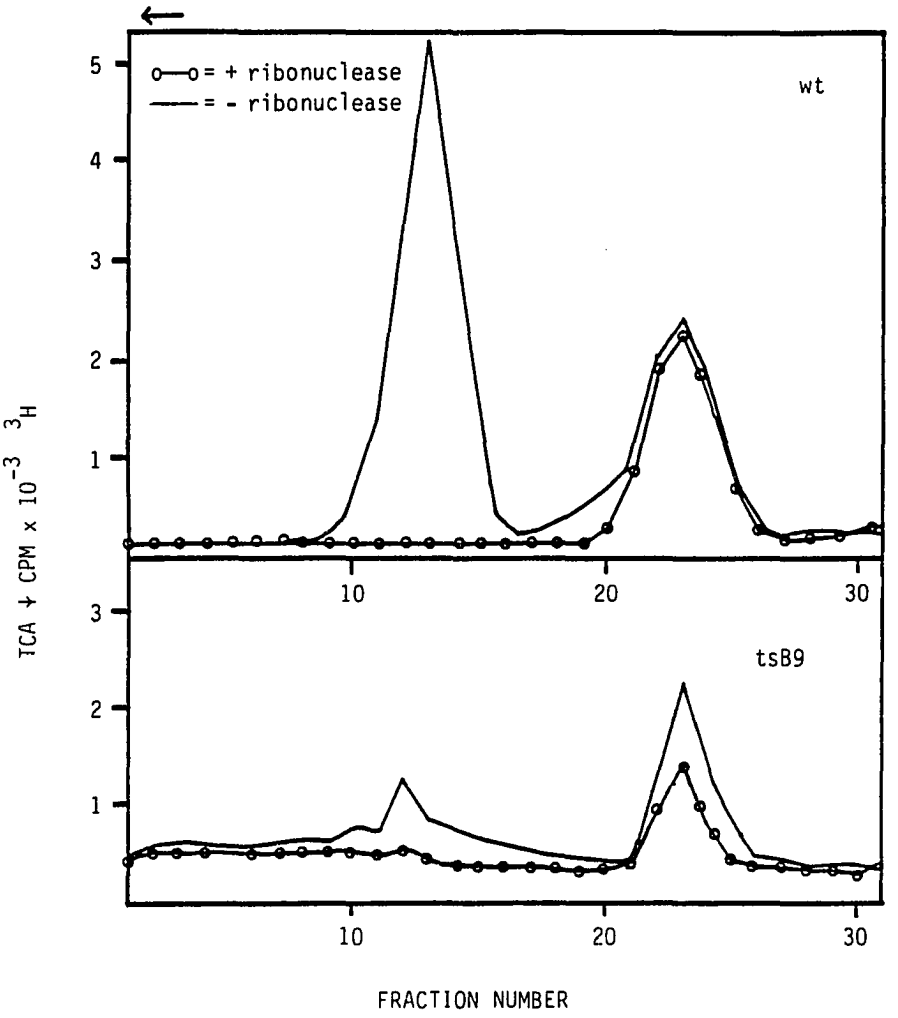
presence of low salt concentrations, the results seen in Table 7 were obtained.

Nature of the Aberrant 20 S RNA of tsB9

Ribonuclease Resistance. The ribonuclease resistance of single- and double-stranded RNA molecules differs, and this difference can aid in the characterization of a given molecular species of RNA. The ribonuclease resistance of the 20 S RNA produced by tsB9 cultures at 39.5°C was examined.

The resistance of the tsB9 aberrant 20 S RNA to ribonuclease A (pancreatic) under high salt conditions was determined by two approaches. Purified cytoplasmic RNA from tsB9 and wt cultures incubated at 39.5°C was sedimented through sucrose density gradients as described in Methods and Materials. Half of each gradient fraction was treated with ribonuclease A while the other half of each fraction was left untreated. The conditions for ribonuclease treatment were described in Methods and Materials. The acid-insoluble radioactivity was then determined for the treated and untreated fractions.

Figure 19 illustrates that 35 S RNA from both wt and tsB9 cultures was completely sensitive to ribonuclease digestion, reflecting their single-stranded nature. The 20 S RNA from wt was 91% resistant to ribonuclease digestion at high salt, reflecting its double-stranded character.

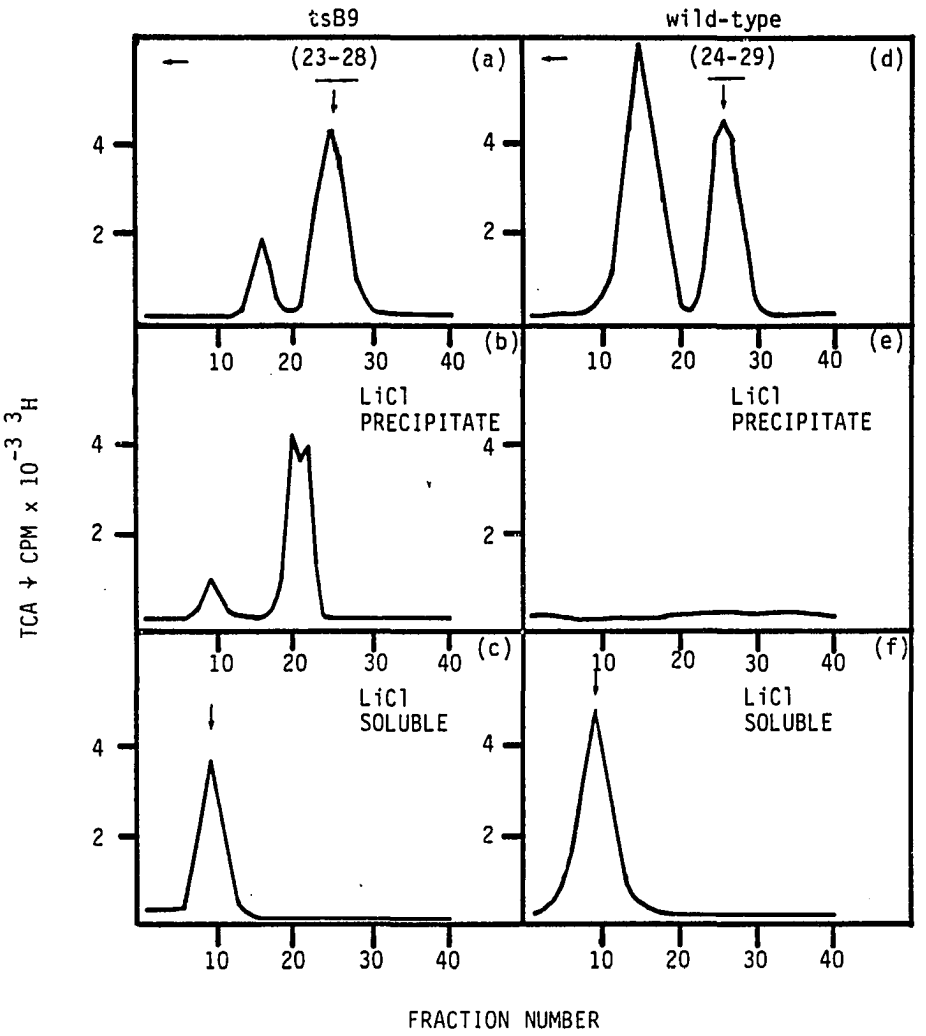


HeLa S-3 cells were infected and labeled as previously described in Methods and Materials. Cytoplasmic RNA was extracted from the wild-type (wt) and tsB9 cultures incubated at 39.5°C. The extracted RNA was subjected to centrifugation through linear gradients of 15% to 30% in TNE + 0.5% SDS (SW 27; 21,000 rpm; 16.5 h; 21°C). The gradients were divided into approximate 1.0-ml fractions. Half of each fraction was treated with ribonuclease A (0.1 mg/ml; 37°C; 15 min) in SCC buffer (0.15 M NaCl; 0.015 M Na citrate) as described in Methods and Materials. The acid-insoluble radioactivity was determined in the ribonuclease-treated and untreated fractions.

Figure 19. Ribonuclease treatment in high salt of sucrose gradient purified RNA produced by wild-type and tsB9 cultures incubated at 39.5°C

The 20 S RNA produced by tsB9 cultures at 39.5°C was 60% resistant to ribonuclease A digestion in high salt, suggesting that it contains some double-stranded RNA or other protected structure but is probably not fully double-stranded.

In the second approach, the 20 S RNA synthesized by tsB9 cultures at 39.5°C was labeled with ^{3}H-uridine, purified, and subjected to sucrose density gradient centrifugation as described in Methods and Materials. The 20 S fractions were pooled and the RNA was ethanol precipitated in the presence of unlabled purified HeLa cell RNA as carrier (Figure 20). The ethanol-precipitated 20 S RNA was treated with 2 M LiCl, and the soluble and insoluble phases were separately subjected to agarose gel filtration as previously described. The RNA from wt cultures was analyzed in parallel as a control. It was anticipated that the LiCl precipitate isolated from the tsB9 20 S fraction would elute as a single species. Instead, it eluted in two distinct fractions. The major portion eluted in the partially included volume. This material was 8% to 9% resistant to digestion by ribonuclease A under high salt conditions and was therefore considered to be single-stranded. The second fraction eluted as a single peak in the excluded volume and was 85% to 90% resistant to ribonuclease A under high salt conditions, and therefore probably contained significant double-stranded tracts.



HeLa S-3 cells were infected with wild-type (wt) or tsB9 and labeled as previously described. Cytoplasmic RNA was phenol-chloroform extracted and ethanol precipitated. The purified RNA was subjected to centrifugation through linear gradients of 15%-30% sucrose in TNE + 0.5% SDS (SW 27; 21,000 rpm; 16.5 h; 21°C). The gradients ((a) and (d)) were divided into approximate 1.0-ml fractions and the acid-insoluble radioactivity of a 0.1-ml aliquot of each fraction was determined. The 20 S fractions (identified by the presence of ^{32}P-replicate form RNA) were pooled as indicated by the bars in (a) and (d), ethanol precipitated, and treated with 2 M LiCl as described in Methods and Materials. The soluble (c) and (f) and insoluble (b) and (e) material were independently subjected to gel filtration through columns of Sepharose 2B. The acid-insoluble radioactivity was determined. The location of the ^{32}P-RF RNA marker is indicated by an arrow.

Figure 20. Sepharose 2B gel filtration of sucrose gradient purified 20 S RNA from tsB9 and wild-type cultures incubated at 39.5°C

The wt 20 S RNA produced the expected patterns. A single peak eluted in the excluded volume from the LiCl soluble phase. This material was 98% resistant to digestion by ribonuclease A under high salt conditions. No radioactivity above background was seen in the wt LiCl precipitate. This reflects the double-stranded nature of the wt normal 20 S (RF) RNA.

The tsB9 RNA in the soluble phase behaved exactly like wt RF RNA.

This procedure was performed twice using ^{3}H-uridine as the RNA label and once using $^{32}PO_4$. The results were as described above in all three experiments.

Effects of Phenol Extraction on RNA. To determine whether the 20 S RNA produced by tsB9 at 39.5°C was an artifact of the phenol extraction-ethanol precipitation procedure, the cytoplasmic extract from a continuously labeled culture (^{3}H-uridine) incubated at 39.5°C was divided into two equal aliquots. One aliquot was phenol extracted and ethanol precipitated as described in Methods and Materials. The other aliquot was deproteinized by exposure to 1% SDS at room temperature. Both samples were analyzed by sucrose density gradient centrifugation (SW 27; 15%-30% sucrose in TNE + 0.5% SDS; 21,000 rpm; 16.5 h; 21°C

The acid-insoluble radioactivity of the fractions of the two gradients was determined as previously described.

The RNA pattern was essentially the same as previously observed for tsB9 at 39.5°C in both samples. It appears that the aberrant 20 S RNA found in tsB9 cultures is not merely an artifact of phenol extraction or ethanol precipitation. It should be noted that the phenol extraction procedure exposes the extract to SDS and possible effects of the detergent cannot be ruled out.

Comparison of the Pulse-labeled and Continuously Labeled RNA at 39.5°C. In a further attempt to determine the nature of the 20 S RNA produced by tsB9-infected cells at 39.5°C, the pattern of RNA produced by short pulse-labeling (2.5 min) with ^{3}H-uridine was examined by agarose gel electrophoresis. Continuously labeled and pulse-labeled wt RNA were examined in parallel as controls. Under pulse-labeling conditions the wt RNA pattern resembled the pattern produced by tsB9 at 39.5°C under continuous-labeling conditions. The amount of 35 S RNA was less than the amount of RNA found in the slower migrating fraction (20 S). The slower fraction migrated more slowly than authentic RF RNA internal markers. Under pulse-labeling conditions, the tsB9 culture produced the same pattern of RNA as it typically produced under continuous-labeling conditions at 39.5°C although the total radioactivity incorporated was less.

A pulse-labeling period of 3 min or less has been shown to label replicative-intermediate (RI) RNA of poliovirus

preferentially (11, 12, 14). It is therefore considered likely that the radioactive peak in wt samples migrating slightly slower than RF RNA is RI, which apparently migrates homogeneously in this gel system. The tsB9 20 S RNA migrates like wt RI RNA with respect to the internal RF RNA marker.

Effects of Temperature Shift on tsB9 RNA

It was shown earlier that the infectivity of tsB9 changed when the cultures were shifted from the restrictive to the permissive temperature. It was decided to determine whether the RNA synthesis responded in a similar fashion.

Standard infections were established at 33°C and 39.5°C. Sample cultures were shifted from 33°C to 39.5°C (shift-up) or from 39.5°C to 33°C (shift-down) at 90 and 240 min p.i. Unshifted control cultures were maintained at 33°C and 39.5°C. The shifted cultures were incubated at the post-shift temperature for 5 min and then exposed to ^{3}H-uridine for 1 h as described in Methods and Materials. The total cytoplasmic RNA was extracted and analyzed by agarose gel elecrophoresis as previously described. The unshifted controls were labeled for 1 h at 245-305 min p.i. Purified $^{32}PO_4$-labeled authentic wt 35 S RNA and RF RNA were added to each sample as internal markers.

The wt cultures produced 35 S RNA and RF RNA in normal proportions at all temperatures and under all shift

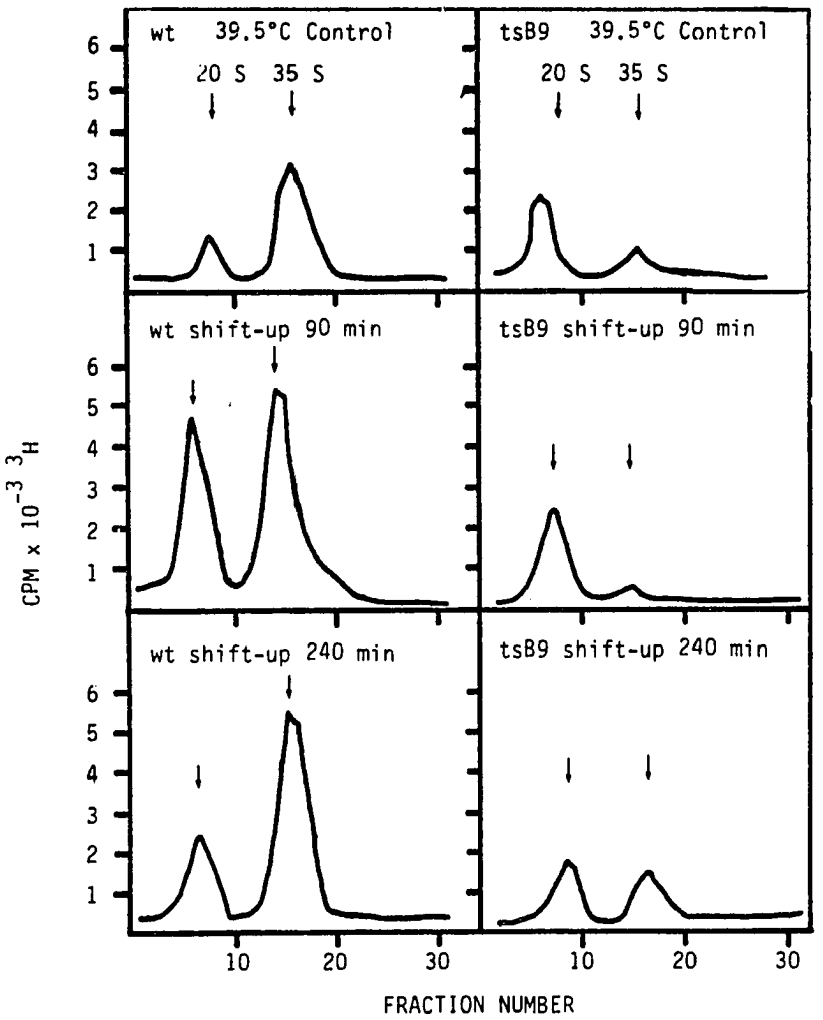
conditions. The tsB9 cultures maintained at 33°C and 39.5°C produced their typical patterns described heretofore. Cultures of tsB9 shifted up at 90 min p.i. presented the same pattern as the unshifted control maintained at 39.5°C. When the tsB9 culture was shifted up at 240 min p.i., a greater amount of 35 S RNA was found than in the 39.5°C or the 90 min shift-up samples, but still markedly less than wt (Figure 21).

The tsB9 culture shifted down at 90 min presented the wt pattern, as did the unshifted 33°C control tsB9 culture (Figure 22). The tsB9 culture down-shifted at 240 min p.i. produced a distinct peak co-migrating with 35 S marker, as well as a broad peak migrating with the RF RNA marker. The amount of material migrating with the 35 S marker was still less than wt.

The temperature-sensitive defect with respect to RNA production appears to be completely reversible early in the infectious cycle. The same results were demonstrated previously in this paper with respect to infectivity. These experiments were repeated twice more with the same results.

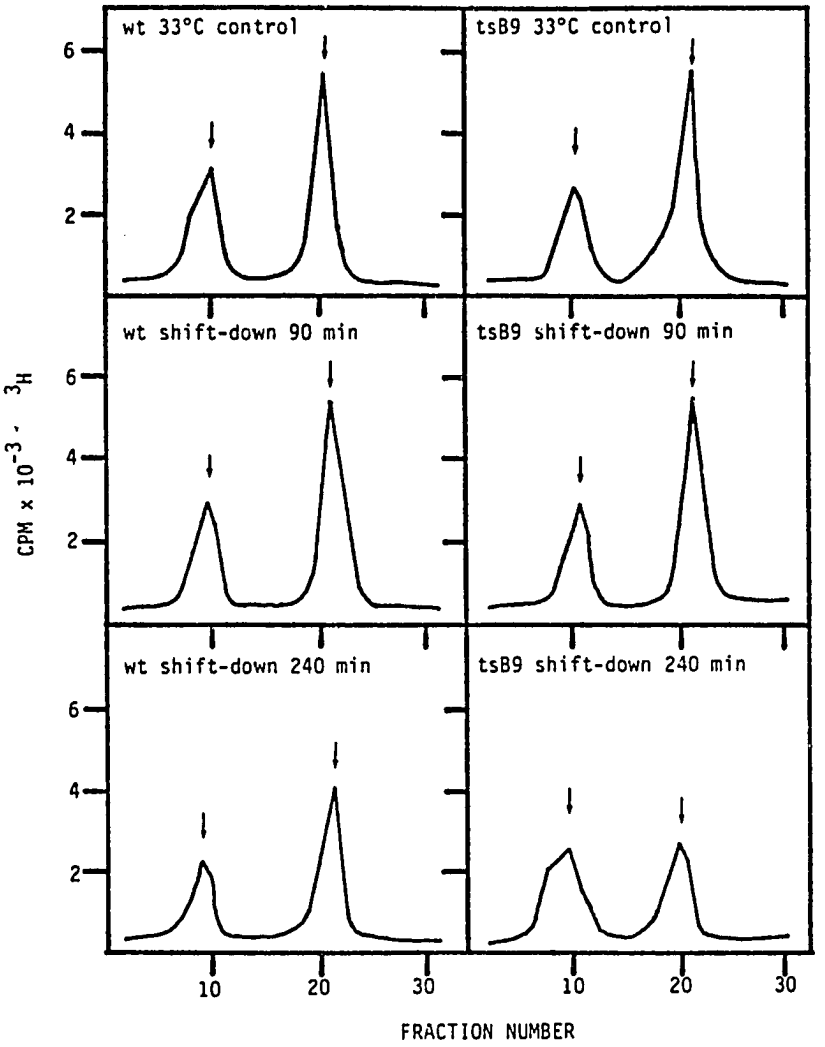
Membrane-associated RNA

Poliovirus RNA synthesis appears to take place in association with virus-induced cytoplasmic membranes (12, 21). A significant proportion of the cytoplasmic viral RNA is found in association with membranes. Using the cytoplasmic extract as previously defined, a significant portion of



HeLa S-3 cells were infected with tsB9 or wild-type (wt) at a multiplicity-of-infection (m.o.i.) of 10 plaque-forming-units/cell in the presence of 5 µg of actinomycin-D per ml of culture as previously described. The cultures were incubated at either 33°C or 39.5°C. Sample cultures were shifted from 33°C to 39.5°C at 90 min and 240 min post-infection (p.i.). Unshifted controls were maintained at 33°C and 39.5°C. The shifted cultures were incubated at 39.5°C for 5 min after shift-up and labeled with 10 µCi/ml ^{3}H-uridine for 1 h. The unshifted controls were labeled for 1 h at 245 to 305 min p.i. Total cytoplasmic RNA was phenol-chloroform extracted, ethanol precipitated, and analyzed by electrophoresis in 1% agarose gels as described in Methods and Materials. Arrows indicate the location of internal wt 35 S and 20 S markers labeled with $^{32}PO_4$. Migration is from left to right.

Figure 21. Effects of temperature shift-up on RNA produced by tsB9 and wild-type cultures



Cultures of tsB9 and wild-type were established in Methods and Materials. At 90 and 240 min post-infection (p.i.), sample cultures were shifted down from 39.5°C, incubated for 5 min and labeled for 1 h with 10 µCi/ml ^{3}H-uridine. Control cultures maintained at 33°C and 39.5°C were labeled from 245 to 305 min p.i.

Cytoplasmic RNA was extracted and analyzed by agarose gel electrophoresis as described in Methods and Materials. Migration is from left to right. Arrows indicate the location of ^{32}P-labeled internal markers of authentic wt 35 S and replicative-form RNA.

Figure 22. Effects of temperature shift-down on RNA produced by tsB9 and wild-type cultures

the acid-insoluble, radioactively labeled RNA is found in the nuclear pellet (about 30%) or in the phenol-chloroform phase (20%). It was therefore of interest to examine the total intracellular RNA produced by tsB9 cultures at 39.5°C.

Two equal aliquots of tsB9-infected cells incubated at 39.5°C were continuously labeled with ^{3}H-uridine and harvested as described in Methods and Materials. One cell pellet was extracted as usual. The second pellet was lysed in RSB + 1% sodium deoxycholate (DOC) + 1% NP-40 and incubated therein for 1 min at 37°C. This sample was then phenol extracted and the RNA ethanol precipitated as usual. Both samples were analyzed by sucrose density gradient centrifugation as described in Methods and Materials. The acid-insoluble radioactivity was determined for each fraction of the gradients.

A greater amount of radioactivity was found in the DOC-treated sample, but the distribution of the label was the same in both samples. The membrane-associated RNA appears to be the same in character as the phenol-extracted RNA from the soluble phase of the cytoplasm. A broad peak was seen in the 20 S region of the gradient in both samples, which is typical for tsB9 RNA produced at the restrictive temperature.

Virus-specific Proteins

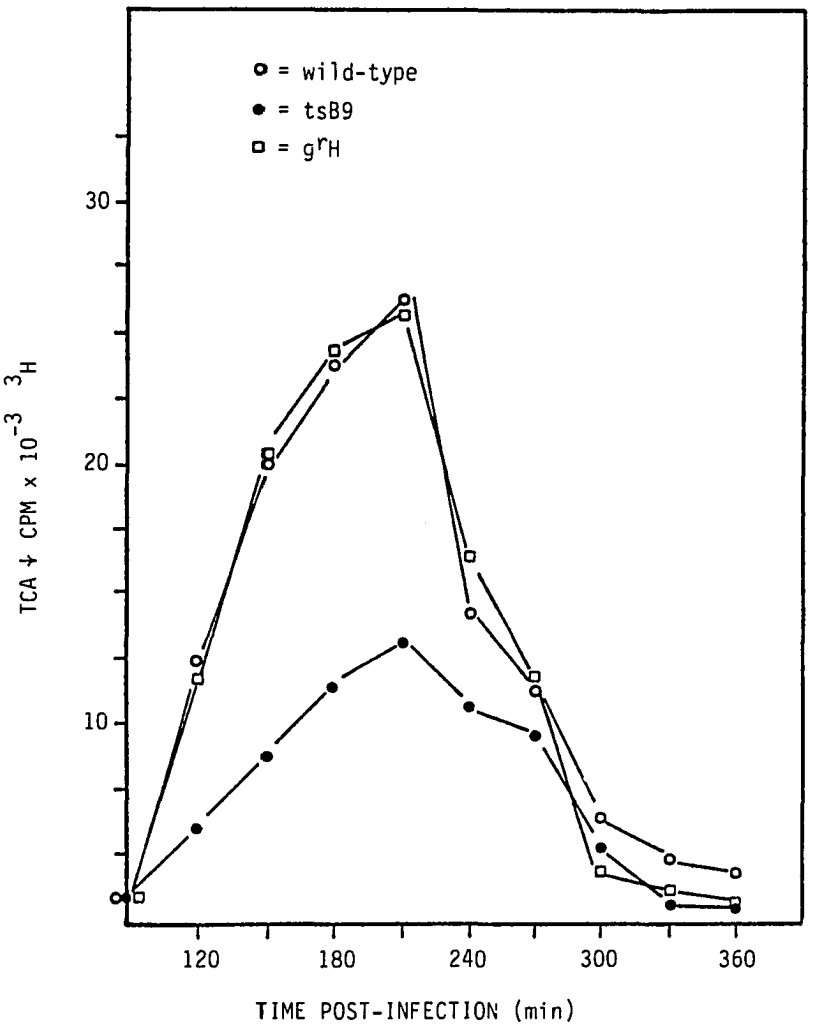
Rate of Protein Synthesis. The picornaviruses are made up of two major components: RNA and protein. It is thus necessary for a complete characterization to examine

protein synthesis of the mutant strains. The rate of protein synthesis in tsB9-, g^rH-, and wt-infected cells incubated at the permissive and restrictive temperatures was examined as described in Methods and Materials.

At 33°C, the rate of protein synthesis in tsB9 and g^rH cultures was essentially the same as that of wt. At 39.5°C, the average rate of synthesis in tsB9-infected cells was approximately 50% of the wt (Figure 23). This was found to be true when ^{35}S-methionine, ^{3}H-lysine, ^{3}H-tyrosine, or ^{3}H-leucine were used as the labeled precursors. The data illustrated in Figure 23 represent an average of 12 experiments in which cultures were labeled with ^{3}H-leucine at 39.5°C.

Effects of Temperature Down-shift on the Production of Virion and Procapsid. The ability of proteins synthesized in tsB9 cultures at 39.5°C to assemble on shift-down was examined.

The standard infection procedure was used to establish wt and tsB9 cultures at 39.5°C. The proteins were labeled with ^{3}H-amino acids. One set of cultures was labeled at 39.5°C, treated with cycloheximide, and shifted down to 33°C. The down-shift occurred at 180 and 300 min p.i. Cultures not treated with cycloheximide were shifted down at 180 and 300 min p.i. and labeled after the down-shift to determine whether incubation at 39.5°C interfered with subsequent



HeLa S-3 cells were infected with tsB9, wild-type (wt), or g^rH at a multiplicity-of-infection (m.o.i.) of 10 plaque-forming-units (pfu)/cell in the presence of 5 g of actinomycin-D per ml of culture and incubated at 39.5°C. At 90 min post-infection (p.i.) and every 30 min thereafter until 360 min p.i., a 0.1-ml sample was withdrawn from each culture and placed in a microcentrifuge tube containing 5 µCi of ^3H-leucine. The 0.1-ml samples were incubated at 39.5°C for 10 min. The reaction was stopped by the addition of 0.1 ml of 2 N NaOH. The samples were further incubated at 39.5°C for 15 min to dissociate charged acyl-tRNA and then precipitated by the addition of 1.0 ml of ice-cold 10% TCA. Samples were chilled for 20 min in ice, the acid-insoluble material was collected on Millipore membrane filters, and their radioactivity determined.

This figure represents an average of 12 replicate experiments.

Figure 23. Rate of protein synthesis by tsB9, g^rH, and wild-type cultures at 39.5°C

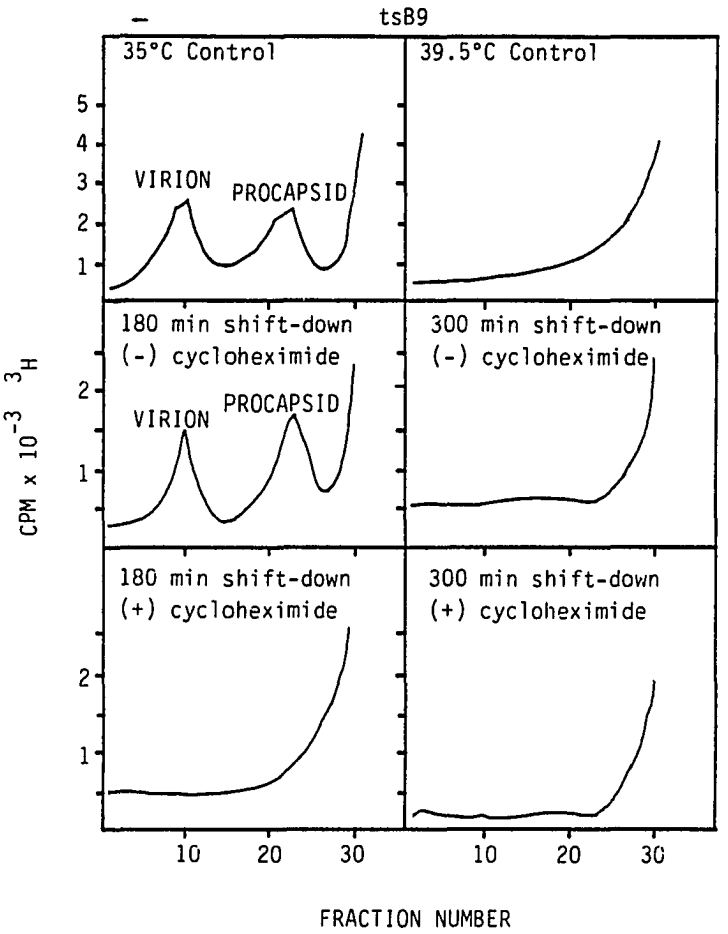
virion production at 33°C. Unshifted controls were maintained at 33°C and 39.5°C, and cycloheximide treatment omitted. Cytoplasmic extracts were prepared as previously described and analyzed in sucrose density gradients (SW 40; 90 min; 39,000 rpm; 10%-22% sucrose in TNE) (Figure 24).

The wt cultures produced both virion and procapsid under all experimental conditions. At 39.5°C, tsB9 cultures produced neither virions nor procapsids. In the presence of cycloheximide, the tsB9-infected cells labeled before down-shift produced no labeled virions or procapsids, whether the down-shift occurred at 180 or 300 min p.i.

The tsB9 culture shifted down in the absence of cycloheximide at 180 min p.i. produced both virions and procapsids, as did the 33°C control culture. The tsB9 culture shifted down at 300 min p.i. in the absence of cycloheximide produced neither virions nor procapsids.

It appears that the proteins produced by the tsB9-infected cells at 39.5°C are defective and unable to assemble when exposed to 33°C. It further appears that the defect in protein synthesis and assembly is reversible early (180 min) in infection but not late (300 min) in infection.

Electron Microscopic Examination of Poliovirus-infected Cells for the Presence of Crystal Lattices of Virions. It has been established that poliovirus-infected cells accumulate virions in crystal lattices in the cytoplasm



HeLa S-3 cells were infected with tsB9 or wild-type (wt) at a multiplicity-of-infection of 10 plaque-forming-units/cell in the presence of actinomycin-D as previously described. Cultures were incubated at 33°C and 39.5°C. Viral proteins were labeled by the addition of 10 Ci/ml each of ^{3}H-leucine. ^{3}H-lysine, and ^{3}H-tyrosine. Cultures were shifted down from 39.5°C to 33°C at 180 and 300 min post-inspection (p.i.). Control cultures were were maintained at 33°C and 39.5°C.

Cycloheximide-minus cultures were labeled from 5 min after shift-down to 360 min p.i. Cycloheximide-plus cultures were labeled from 90 min p.i. until shift-down. When cycloheximide-plus cultures were shifted down, cycloheximide was added to a final concentration of 0.1 mg/ml. Control cultures were labeled from 90 to 360 min p.i. in the absence of cycloheximide.

Cytoplasmic extracts prepared by lysis in RSB +1% NP-40 were analyzed by sucrose density gradient centrifugation (SW 41; 90 min; 30,000 rpm; 10%-22% sucrose in TNE). The radioactivity in each fraction was determined.

Figure 24. Formation of virions by tsB9-infected cells after temperature shift-down in the presence and absence of protein synthesis

before cell lysis. These lattices can easily be detected by electron microscopy (1, 37, 38, 88). Since tsB9-infected cells incubated at 39.5°C produce little or no biochemically detectable virions, it was of interest to determine whether they produced the crystal lattices found in wt.

Cultures of tsB9 and wt incubated at 33°C and 39.5°C and mock-infested HeLa cells incubated at 39.5°C were examined by electron microscopy as described in Methods and Materials. The wt-infected cells contained visible lattices when incubated at either temperature. The tsB9-infected cells incubated at 33°C also contained crystal lattices. No virion lattices were seen in the tsB9-infected cells incubated at 39.5°C that were indistinguishable from the mock-infected cells incubated at the same temperature. No micrographs are included here.

Sodium Dodecyl Sulfate-polyacrylamide Gel Electrophoresis of Proteins. To explore the defect in tsB9 further, the proteins produced by tsB9-infected cells at 39.5°C were examined. The proteins of tsB9 and wt were labeled with ^{35}S-methionine using the standard infection procedure and incubating the cultures at either 33°C or 39.5°C. Cytoplasmic extracts were prepared and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described.

The tsB9 cytoplasmic proteins labeled at 33°C were indistinguishable from wt by SDS-PAGE. The tsB9 proteins

labeled at 39.5°C exhibited a consistently different pattern. No VP 2 was seen. The migration of NCVP 1a and VP 1 was consistently 6%-7% and 4%, respectively, slower in migration than the corresponding wt proteins. This procedure was performed three times with the same results. Average values are presented in Table 8.

Isolation and Characterization of Additional Mutants

Once the strains tsB9 and g^rH had been generally characterized, it became possible to utilize them along with the well-characterized wt and DI strains as model strains in the development of a physiological screening procedure for poliovirus mutants. A screening procedure was developed with the aim of quickly characterizing a large number of virus mutants. The techniques finally incorporated into the screening procedure were chosen because they proved to be extremely suitable for the phenotypic analysis of large numbers of mutants with a minimal expenditure of time, money, and virus stock. The procedures adopted were individually described in Methods and Materials.

Generation and Isolation of Poliovirus Mutants

Mutagenesis of type 1 Mahoney poliovirus by hydroxylamine was used to generate a number of mutants from which the temperature-sensitive strains were selected. Hydroxylamine mutagenesis was described in Methods and Materials.

Table 8. SDS-PAGE of cytoplasmic proteins of poliovirus-infected cells labeled with ^{3}H-tyrosine, ^{3}H-lysine (tsB9), and ^{35}S-methionine (wild-type).--Gels were cut and counted. Both tsB9 and wild-type (wt) samples were mixed and run on the same gels. Numbers indicate average distance migrated from the origin in three replicate runs (cm).

Protein	wt Virion Proteins (cm)	wt Cytoplasmic Proteins (cm)	tsB9 33°C (cm)	tsB9 39.5°C (cm)
NCVP 1A	not present	4.5	4.5	4.3
VP 0	not present	8.0	7.9	8.0
VP 1	10.1	10.1	10.0	9.7
VP 2	10.3	10.3	10.3	not present
VP 3	10.7	10.7	10.7	10.7

Out of 2,000 randomly selected plaques, 14 ts mutants were isolated and stocks were prepared as previously described. One of these mutants appeared to revert to wt growth and was discarded. Another mutant was eliminated when its apparent titer dropped to zero during stock preparation.

The method of Korant (79) was used to isolate guanidine-resistant (g^r) mutants without chemical mutagenesis. Out of 200 plaques selected from virus grown in the presence of guanidine, only 3 exhibited equal infectivity in the presence and absence of guanidine. Five of the 200 mutants appeared to be guanidine dependent (g^d). During the growth of stocks, one of the g^r mutants appeared to become g^d and was discarded, as were all g^d mutants. Guanidine dependency is believed to result from a double mutation (Holland, personal communication, 1978 (64)). Only two g^r mutants were retained for further characterization; they were designated g^r1 and g^r2.

Characterization of Isolated Mutants

The mutants selected were examined by a number of methods in order to determine their physiological characteristics. All four prototype virus strains (wt, DI, tsB9, and g^rH) were analyzed in parallel with the 14 newly isolated mutants.

The virus stocks were divided into small aliquots and kept frozen (-20°C) or on ice at all times. The plaque size, titer, and e.o.p. 33/39.5 were determined for all 18 virus strains. The e.o.p. g-/g+ was determined only for the g^r strains as part of the isolation and purification procedure (Table 9).

All 14 of the new mutants were screened for the following characteristics at 33°C: 150 S virion, 80 S RNP, RNA synthesis, and protein synthesis. In every case, the mutants were wt with respect to these characteristics at 33°C.

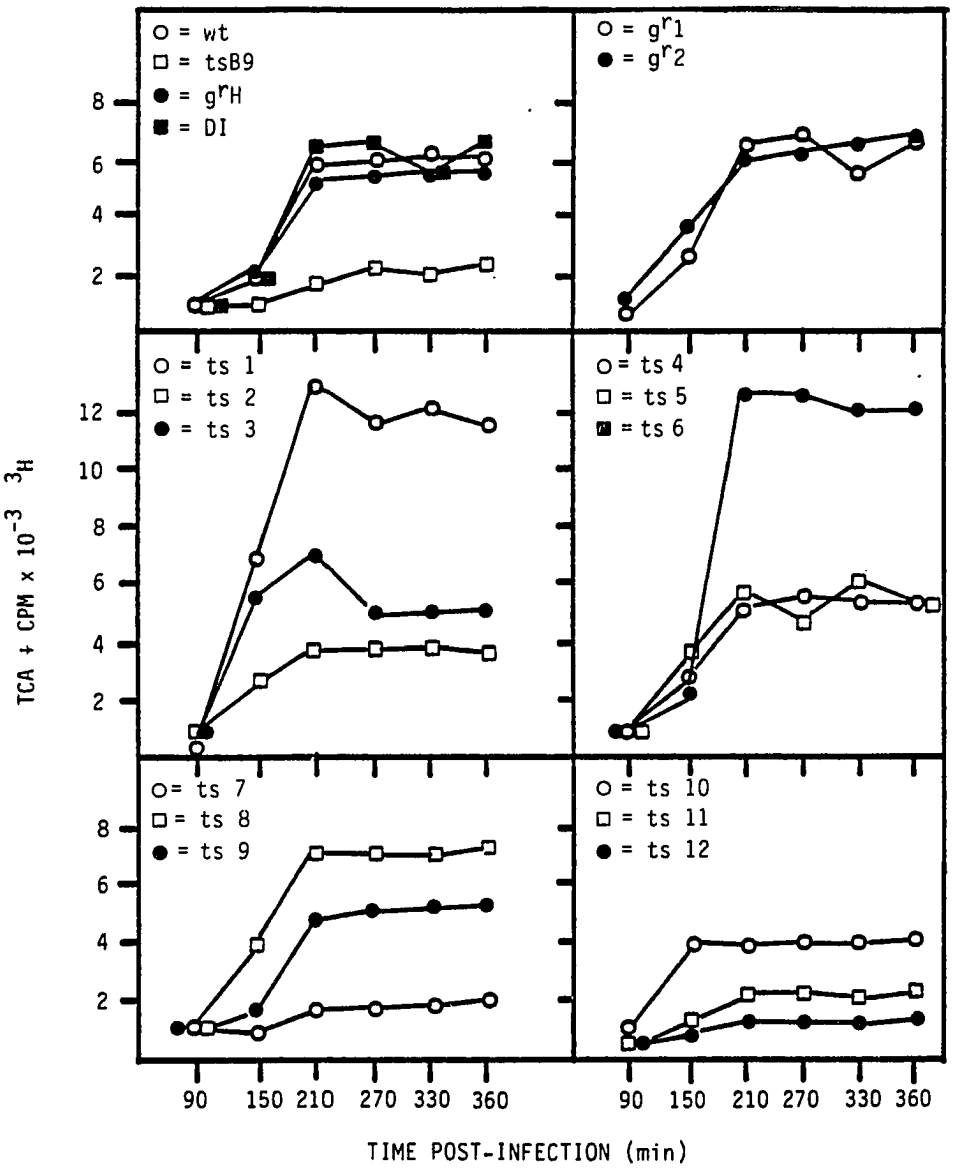
Synthesis of Actinomycin-D-resistant RNA. The accumulation of acid-precipitable material labeled with ^3H-uridine at 39.5°C was determined for all 18 virus strains as a measure of RNA synthesis.

Two of the ts mutants (ts 1 and ts 6) accumulated more labeled RNA than wt at 39.5°C and were scored ">+". Three ts mutants (ts 7, ts 11, and ts 12) were clearly defective "-" in RNA synthesis at 39.5°C. Three ts mutants (ts 2, ts 9, and ts 10) accumulated somewhat less RNA than wt at 39.5°C and were accordingly scored "+/-". The remaining ts mutants and the two g^r mutants were wt in accumulation of RNA at 39.5°C and were scored "+" (Figure 25).

Guanidine Sensitivity of RNA Synthesis at 33°C. In guanidine-sensitive (g^s) strains, RNA synthesis is decreased in the presence of guanidine. The synthesis of RNA was

Table 9. Growth characteristics of virus strains

Virus Strain	Titer at 33 (-) g	Titer at 39.5°C	e.o.p. 33/39.5	Titer 33 (+) g	e.o.p. -g/+g	Plaque Size
wt	1.0×10^{10}	1.1×10^{9}	0.9×10^{1}	6.3×10^{6}	1.6×10^{3}	wt
DI	3.0	0	-	-	-	wt
$g^{r}H$	8.7×10^{9}	8.0×10^{8}	10.9	8.3×10^{9}	1	wt
tsB9	6.0×10^{9}	3.2×10^{4}	1.9×10^{5}	7.3×10^{6}	8.2×10^{2}	wt
$g^{r}1$	8.0×10^{9}	7.1×10^{8}	11.3	7.6×10^{9}	1	wt
$g^{r}2$	7.3×10^{9}	6.9×10^{8}	10.6	7.0×10^{9}	1	wt
ts 1	2.2×10^{8}	4.1×10^{3}	5.4×10^{4}	-	-	wt
ts 2	3.0×10^{8}	2.9×10^{3}	1.0×10^{5}	-	-	m
ts 3	1.0×10^{9}	1.2×10^{4}	8.3×10^{4}	-	-	m
ts 4	1.6×10^{8}	7.0×10^{2}	2.3×10^{5}	-	-	m
ts 5	1.4×10^{8}	6.4×10^{3}	2.2×10^{4}	-	-	m
ts 6	1.1×10^{9}	1.0×10^{4}	2.2×10^{5}	-	-	m
ts 7	1.5×10^{8}	4.0×10^{3}	3.8×10^{4}	-	-	m
ts 8	8.0×10^{7}	1.0×10^{2}	8.0×10^{5}	-	-	m
ts 9	5.0×10^{8}	3.1×10^{3}	1.6×10^{5}	-	-	m
ts 10	2.6×10^{8}	1.0×10^{4}	2.6×10^{4}	-	-	m
ts 11	3.1×10^{9}	2.1×10^{4}	1.5×10^{5}	-	-	lg
ts 12	1.5×10^{8}	3.2×10^{3}	4.7×10^{4}	-	-	m



HeLa S-3 cells were infected at a multiplicity-of-infection of 10 plaque-forming-units/cell with each of the 14 mutants, tsB9, wt, DI, and g^rH and incubated at 39.5°C in the presence of 5 μg of actinomycin-D per ml of culture. At 90 min post-infection (p.i.), 10 μCi/ml of ^3H-uridine was added to each culture. At 90 min p.i. and each 30 min thereafter until 360 min p.i., a 0.1-ml aliquot was withdrawn from each culture and precipitated in 1.0 ml of ice-cold 10% TCA. Insoluble material was collected on Millipore membrane filters and the radioactivity was measured.

Figure 25. Accumulation of acid-insoluble RNA at 39.5°C

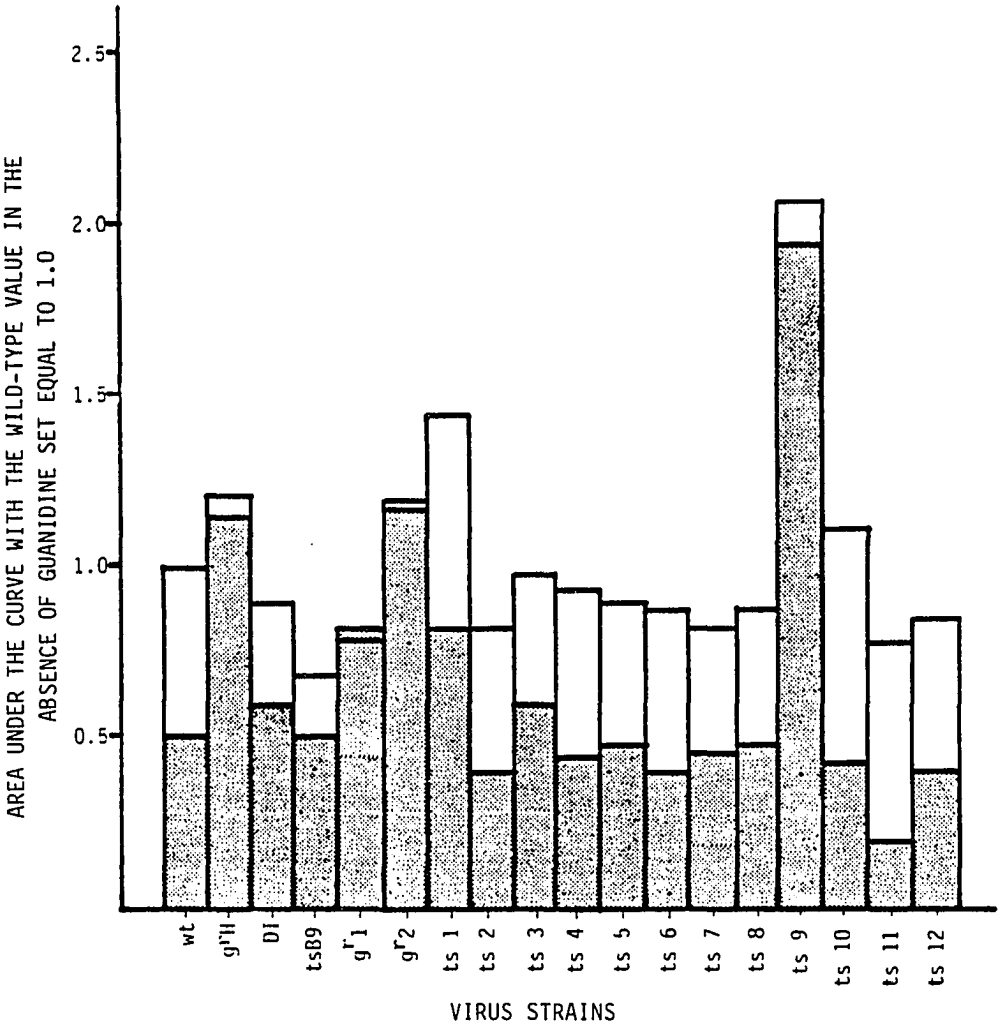
used as the parameter to determine the guanidine sensitivity or resistance of the new mutants.

The guanidine sensitivity of RNA synthesis was determined for all three strains at 33°C by measuring the rate of RNA synthesis in the presence and absence of guanidine (Figure 26). One of the ts mutants (ts 9) was found to be g^r. The g^r strains 1, 2, and H all exhibited approximately equal RNA synthesis in the presence and in the absence of guanidine. The remaining ts mutants and three model strains (wt, DI, and tsB9) were g^s to varying degrees with respect to RNA synthesis.

Protein Synthesis at 39.5°C. The rate of protein synthesis was determined for each of the 18 strains at 39.5°C. The procedure for monitoring the rate of protein synthesis was described in Methods and Materials.

Seven of the ts mutants (ts 2, ts 3, ts 5, ts 7, ts 9, ts 11, and ts 12) were found to be defective in protein synthesis and were scored "-" (Figure 27). Four ts mutants (ts 1, ts 4, ts 6, and ts 10) synthesized slightly less protein than wt at 39.5°C and were scored "+/-". One ts mutant (ts 8), g^rH, g^r1, g^r2, DI, and wt produced wt amounts of protein at 39.5°C and were scored "+".

Development of the "In Situ Lysis" Technique. A number of characteristic structures exist in the poliovirus-infected cell that can be seen in sucrose density gradient

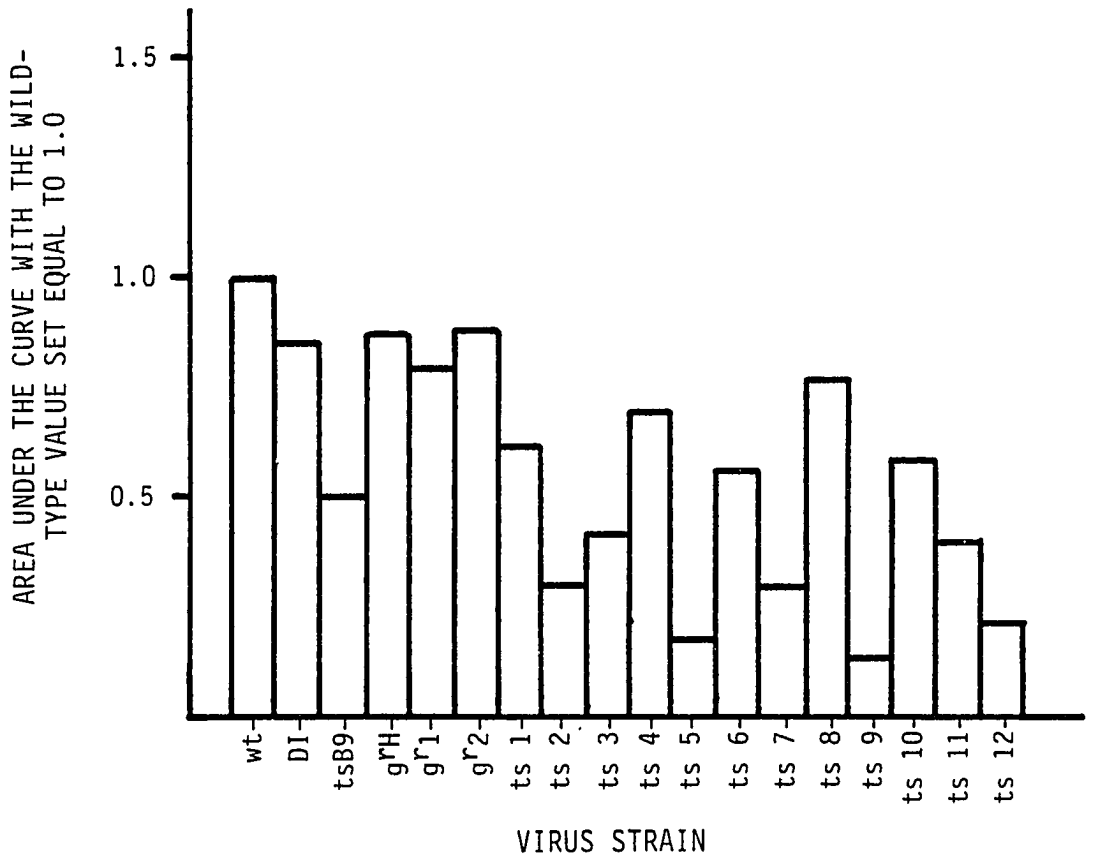


The sensitivity of RNA synthesis to guanidine (3 mM) at 33°C was determined for all virus strains. HeLa S-3 cells were infected at a multiplicity-of-infection of 10 plaque-forming-units/cell in the presence of actinomycin-D and incubated at 33°C. At 120 min post-infection (p.i.) and every 30 min thereafter, a 0.1-ml aliquot was withdrawn from each culture and placed in a microcentrifuge tube containing 2 Ci of ^{3}H-uridine and incubated at 33°C for 10 min. The samples were then chilled and treated with 0.5 ml of ice-cold 10% TCA. Insoluble material was collected on Millipore membrane filters and the radioactivity was measured.

Replicate cultures were incubated in the presence and absence of 3 mM guanidine. The guanidine was added to the (+) cultures at 110 min p.i.

The areas under the rate curves were determined. The value of the wild-type (wt) area in the absence of guanidine was set equal to 1.0. The total bar represents the value in the guanidine. Shading indicates the value in the presence of guanidine.

Figure 26. Guanidine sensitivity of RNA synthesis at 33°C



All 18 virus strains were examined with respect to the rate of protein synthesis at 39.5°C. The rate of protein synthesis was determined as described in Methods and Materials with the exception that the cultures were labeled by the uptake of a ^{3}H-amino acid mixture.

Figure 27. Rate of protein synthesis at 39.5°C

centrifugation due to their distinctive S values. With the techniques most often used, relatively large numbers of infected cells are used to make a cytoplasmic extract that is then cleared of nuclei by low-speed centrifugation and then subjected to ultracentrifugation on sucrose density gradients. This requires use of large amounts of cells and virus and involves a number of different steps for each structure to be examined. These factors limit the characterization of virus strains due to time and cost constraints. The need to conserve virus stock to minimize the risk of reversion to wt places further limits on these types of experiments.

In order to circumvent these problems, I developed an "in situ lysis" procedure that was used to characterize a number of different poliovirus molecular species and structures. The in situ lysis procedure uses small culture volumes (1.0 ml) that require small amounts of virus stock. It is a one-step procedure which dramatically reduces the time between inception of the experiment and having the data in hand. The infected cells are pelleted and resuspended in a small volume of buffer (0.25 ml of RSB). The intact cells are layered onto composite gradients for ultracentrifugation.

The composite gradients consisted of linear sucrose density gradients overlaid with 0.25 ml of detergent in low percentage sucrose (1% NP-40 and 1% DOC). The initial centrifugation was at 1,000 rpm for 2 min, which moved the intact

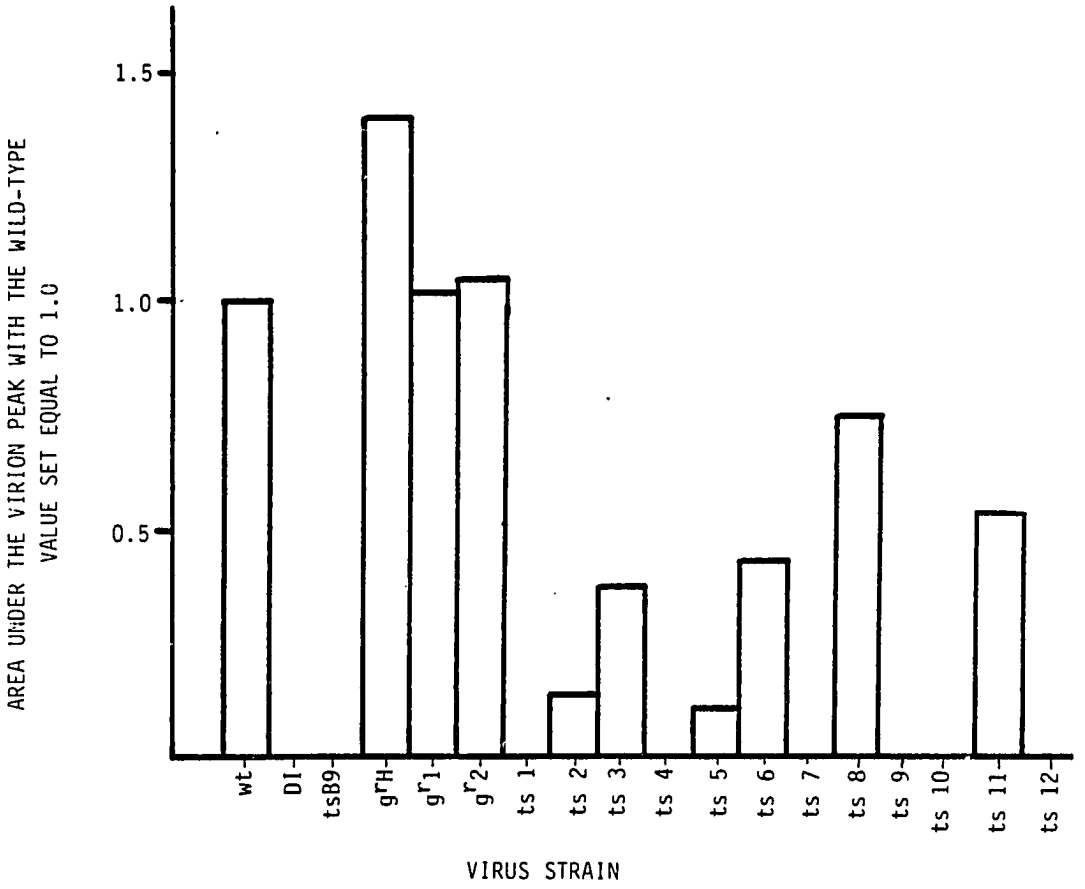
cells into the detergent layer where they underwent lysis. The centrifugation speed was then increased to 39,000 rpm for a length of time dependent upon the S value of the structure to be examined.

This single, simple technique allowed the virus-infected cells to be examined for the presence of 80 S procapsid, 150 S virion, and 80 S RNP under various culture conditions by varying the radioactive label and the duration of centrifugation. It could easily be adapted to detect other structures in other systems.

The details of the in situ lysis experiments were described in Methods and Materials.

Production of Virions and Procapsids at 39.5°C. All 18 strains were examined for production of virion at 39.5°C by labeling with ^{3}H-amino acids and examining the harvested cells by the in situ lysis procedure described in Methods and Materials.

Six of the ts mutants (ts 1, ts 4, ts 7, ts 9, ts 10, and ts 12) failed to produce detectable levels of virions at 39.5°C (Figure 28). The mutants ts 2 and ts 5 produced reduced, but still detectable, amounts of virion. The mutants ts 8, $g^{r}1$, and $g^{r}2$ all produced wt amounts of virion at 39.5°C. The three mutants ts 3, ts 6, and ts 11, produced slightly less virion than wt at the restrictive temperature.



HeLa S-3 cells (6 x 10^6 cells/ml) were infected with each of the 18 virus strains at a multiplicity-of-infection of 10 plaque-forming-units/cell in the presence of 7 µg of actinomycin-D per ml of culture and incubated at 39.5°C. At 90 min post-infection (p.i.), 60 µCi/ml of ^{3}H-uridine was added to each culture. At 360 min p.i., the cultures were harvested. The infected cells were resuspended in 0.25 ml of RSB and layered onto linear gradients of 14%-30% sucrose in TNE overlaid with 0.25 ml of 5% sucrose in RSB + 1% NP-40 + 1% DOC. The gradients were subjected to centrifugation (SW 50; 39,000 rpm; 1.5 h; 4°C). Two-drop fractions were collected directly into liquid scintillation minivials and the radioactivity was determined.

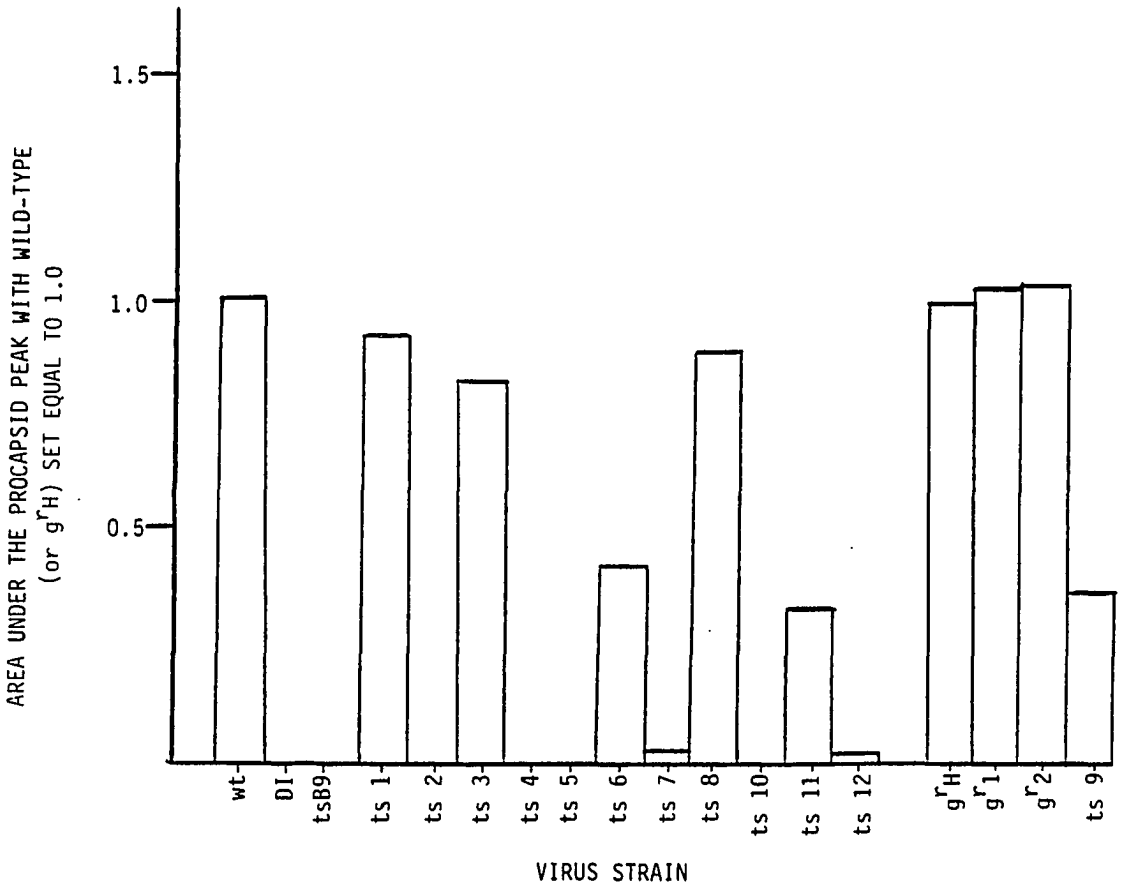
The area under the virion peaks was determined and the wild-type (wt) value was arbitrarily set equal to 1.0.

Figure 28. Virion production at 39.5°C examined by in situ lysis

In order to assess the production of procapsid, the guanidine-sensitive strains were examined by the in situ lysis procedure. The infected cells were labeled by the uptake of ^{3}H-amino acids after guanidine reversal. They were layered on in situ lysis gradients and analyzed as for virions with the exception that the centrifugation time was shortened. Guanidine-sensitive strains were compared to wt as the standard strain. Guanidine-resistant strains were examined without guanidine treatment or reversal and were compared to g^{r}H as the standard strain.

Six of the ts mutants produced little or no procapsid: ts 2, ts 4, ts 5, ts 7, ts 10, and ts 12 (Figure 29). These six also failed to produce virion. The ts mutant (ts 1) produced normal amounts of procapsid but no virion. The mutant ts 9 produced reduced amounts of both procapsid and virion. Both $g^{r}1$ and $g^{r}2$ produced wt amounts of both virion and procapsid. These three strains (ts 9, $g^{r}1$, and $g^{r}2$) were compared to wt levels of virion and g^{r}H levels of procapsid. The mutant ts 8 was equal to wt in production of virion and procapsid The remaining ts mutants produced varying patterns: ts 3 produced wt amounts of procapsid, but slightly less than wt amounts of virion; ts 6 and ts 11 produced slightly reduced amounts of both virion and procapsid.

Production of 80 S RNP at 39.5°C. An 80 S RNP is characteristically found in poliovirus-infected cells (65).



HeLa S-3 cells (6 x 10^6 cells/ml) were infected at a multiplicity-of-infection of 150 plaque-forming-units (pfu)/cell in the presence of 7 µg of actinomycin-D per ml of culture and incubated at 39.5°C. At 150 min post-infection (p.i.), guanidine hydrochloride was added to a final concentration of 3 mM to all guanidine-sensitive cultures. At 210 min p.i., 60 µCi of ^3H-amino acid mixture was added to each culture. The cultures were harvested at 270 min p.i. and analyzed on in situ lysis gradients centrifuged for 3.5 h. The guanidine-resistant strains (g^rH, g^r1, g^r2, and ts 9) were infected at 150 pfu/cell in the absence of guanidine. The area under the procapsid peak was determined and the wild-type (wt) value was set equal to 1.0 for all guanidine-sensitive strains, while the g^rH value was set equal to 1.0 for all guanidine-resistant strains.

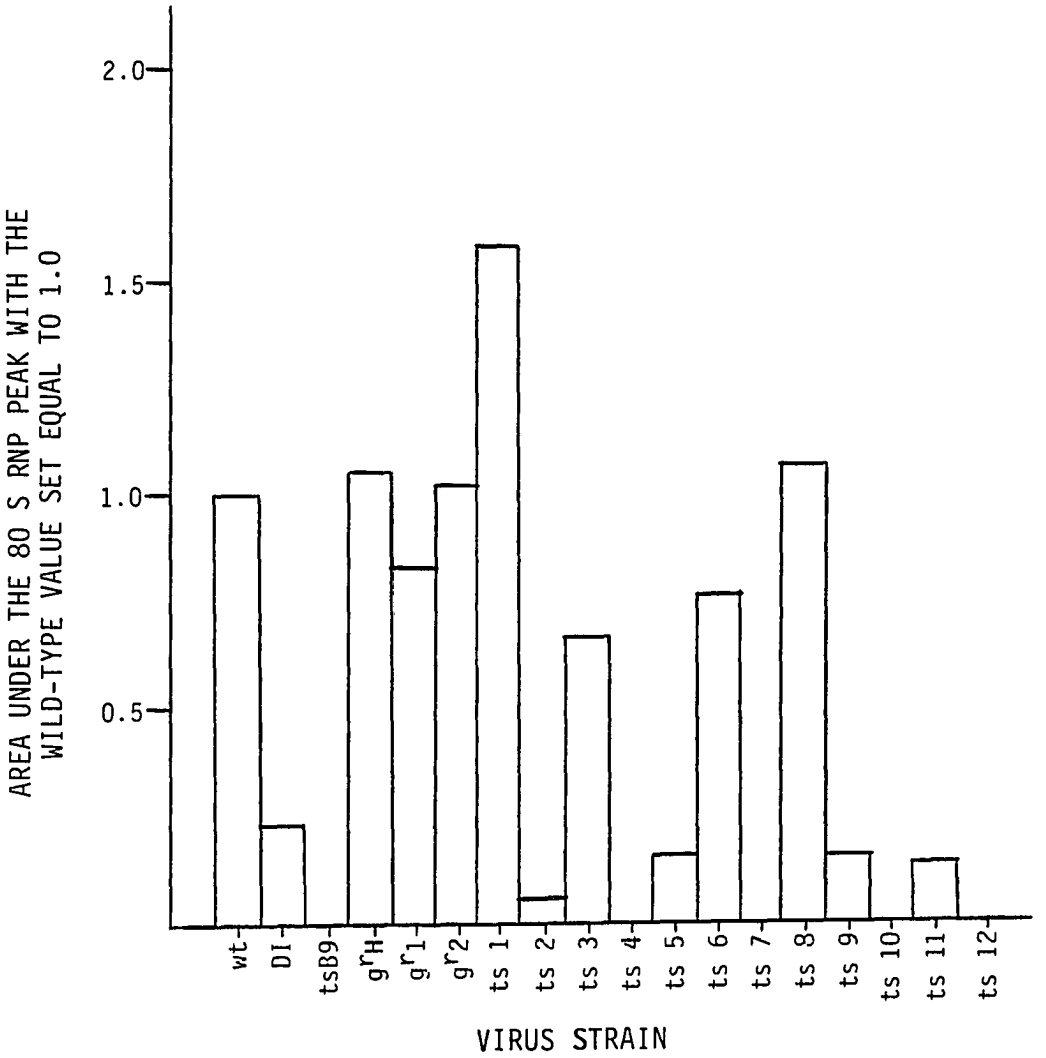
Figure 29. Procapsid production by infected cells at 39.5°C

This structure probably consists of the 35 S single-stranded RNA associated with various viral proteins. The 80 S RNP was examined at 39.5°C in cells infected with each of the 18 virus strains. The complex was labeled by the uptake of ^{3}H-uridine and subjected to centrifugation on in situ lysis gradients as described in Methods and Materials.

Eight of the ts mutants (ts 2, ts 4, ts 5, ts 7, ts 9, ts 10, ts 11, and ts 12) were found to be defective in the production of 80 S RNP when compared to wt at 39.5°C. The prototype strains tsB9 and DI were also defective in production of 80 S RNP.

Two of the mutants (ts 3 and ts 6) produced somewhat less 80 S RNP than wt. All other strains, except ts 1, were wt in 80 S RNP production. The mutant ts 1 produced more 80 S RNP than wt (Figure 30).

Production of 35 S and 20 S RNA at 39.5°C. The production of 35 S and 20 S RNA was examined by a variation of the in situ lysis procedure. Infected cells were labeled by the uptake of ^{3}H-uridine and harvested as previously described. The harvested cells were lysed in RSB buffer and the nuclei were pelleted by centrifugation. The cytoplasmic extract thus formed was treated with 1% SDS and subjected to centrifugation as for all in situ lysis procedures, using a centrifugation time optimized for displaying structures with S values of 35 and 20 (5 h).



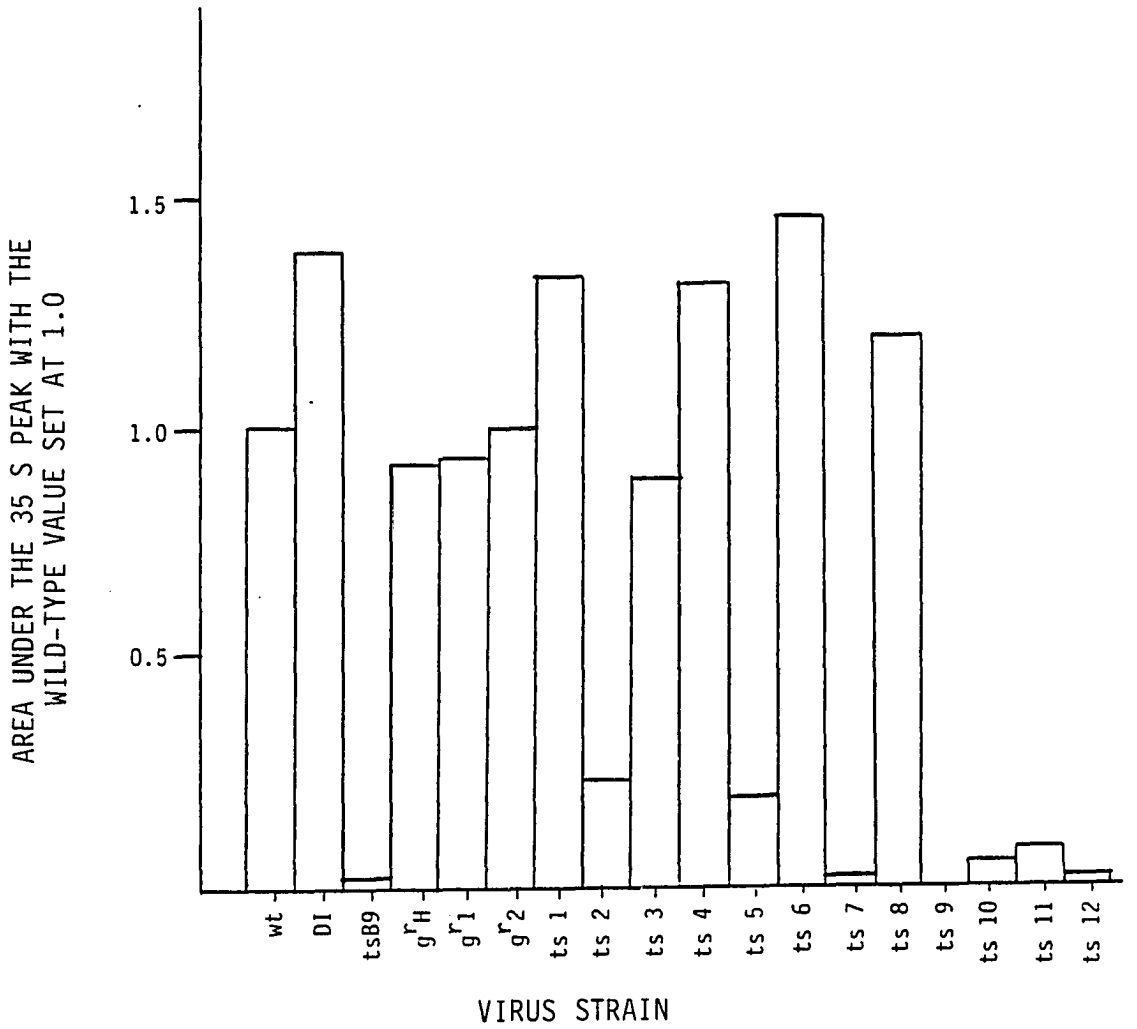
HeLa S-3 cells (6 x 10^6 cells/ml) were infected with each of the 18 strains at a multiplicity-of-infection of 10 plaque-forming-units/cell in the presence of actinomycin-D and incubated at 39.5°C. At 90 min post-infection (p.i.), 60 µCi/ml of ^{3}H-uridine was added to each culture. The cultures were harvested at 240 min p.i. and analyzed as described in Methods and Materials using the in situ lysis technique except that centrifugation was for 3.5 h. The area under the 80 S RNP peak was determined and the wild-type value was arbitrarily set equal to 1.0.

Figure 30. Presence of 80 S RNP in cultures incubated at 39.5°C using in situ lysis

Three ts mutants (ts 7, ts 9, and ts 12) were found to be defective in the production of 35 S RNA. Of these, only ts 12 was also defective in the production of 20 S RNA, ts 7 produced wt amounts of 20 S, and ts 9 somewhat less than wt. In view of the results described earlier in the analysis of tsB9 20 S RNA, no assumption can be made that the 20 S RNA produced by ts 7 and ts 9 are authentic RF RNA. While this would be an interesting avenue for further research, it is beyond the scope of the present paper.

Four ts mutants (ts 2, ts 5, ts 10, and ts 11) produced detectable, but less than wt, amounts of 35 S RNA at 39.5°C. Three of these (ts 2, ts 10, and ts 11) also produced less than wt amounts of 20 S RNA. The fourth (ts 5) produced wt amounts of 20 S RNA. Seven mutant strains (g^r1, g^r2, ts 1, ts 3, ts 4, ts 6, and ts 8) produced wt amounts of both 35 S and 20 S RNA at 39.5°C (Figures 31 and 32).

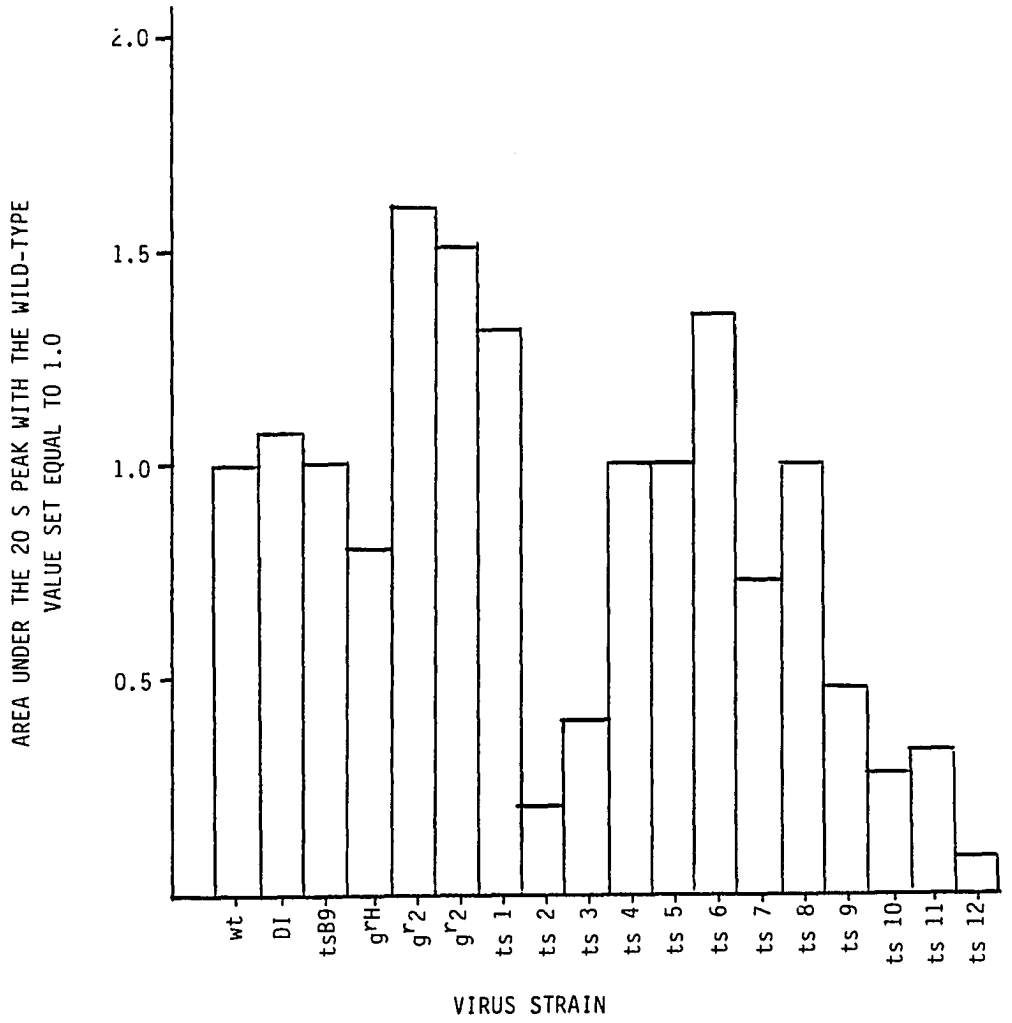
Inhibition of Host Protein Synthesis. Since inhibition of host-cell protein synthesis is an important characteristic of poliovirus infection, all of the 18 strains were examined for their ability to inhibit host-cell protein synthesis at 39.5°C. All of the strains caused the typical decline in total protein synthesis due to inhibition of host-cell protein synthesis by the virus. This was followed in all cases by an increase in total protein synthesis, probably due to virus-specific protein synthesis. The level of



HeLa S-3 cells (6 x 10^6 cells/ml) were infected at a multiplicity-of-infection of 10 plaque-forming-units/cell in the presence of actinomycin-D and incubated at 39.5°C. At 90 min post-infection (p.i.), 60 Ci/ml of ^{3}H-uridine was added to each culture. The cultures were harvested at 240 min p.i. The cells were lysed in 0.25 ml of RSB + 1% NP-40 + 1% DOC and cleared of nuclei by centrifugation (2,000 x g; 10 min; 4°C). The cytoplasmic extracts were treated with SDS at a final concentration of 1% and analyzed on linear gradients of 14%-30% sucrose in TNE (SW 50; 39,000 rpm; 5 h; 4°C).

The area under the 35 S peak was determined and the wild-type value was set equal to 1.0.

Figure 31. Presence of 35 S RNA in the cytoplasm of infected cells at 39.5°C



The gradients described in Figure 31 were analyzed for the amount of 20 S RNA. The area under the 20 S peak was determined and the wild-type value was set equal to 1.0.

Figure 32. Presence of 20 S RNA in the cytoplasm of infected cells at 39.5°C

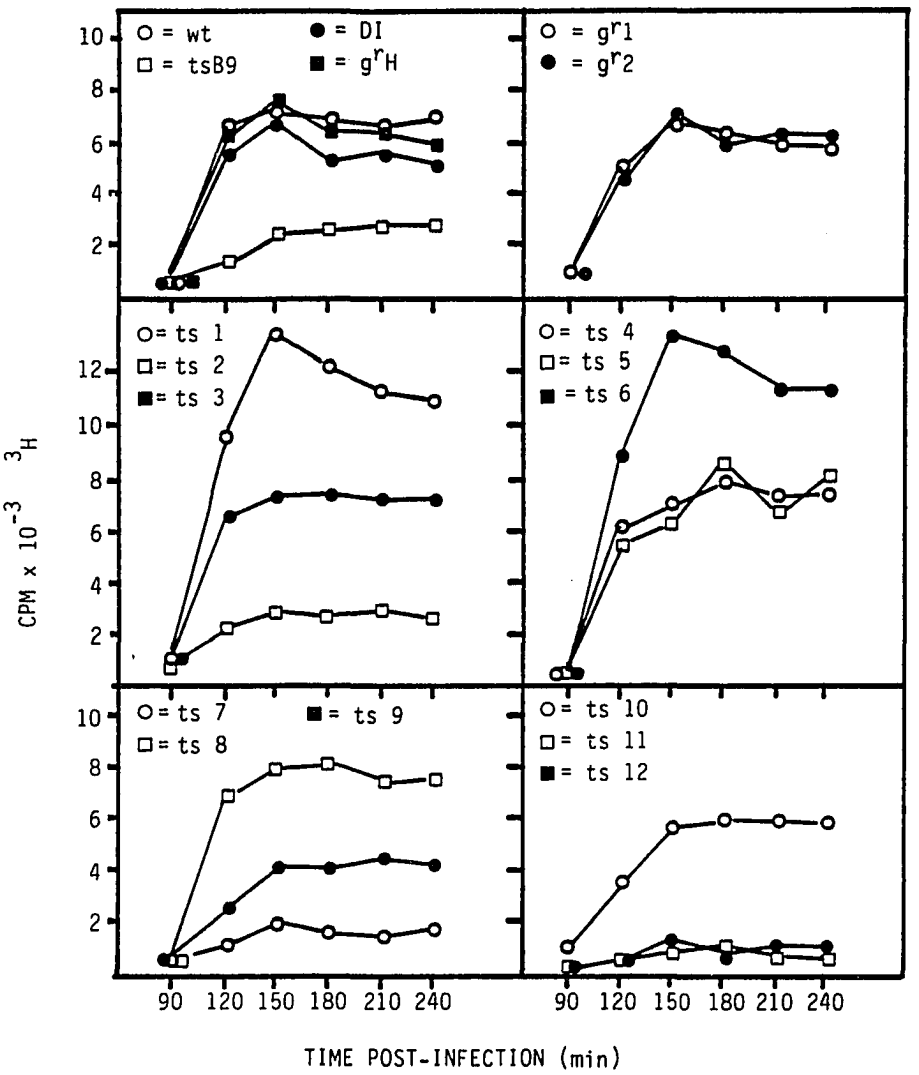
synthesis due to virus-directed translation never reached the level seen in the uninfected cell. The level of virus-directed protein synthesis was characteristic of the virus strain and reflected its phenotype with respect to protein synthesis which was discussed earlier in this section.

Incorporation of ^{3}H-choline into Membranes. Virus-induced membranes appear to play an important part in poliovirus infection. The level of synthesis of membranes after infection of each of the virus strains was examined by determining the incorporation of ^{3}H-choline into acid-precipitable material at 39.5°C (Figure 33).

Four ts mutants (ts 2, ts 7, ts 11, and ts 12) were defective in choline incorporation, as was the reference strain tsB9. The strain ts 9 stimulated intermediate levels of choline incorporation. One mutant (ts 10) produced just slightly less labeled membrane than wt. Two ts mutants (ts 1 and ts 6) stimulated greater incorporation of choline than did wt. The remaining ts mutants, as well as $g^{r}1$ and $g^{r}2$, stimulated choline incorporation to the same extent as wt at 39.5°C. The reference strains DI and $g^{r}H$ also stimulated wt levels of choline incorporation.

Correlation of Phenotypic Characters

The phenotypes of the 18 virus strains examined are shown in Table 10.



HeLa S-3 cells were infected at a multiplicity-of-infection of 10 plaque-forming-units/cell with each of the 18 virus strains and incubated at 39.5°C in the presence of actinomycin-D. At 90 min post-infection (p.i.), 10 μCi/ml of ^{3}H-choline was added to each culture. At 90 min p.i. and every 30 min thereafter, a 0.1-ml aliquot was withdrawn from each culture and precipitated in 1.0 ml of ice-cold 10% TCA. The acid-insoluble material was collected on Millipore membrane filters and the radioactivity was measured.

Figure 33. Incorporation of ^{3}H-choline into acid-insoluble material at 39.5°C

Table 10. Phenotypic groups of poliovirus and their characteristics

Virus Groups								
I	II	III	IV	V	VI	VII	VIII	IX
wt	ts 1	DI	ts 3	ts 4	ts 5	ts 10	ts 2	ts 7
g^r_H				ts 6			ts 9	tsB9
g^r_1							ts 11	
g^r_2							ts 12	
ts 8								
RNA+	RNA+	RNA+	RNA+	RNA+	RNA+	RNA+/-	RNA-	RNA-
chol+	chol+	chol+	chol+	chol+	chol+	chol+/-	chol-	chol-
80 S RNP+	80 S RNP+	80 S RNP-	80 S RNP-	80 S RNP-	80 S RNP-	80 S RNP-	80 S RNP-	80 S RNP-
35 S RNA+	35 S RNA+	35 S RNA+	35 S RNA+	35 S RNA+	35 S RNA+/-	35 S RNA-	35 S RNA-	35S RNA-
20 S RNA+	20 S RNA+	20 S RNA+	20 S RNA+	20 S RNA+	20 S RNA+	20 S RNA-	20 S RNA-	20 S RNA+
pro+	pro-	pro+	pro-	pro-	pro-	pro-	pro-	pro-
V+	V-	V-	V-	V-	V-	V-	V-	V-
PC+	PC+	PC-	PC+	PC-	PC-	PC-	PC-	PC-

It can be seen that g^r1 and g^r2 are not ts in any characteristic. They were found to differ by the criteria used herein from wt only by the resistance of their RNA synthesis and infectivity to guanidine.

No strains were found to be defective in the ability to cause inhibition of host-cell protein synthesis.

All of the virus strains that synthesized virions at the restrictive temperature were also found to produce procapsid, 80 S RNP, 35 S RNA, and 20 S RNA. The strains wt, g^rH, g^r1, g^r2, and ts 8 fell into this group. By all characteristics except titer, e.o.p. 33/39.5, and plaque size, ts 8 was essentially the same as wt. It showed, however, a marked decrease in infectivity at 39.5°C, and produced very small distinct plaques.

It was found that any strain that produced 80 S RNP also produced both 35 S and 20 S RNA.

There was a high degree of correspondence between RNA synthesis and incorporation of ^3H-choline into acid-insoluble material at 39.5°C. Strains that were defective in one feature were also defective in the other to approximately the same degree with the single exception of ts 10, which produces 80% as much RNA as wt. This is not unexpected in view of the close association between RNA synthesis and cytoplasmic membranes in poliovirus infection.

DISCUSSION

The current research was carried out in two discrete sections:

1. The physiological characterization of the mutant strains tsB9 and g^rH.
2. The development, validation, and application of a screening procedure that quickly and efficiently demonstrated the physiological character of a large number of poliovirus mutants in a short period of time.

After the virus strains tsB9 and g^rH had been characterized, they were used, along with wild-type (wt) and defective-interfering (DI) strains, to serve as model strains for the validation of the screening procedures. The validated screening procedure was then used to quickly evaluate the physiological characteristics of 14 newly isolated mutants of poliovirus type 1.

Characterization of tsB9 and g^rH

The model guanidine-resistant (g^r) strain, g^rH, was found to differ from wt only in its resistance to guanidine. It did not exhibit any other defects nor any temperature-sensitive (ts) characteristics.

The g^rH strain was essentially wt in its ability to produce cytopathic effects, in the stability of its infectivity to acid and heat, and in the production of RNA, virion, and procapsid.

It might be expected that g^rH would exhibit a difference from wt in the electrophoretic mobility of its structural proteins (79), which were not examined in the current research.

The virus strain tsB9 was found to be a temperature-sensitive mutant that expressed its defects in a narrow range around the restrictive temperature. It was defective in both RNA and protein synthesis and did not produce virions or procapsids at the restrictive temperature. Both the RNA(-) phenotype and the temperature sensitivity of infectivity were fully expressed at 38.5°C to 39.5°C.

A number of findings suggest that tsB9 might have a defect in its structural proteins. The synthesis of protein by tsB9 at the restrictive temperature was approximately 50% of wt. No virions or procapsids can be detected, by electron microscopy or radiochemical labeling procedures, in tsB9-infected cells at the restrictive temperature. The infectivity of tsB9 is more sensitive to acid pH than wt, as is often true of structural mutants (90). The electrophoretic mobility of the structural proteins NCVP 1a and VP 1 are 4% to 7% less in tsB9-infected cells at the

restrictive temperature than in wt. Structural protein precursors synthesized in tsB9-infected cells at the restrictive temperature are not able to assemble into virions or procapsids when the cultures are shifted down to the permissive temperature under conditions preventing further protein synthesis. It is consistent with the results that tsB9 was also unable to complement DI infections in any respect at the restrictive temperature, which would be expected if both mutants express defects in the structural protein region.

In addition to its defect in protein synthesis, tsB9 was demonstrated to be ts with respect to RNA synthesis. The synthesis of RNA in tsB9-infected cells at the restrictive temperature was about half of that of wt at 39.5°C. Cells infected with tsB9 synthesized little or no 35 S single-stranded RNA at the restrictive temperature. The predominant species of RNA produced by these cells sedimented slightly faster than authentic replicative-form (RF) RNA in sucrose density gradients and had a lower electrophoretic mobility in agarose gels. This anomalous RNA appeared to be a partially single-, partially double-stranded species by the following criteria.

1. It consisted of species that were soluble (double-stranded) and species that were insoluble (single-stranded) in 2 M LiCl.

2. It was about 60% resistant to ribonuclease A at high salt, which is intermediate between the RNAse resistance of single- and double-stranded RNA.
3. It did not exhibit the A = U, G = C base composition of double-stranded RNA nor the typical base composition of poliovirus single-stranded RNA.
4. It remained at the origin during high-voltage ionophoresis after digestion in high salt with single-strand ricobnucleases, and it mgirated with the nucleotides after digestion in low salt.
5. It migrated exactly with replicative-intermediate (RI) RNA of wt, which itself is a partially single-, partially double-stranded molecule, in agarose gel electrophoresis.

It is apparent that this anomalous "20 S" RNA is not identical with authentic RF RNA. The exact nature and function of this RNA, however, could not be established.

The fact that tsB9 expressed ts defects in both protein and RNA synthesis is open to a number of interpretations. Since both the RNA(-) and virion(-) phenotypes co-reverted to wt, it is suggested that the occurrence of both defects is not due to a double mutation. It follows that RNA(-) and virion(-) phenotypes are related genetically, functionally, or by the conformation of a precursor protein.

In a virus like polio, where the proteins are generated by post-translational cleavage of a single precursor, the interrelationship of the various coding regions is complex. If the ts defect results in an altered conformation of the large precursor (NCVP 00), such that its cleavage is altered, it is conceivable that several coding regions could be affected by a single mutation. In such a case, the location of the mutation might not be correctly inferred from phenotypic analyses. The phenotypic defects could occur in functions coded by regions distal to the mutation.

There is also the possibility that the RNA(-) and virion(-) phenotypes are functionally related. It has been suggested that RNA synthesis in poliovirus-infected cells involves structural proteins in an as yet undefined role (140). If this is true, then a defect in structural proteins could result in decreased RNA synthesis. Mutants such as DI, which have normal RNA synthesis in the presence of defective structural proteins, would be presumed to have still intact those regions of structural protein which are involved in RNA synthesis.

Recently, techniques have been developed that allow picornavirus RNA sequences to be determined (113). To fully elucidate the nature of the defects in virus mutants such as tsB9 and g^rH, I would recommend that their RNA sequences be determined and compared to that of their wt parent. It would

then be possible to correlate the observed physiological characteristics with the altered sequences. A great deal of insight into poliovirus macromolecular synthesis would thus be made available.

Isolation and Characterization of Additional Mutants

Twelve ts mutants of poliovirus type 1 were generated by hydroxylamine mutagenesis and isolated by replicate plating at 33°C and 39.5°C. Two g^r mutants were isolated from stocks of wt after repeated passage in the presence of guanidine.

The developed screening procedure proved to be quite suitable for quickly characterizing the mutant strains with the expenditure of minimal amounts of time, money, and virus stock. The individual techniques used provided clear and meaningful results which were able to differentiate the four model strains, wt, DI, tsB9, and g^rH. All of these techniques utilized small-scale infection and analytic methods, thereby requiring only small amounts of materials and virus stock. The small scale of the analytic methods allowed the experiments to be carried out in a short period of time.

With the exception of the plaque assays and the examination of 35 S and 20 S RNA, all of the methods used allowed the analysis of a single parameter for all 18 strains in a single day.

Once the analysis procedures have been validated for the virus system in question, the screening of large numbers of virus mutants can be done in a short period of time.

All 12 of the newly generated ts mutants were examined and found to be essentially wt at the permissive temperature.

The g^r strains, g^r1 and g^r2, like the model strain g^rH, differed from wt only in their resistance to guanidine. In view of the previous research (79), these mutants might be expected to show alterations in structural proteins. An in-depth analysis of the viral proteins was beyond the scope of the current research. Such an analysis would be appropriate if these mutants were retained for more detailed examination.

The 18 virus strains fell into 9 groups according to differing phenotypes expressed at the restrictive temperature. These groups are the same as those found by various other investigators (32, 33, 34, 35, 138). Groups I-VI were RNA(+), producing wt or greater levels of total RNA as measured by the accumulation of acid-precipitable material labeled with ^{3}H-uridine. Groups VII-IX were RNA(-) or RNA(+/-), producing less than wt levels of total RNA.

Incorporation of ^{3}H-choline into membranes correlated closely with the RNA phenotype. Groups I-VI incorporated

wt or greater levels of choline while Groups VII-IX incorporated less than wt levels.

The strains in Group I were also wt with respect to the presence of 80 S RNP in the cytoplasm, synthesis of 35 S and 20 S RNA, total protein synthesis, and the production of virion and procapsid in addition to RNA synthesis, as well as choline incorporation. Group I includes wt, g^rH, g^r1, g^r2, and ts 8. The g^r mutants differed from wt only in their resistance to guanidine. The mutant ts 8 was different from wt only in the ts character of its infectivity and the small size of its plaque.

Group II contained only one strain, ts 1. It was defective in virion production, and total protein synthesis produced small plaques, and was ts in infectivity. In all other respects it was wt.

Group III included one mutant strain, DI. It was defective in the production of virions, procapsids, and 80 S RNP, and produced no plaques. No ts characteristics were found in DI.

Group IV contained only the strain ts 3, which was defective in virion production, 80 S RNP production, and total protein synthesis. It was also ts in infectivity and produced smaller than wt plaques. It was wt with respect to procapsid production, total RNA synthesis, choline incorporation, and the synthesis of 35 S and 20 S RNA.

Two strains, ts 6 and ts 4, fell into Group V. These strains were defective in virion, procapsid, 80 S RNP, and total protein synthesis. The plaques produced were smaller than wt. These strains produced wt amounts of total RNA, 35 S RNA and 20 S RNA, and incorporated wt amounts of choline.

The last RNA(+) group, Group VI, contained the strain ts 5. It was defective in virion, procapsid and 80 S RNP production, as well as total protein synthesis. It synthesized slightly less 35 S RNA than wt but fully wt levels of 20 S RNA.

The last three groups were all defective in total RNA synthesis and incorporation of choline. Group VII contained the strain ts 10, which was +/- in both RNA synthesis and choline uptake. It was defective in virion, procapsid, 80 S RNP, 35 S RNA, 20 S RNA, and total protein production. Its plaques were smaller than wt and it was ts in infectivity.

Group VIII included ts 2, ts 9, ts 11, and ts 12, which were defective in virion, procapsid, and 80 S RNP production, total protein synthesis, and synthesis of 35 S and 20 S RNA. Strains ts 2, ts 9, and ts 12 produced small plaques, while ts 11 produced large plaques; all exhibited ts infectivity.

Group IX included tsB9 and ts 7. These two strains were very similar under all conditions. They were defective in total RNA and protein synthesis, virion, procapsid, and 80 S RNP production, and in the synthesis of 35 S RNA. They both produced wt levels of a 20 S RNA. The nature of this 20 S RNA is not known, but in view of the previously described work with tsB9, it cannot be assumed to be authentic RF RNA.

It is interesting to note that all strains which produced wt amounts of virion also produced wt amounts of procapsid, 80 S RNP, 35 S RNA, and 20 S RNA. All strains with wt levels of 80 S RNP also produced wt levels of procapsid, 35 S RNA and 20 S RNA.

It is known that the synthesis of virion requires the presence of both virion RNA (35 S) and capsid proteins. Structural proteins and 35 S RNA are also major components of the 80 S RNP (66) and may be necessary for its formation.

The function of the 80 S RNP is not known. Indeed, it has not been fully established that it has any function and could conceivably be an association of viral proteins with virion RNA for which they have an affinity. On the other hand, the 80 S RNP may be involved in transcription, translation, and/or assembly. The exact composition and function of this complex would offer interesting avenues for further exploration.

All strains which were wt in the production of 35 S RNA were also wt in the production of 20 S RNA.

Two strains produced reduced amounts of both procapsids and virions (ts 6 and ts 11) while one produced wt amounts of procapsids but slightly reduced amounts of virions (ts 3).

Several of the virus strains were found to be defective in production of both RNA and protein species. Such mutants are found regularly and are dismissed as double mutants especially if the protein defect appears to fall in the structural proteins (32, 33, 34, 35, 138). This may be true in some cases. It became clear, however, in examining tsB9 that it is not necessarily true in every instance. When the co-reversion of RNA synthesis and virion production of tsB9 to wt was examined, the two characteristics were found to co-revert in every case. Since the stock examined was plaque-purified, it is doubtful that these revertants were simply wt contaminants. Therefore, it appears that the RNA(-) and virion(-) phenotypes of tsB9 are related. The nature of that relationship needs to be further explored.

The availability of rapid sequencing techniques applicable to poliovirus has made it possible to more clearly define the correlation between RNA sequence changes (mutations) and physiological functions. The physiological screening procedure used in the current research could

prove valuable in this regard. Once a mutant is isolated, it could be cloned and its RNA sequence determined (113). The sequence of the mutant could then be compared to that of the wt parent strain and the exact location and nature of the mutation determined. The physiological effects of this mutation would then be determined using the phenotype screening procedure. Appropriate avenues for more detailed exploration would thus be discovered.

A valuable technique to add to a screening procedure such as the one developed herein would be slab-gel polyacrylamide gel electrophoresis. Equipment now commercially available makes it possible to run up to 12 vertical slab gels simultaneously (Hoefer, Bio-Rad). Each slap potentially accommodates 20 samples, thus allowing the rapid examination of 240 virus strains at one time. The major limitation of this technique would be the expense of the electrophoresis equipment and chemicals. It would be amenable to small-scale experiments, and would therefore retain the advantage of quick anlysis using minimal time, virus stock, and materials.

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