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COMPLEMENTATION BETWEEN TEMPERATURE-SENSITIVE MUTANTS OF POLIOVIRUS

by

LAURA WAKEFORD

A thesis submitted to the faculty of the

DEPARTMENT OF MOLECULAR AND CELLULAR BIOLOGY

In Partial Fullfillment of the Requirements

for the degree of

MASTER OF SCIENCE

In the Graduate College

THE UNIVERSITY OF ARIZONA

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STATEMENT BY THE AUTHOR

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This thesis has been approved on the date shown below:

Martinez J. Hewlett
Associate Professor of
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9-22-87
Date

This work is dedicated with much love
to ANDY and APRIL.

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ACKNOWLEDGEMENT

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ABSTRACT

Conditional lethal mutants of poliovirus type 1 (Mahoney) were generated by treatment with the mutagen hydroxylamine. Temperature-sensitive mutants were selected by the replica plating technique at temperatures of 33°C (permissive) and 39°C (restrictive). New mutants were generated to acheive a larger population of mutnats and also to generate additional RNA- mutants in this population.

These mutants were characterized by two criteria: RNA synthesis and thermal stability. RNA synthesis is measured by the accumulation of labeled uridine incorporation into trichloroacetic acid (TCA) insoluble material. The thermal stability is determined by the difference in plaque forming units before and after treatment of the virion at 45°C.

Complementation co-infections (5 MOI for each virus stock) were analyzed for the presence of the 150S virion particle of poliovirus after sedimentation through a linear sucrose gradient. Complementation is observed between RNA(+) mutants v.s. RNA(-) mutants, and between two RNA(-) mutants, but not between two RNA(+) mutants. Although reciprocal complementation has not been documented in this study some speculation on complementation is presented in this thesis.

CHAPTER 1

INTRODUCTION

Poliovirus is a member of the Picornaviridae family. These are among the smallest animal RNA viruses. The Picornaviridae family of viruses is divided into four genera on the basis of pH stability, bouyant density, sedimentation coefficient and serological properties. The Picornaviridae genera are: Enterovirus, Cardiovirus, Rhinovirus, and Aphthovirus (Table 1). Poliovirus is included in the enterovirus group which inhabit the alimentary tract (Reuckert, 1985). Also included in this group are the coxsackieviruses (23 serotypes), the echoviruses (32 serotypes), the human enteroviruses (4 serotypes), human hepatitis virus A (1 serotype, and a number of nonhuman enteric viruses (37 serotypes). The cardioviruses have a single serotype but includes Columbia SK, encephalomyocarditis (EMC) virus, Maus Elberfeld (ME) virus, MM virus and mengovirus. The rhinovirus group is now approaching 113 serotypes. These human rhinoviruses have developed a special adaptation to the nasopharyngeal region and are agents of the common cold in adults and children. Foot-and-mouth disease virus (7 serotypes) is the most important single pathogen of livestock and is the protoype for the Aptho-

TABLE 1. *The picornavirus family*[a]

Genus	Number of serotypes	Members
Enterovirus	3	Human polioviruses 1–3
	23	Human coxsackieviruses A1–22, 24 (A23 = echovirus 9)[b]
	6	Human coxsackieviruses B1–6[c] (swine vesicular disease virus is very similar to coxsackievirus B5) (112)
	32	Human echoviruses 1–9, 11–27, 29–34; echo 10 = reovirus type 1[b,d] echo 28 = human rhinovirus 1A[b]
	4	Human enteroviruses 68–71
	1	Human hepatitis virus A (human enterovirus 72)
	1	Vilyuisk virus
	18	Simian enteroviruses 1–18
	7	Bovine enteroviruses 1–7
	11	Porcine enteroviruses 1–11
	1	Murine poliovirus (Theiler's virus TO, FA, GD7)
Cardiovirus	1	Encephalomyocarditis (EMC), mengovirus, Maus Elberfeld (ME) virus, Columbia SK, MM
Rhinovirus	113?	Human rhinoviruses 1–89
	2	Bovine rhinoviruses 1–2
Aphthovirus	7	Foot-and-mouth disease virus 1–7 (serotypes A, C, O, SAT 1, 2, 3, Asia 1)
Unassigned	>3	Equine rhinoviruses 1, 2, cricket paralysis virus, drosophila C virus, tussock moth picornavirus
	>231	

[a] The so-called feline picornaviruses are now classified in the family Caliciviridae (194).

[b] Vacated numbers are now unused.

[c] Coxsackieviruses are named after Coxsackie, New York, the town from which the initial isolates were made.

[d] Echo is an acronym for enteric cytopathic human orphan.

virus genus. There is now an unassigned group of picornaviridae which includes the equine rhinoviruses 1 and 2 and a collection of insect picornaviruses.(Rueckert,1985) Typically, these viruses are composed of a single molecule of single-stranded RNA(MW=2.5 X 10^6) inside the cavity of a thin protein shell(MW=5.4 X 10^6). The diameter of the virion is between 27 and 28nm as measured through the electron microscope (Rueckert,1985). X-Ray diffraction analysis of poliovirus crystals indicates icosohedral symmetry due to the regular organization of 60 identical protein subunits arrange in axes of 5-fold, 3-fold, and 2-fold symmetry (Finch and Klug, 1959).

The genome of poliovirus type 1 has been completely sequenced (Racaniello and Baltimore,1982; Kitamura et al,1981) by the construction of cDNA into plasmids of E. coli. The cDNA clone containing the entire genome has been shown to be infectious when transfected into mammalian cells. Although the mechinism of virus replication is unclear it is possible that the plasmid enters the nucleus and RNA synthesis may initiate at one of the areas in pBR322 which functions as a promoter in <u>in vitro</u> reactions (Racaniello and Baltimore, 1981).

The poliovirus genome is characterized as a single strand of RNA 7433 ribonucleotides long (Kitamura et al,1981) with a viral protein (designated VPg) linked by an

O^4(5'uridyl)-tyrosine at the 5'terminus of this genome (Lee et al,1976; Nomoto et al,1977; Rothberg et al,1978; Ambros and Baltimore,1978). The poly(A) tail located at the 3'terminus is genetically encoded by a poly(U) series of ribonucleotides (Armstrong,1972; Yogo and Wimmer,1972). The viral RNA is the message RNA which is transcribed into a single polyprotein (Summers and Maizel,1968; Jacobson and Baltimore,1968b). Protein synthesis of this polyprotein is initiated at an AUG codon at ribonucleotide 741. This is the nineth AUG codon that the ribosome traverses which is in the appropriate reading frame, and also not followed by a termination codon (Kitamura et al,1981; Racaniello et al,1981).

The virion has been studied by electron microscope in vivo within the cellular cytoplasm (Dales et al,1965). The capsid structure was observed to be a perfect icosohedron with 12 vertices. Each vertex is composed of 60 subunits organized in an arrangement of pentamers. These pentamers are structural proteins (VP0, VP1, VP2, VP3,and VP4) genetically coded by the virus.

The viral RNA that has been released from the capsid has been translated in cell-free systems (Villa-Komaroff et al,1974; Smith,1973) and when it is purified away from the capsid it is infectious (Colter et al,1957; Alexander et al,1958). This RNA genome has been defined as

plus sense because it serves as message within the infected cell to translate the virus specific replicase and other proteins necessary for virus replication.

Host Cell-Virus Interaction

Specific cell surface interactions occur for each of the major species and genera within the picornavirus family (Crowell,1976). Poliovirus attaches to the surface of specific human or primate target cells through enterovirus cell surface receptors (Holland,1964b; Mandel,1967; Lonberg-Holm and Korant,1972). Polioviruses of each of the three serotypes bind to the same receptor on cells of primate origins, but other members of the picornavirus family do not compete for this receptor (Crowell and Siak, 1978). Analysis of mouse-human hybrids has shown that the information for the poliovirus receptor is located on human chromosome number 19 (Medrano and Green,1973; Miller et al,1974). Isolated plasma membrane components can still specifically bind poliovirus (Krah and Crowell, 1982). Crowell and Landau (1983), suggest that the receptor is a glycoprotein based on studies on receptor blockage and inactivation. Nobis et al (1985), prepared human-mouse cell hybrids which produced a specific monoclonal antibody (D171). The antibody D171 recognizes essentially all of the epitopes blocked by poliovirus. These researchers have concluded that idiotypic antibodies

to D171 might be useful in the elucidation of the receptor recognition site of poliovirus (Nobis et al, 1985).

After picornavirus attachment to the cell surface the virus undergoes preliminary and irreversible modifications which designate the "eclipse" stage of virus infection (De Sena and Mandel,1977). These modifications are characterized by (1) loss of the VP4 capsid polypeptide, (2) a slight reduction in S value (130S),(3) acquired sensitivity to proteases and detergents, and (4) loss of infectivity (although the particle still contains a functional genome which is inaccessible to nucleases; Crowell and Philipson,1971; Fenwick and Wall,1973;Lonberg- Holm, Gosser, and Kauer,1975 ; Crowell et al, 1981).

The eclipse stage is a prerequisite to "uncoating" of the virus. "Uncoating" is the release of the viral genome from the enveloping capsid (De Sena and Mandel,1977). The experimental evidence recounted above allows that two alternate pathways may be hypothesized for virus uncoating, (1) the M (modified) particles are uncoated directly by a detergent-like cellular constituent or (2) the M particles are further modified by an intracellular protease prior to uncoating (De Sena and Mandel,1977). However, the actual cellular processes and events between modification and the final stage of uncoating are virtually unknown (De Sena and Mandel,1977).

Poliovirus Protein Synthesis

Following uncoating of the virion, the viral RNA genome is translated to provide the necessary proteins for self-replication. When extracted from the cytoplasm the poliovirus message is closely associated with polyribosomes attached to the rough endoplasmic reticulum (Penman et al,1964; Caliguiri and Tamm,1970). It is distinct from the genomic 35S RNA because it is isolated from the polyribosomes with a 5'pUp terminal nucleotide (Hewlett et al,1976; Nomoto et al, 1976; Fernandez-Munoz and Darnell,1976). It appears that the genome linked protein VPg is enzymatically cleaved by a protease present in the uninfected cell (Ambros et al,1978). This difference between the virion RNA (5'VPg) and the mRNA (5' pUp) of poliovirus might be a method to regulate the types of RNA's synthesized during the course of an infection.

Poliovirus contains a highly conserved 741 nucleotide untranslated sequence 5' of the AUG initiation codon used for synthesis of the of the poliovirus polyprotein. Semler et al (1986) constructed a recombinant virus from a chi-meric plasmid between poliovirus and a temperature sensitive coxsackie virus. However, he only transfered a 405-base pair cDNA fragment from the 5' untranslated region of the coxsackie virus into its corresponding precise location in the poliovirus cDNA

clone. The resultant infectious virus displayed a temperature sensitive phenotype in the production of viral proteins as well as in the synthesis viral RNA's. These data indicate that the 5' untranslated region may function in the regulation of protein synthesis, RNA synthesis or both. Additionally, it has been discovered that this noncoding region contains hybridizable regions to the 18S and 28S ribosomal RNA's (McClure and Perrault,1985).

The poliovirus message is translated into a single polyprotein 247 kd called NCVP00. This protein was first visualized with the use of amino acid analogs which alter protease recognition of cleavage in the 247 kd polyprotein (Jacobson et al,1970; Jacobson and Baltimore,1968b). Shortly, thereafter, protein synthesis inhibitors were used to corroborate the appearance of the 247 kd polyprotein (Summers and Maizel,1971). The NCVP00 protein is cleaved into three main functional regions (by convention from 5'to 3'; Putnak and Phillips,1981; Kitamura et al,1981). P1 contains capsid polypeptides and their precursor (P1-1a), P2 in the central portion contains the P2-3b precursor to the P2-X and P2-5b proteins, and the P3 portion codes for the precursor polyprotein of the proteins responsible for virus replication, P3-1b (Figure 1). Each proteolytic cleavage which separates these regions of the polyprotein is a primary cleavage site. The cleavage

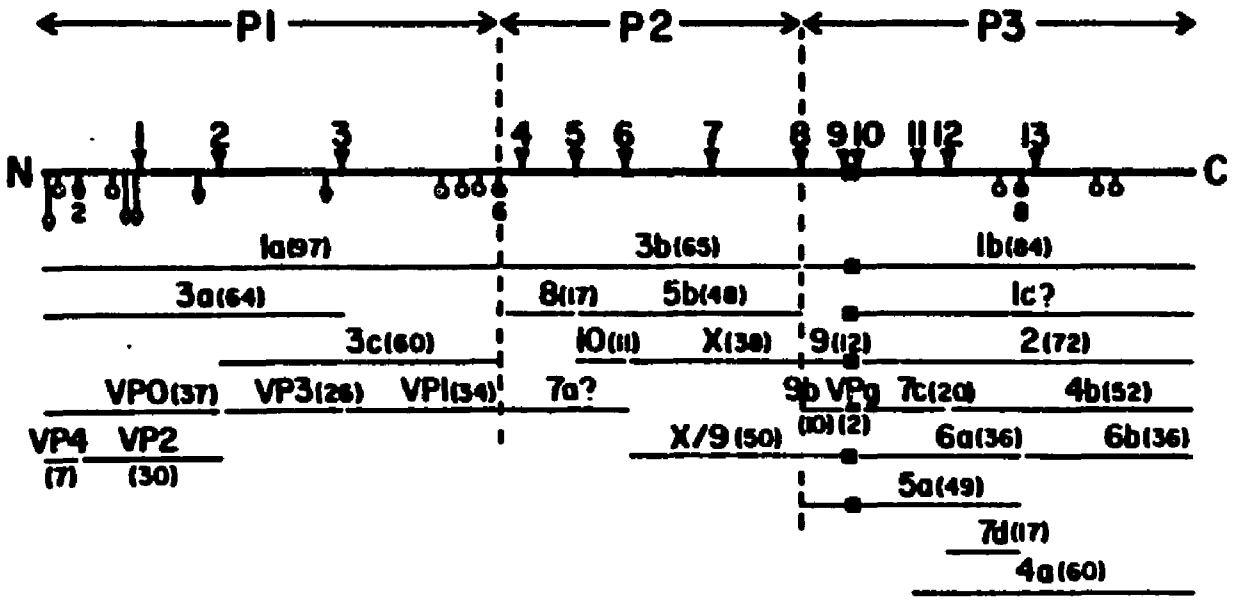

Figure 1. Processing Map of the Polioviral Polyprotein.

between P1-1a and P2-3b occurs at a unique site between the amino acid pair tyrosine-glycine. For this reason it is believed to be the only host protease cleavage site (Hanecak,1982). The second primary cleavage between the carboxyl terminus of the P2-3b and the amino terminus of the P3-1b is between a glutamine-glycine amino acid pair which is the same cleavage site used in almost all of the remaining secondary cleavages (these produce the mature proteins). There is evidence that the P3-7c protein is a protease specific for this site (Figure 1; Hanecak et al,1982). Another unique cleavage site is between the asparagine and serine amino acids which separate VP0 into the mature proteins VP2 and VP4 late in infection (Kitamura et al,1981). The protease responsible for this cleavage may be either the P2-X protein or it may be contained in the VP3 structural protein (Korant,1979; Agol,1980; Sangar,1979). However, Kitamura et al (1981) believe that this is another site recognized by the P3-7c protease. P1-1a is the precursor polyprotein for the four structural proteins, VP1-VP4, which form the capsid and may be partially responsible for virus morphogenesis. Studies indicate that the capsid structure must be present in the infection to allow encapsidation (Putnak and Phillips,1981).

The precursor proteins and the mature proteins of the P2 region have been implicated recently to possess a virus specific function. The P2-X protein may have a protease activity (Korant,1979) but Hanecak et al (1982) have information denouncing this function. Bienz et al (1983) have evidence that the P2-5b virus protein is responsible for the proliferation of the smooth membranes into vesicles in the infected cells as is demonstrated with anti-P2-5b antibodies.

The P3-1b polyprotein contains pre-VPg precursors, and a P3-2 precursor protein of the P3-7c protease and the P3-4b (p63) polymerase (Kitamura et al,1981). This P3-2 polyprotein may act in the initiation of viral RNA replication upon cleavage by the P3-7c protease (Morrow and Dasgupta,1983).

Ruekert and Wimmer, (1984) have devised a systematic nomenclature designated the L434 convention. The pattern is L-ABCD-ABC-ABCD. L represents the leader protein (not found in the enteroviruses) and A,B,C,D represents end products of a capsid piece (P1), a midpiece (P2), and a right piece (P3). Table 2 compares the new nomenclature with the old. This systematic nomenclature simplifies the teaching of picornavirology and also the communications between independent laboratories working on different picornaviruses. As a result of the change in

TABLE 2

Relationship of picornavirus proteins

Previous name					L434 name
Poliovirus		EMC virus[c]	FMDV		
Reference 12[a]	Reference 7[b]		Reference 4[d]	Reference 3[e]	
		p12/p14	P16	p16/20a	L
		pre-A or A1			L-1-2A
		A			1-2A
1a	P1-1a	B	P91	P88	1 or P1
			P56	P52	2AC
3b	P2-3b				2 or P2
5b	P2-5b				2BC
8	P2-8	G	P14	P20c	2A
10	P2-10	I	Deleted?		2B
X	P2-X	F	P41	P34	2C
7a	P2-7a				2AB
	X/9				2C-3AB
1b	P3-1b	C	P102	P100	3 or P3
9	P3-9	H	P19	P14	3AB
7c	P3-7c	P22	P18	P20b	3C
4	P3-4b[f]	E	P61	P56a	3D
2	P3-2	D	P81	P72	3CD
			VPgP81		3BCD
VP0	VP0	ε	VP0	VP0	1AB
VP1	VP1	α	VP1[g]	VP1	1D
VP2	VP2	β	VP2	VP2	1B
VP3	VP3	γ	VP3	VP3	1C
VP4	VP4	δ	VP4	VP4	1A

[a] Summers et al. (12) as modified by Butterworth (1).
[b] Kitamura et al. (7).
[c] See references 2, 5, 10, and 11.
[d] Plum Island nomenclature (4).
[e] Pirbright nomenclature (3).
[f] Also known as NCVP4, p63, poly(U) polymerase, and RNA polymerase.
[g] Formerly called VP3.

nomenclature most of the recent papers (after 1984) have conformed to the new names.

Virus Replication

The initiation of replication and the elongation of the complementary RNA during an infection with a single infectious vRNA (MOI=1) has been a difficult problem in poliovirus research. For example, the single vRNA must act as a messenger in the cytoplasm before RNA can be repli cated, but how does the replicase elongate the template without encountering a ribosome moving along the mRNA from the 5'direction? Do the ribosomes translating from the 5' pUp terminus fall off the RNA to allow the replicase to elongate the RNA template? Or is there a limited number of ribosomes which translate a single strand of mRNA,i.e, 35 (Levy, 1974)? If this were the case then the complementary RNA could be initiated and elongated without interruption. Secondly, this complementary RNA must continue into the replicative intermediate form of RNA synthesis (see below). Some investigators postulate that the replicase must initiate and elongate within the 5-12 minutes it takes for a ribosome to migrate along the RNA (Baltimore,1969). This problem of the "initiation event" of RNA replication has not been approached in the current literature. However, a break through in the elucidation of RNA replication appears emminent with the use of antibodies to virus specific

polyproteins and their mature proteins.

Viral RNA has been isolated from the cytoplasm in association with smooth endoplasmic reticulum membranes (Penman et al,1964; Caliguiri and Tamm,1970) in the form of a "replication complex" (Girard et al,1967; Petterson et al,1977; Nomoto et al,1977). Bienz et al (1983) identify these smooth endoplasmic membranes as vesicles induced to proliferate by the virus-specific protein, P2-5b. They define their function to be the separation of the virus replication machinery from that of the other RNAs in the cytoplasm.

The replication complex sediments at 250S in a linear sucrose gradient. RNA and protein purification of the complex showed that it contained a replicative intermediate RNA species and the viral replicase (Girard et al,1967). The replicative intermediate (RI) sediments heterogenously in a sucrose gradient between 70S and 20S. Purification of the replicative intermediate into its com ponent parts shows that it is composed of a single negative (complementary) RNA and from 1-6 nascent plus strand RNA's which retain single strand properties (Figure 2; Baltimore,1969). All RNA, plus and minus sense, associated with replication complexes have a VPg linked 5' to an O_4-tyrosine (Petterson et al,1977). The replication complex contains all the active replicase in the infected cell

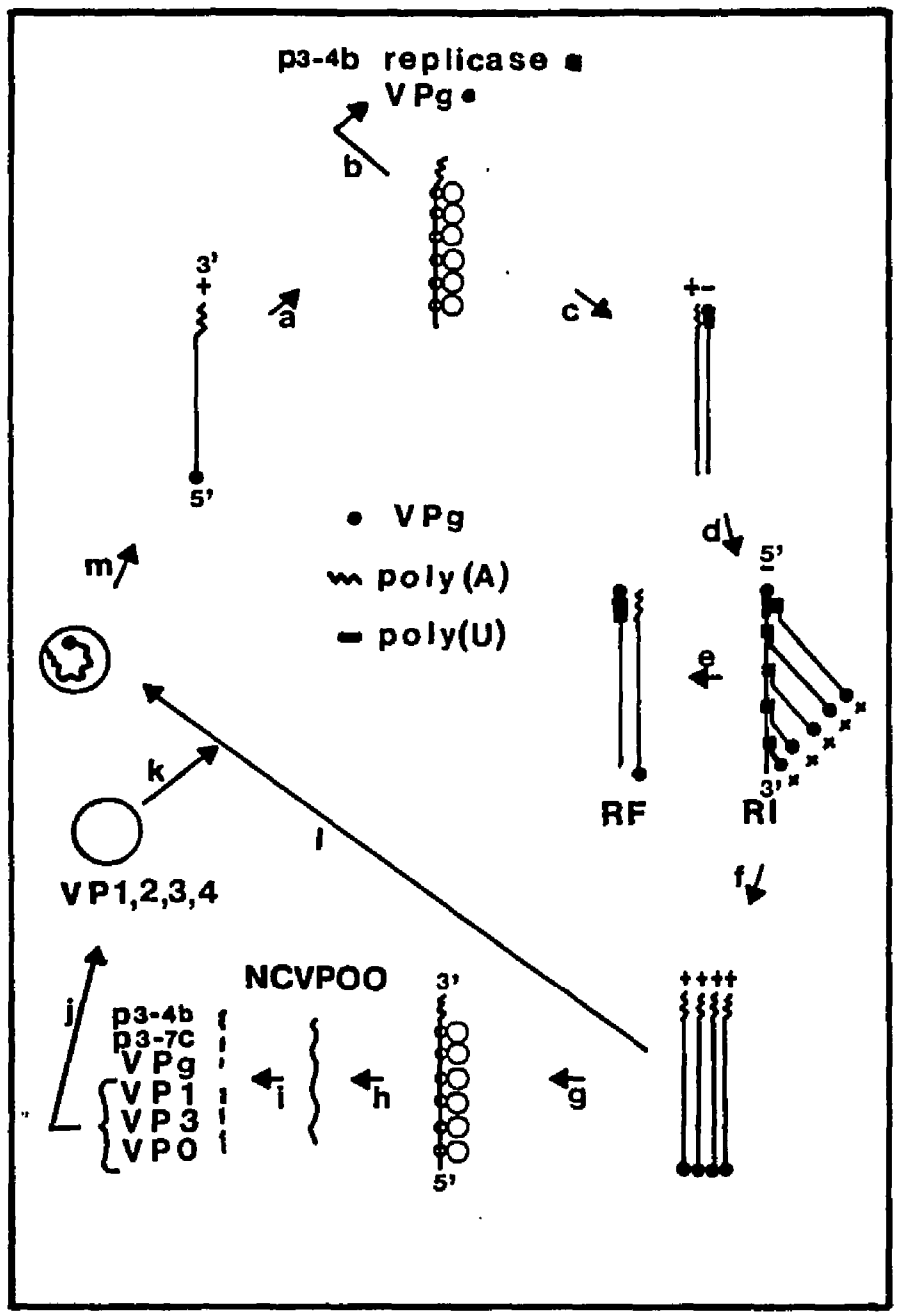

Figure 2. Replication scheme of poliovirus RNA.

(Baltimore,1969). Therefore, the replicative intermediate is the site of all RNA synthesis within the infected cell.

A double strand form of RNA (20S) and the genome RNA (35S) are also found in the infected cell. The ds RNA is a replicative form (RF;Figure 2e). It appears to be a by-product of RNA replication and not an intermediate in replication, since it increases in the later stages of the infection (Baltimore,1969). The ssRNA (35S; Figure 2f) is the product of replication. This is nascent RNA. There are three possible pathways nascent RNA can take (1) to become message (early in infection;figure 2g), (2) to become transcript (to make additional message; figure 2d) and (3) to become virion RNA (late in the infection; figure 2i; Baltimore,1969; Putnak and Phillips,1981). How this nascent RNA is regulated during the course of an infection is unknown.

The viral replicase was first isolated from the replication complex as a [poly(A)]-[oligo(U)]-dependent poly(U) polymerase (Flanegan and Baltimore,1977). This activity co-purifies with a template dependent replicase activity (Dasgupta et al,1979) and was associated with the viral protein p63 (Van Dyke and Flanegan,1980).

The activity of the viral replicase (p63) has been measured in two in vitro assays-- the poly(U) assay and the replicase assay. In the poly(U) assay poly(A) is the

template, oligo(U) supplies the primer activity, and poly(U) is synthesized in the presence of uridine and the purified replicase protein which contains some host factor protein. In the replicase assay poliovirion RNA is the template to synthesize complementary RNA in the presence of the four ribonucleotides, the replicase protein (free of the HF protein) and host factor protein (purified from uninfected cells). The replicase protein can only elongate an already initiated length of poly(A) or genome RNA (Baron and Baltimore,1982; Dasgupta,1982; Dasgupta et al,1979). A primer, or catalyst, is necessary for the initiation of RNA synthesis. The primer function can be added to the system in the form of a synthesized oligo(U) ribonucleotide (Baron and Baltimore,1982; Dasgupta,1983a; Dasgupta et al,1979) or by host factor purified from the ribosomal salt wash of uninfected HeLa cells (Baron and Baltimore,1982; Dasgupta,1980).

The host factor also purifies with the viral replicase in the infected cell but it is separated from the replicase for the replicase assays by a gradient salt elution (Dasgupta et al,1980). Anti-host factor antibodies inhibit RNA initiation in in vitro replicase assays stimulated by host factor but do not affect assays using oligo(U) as a primer for initiation (Dasgupta et al,1980). Host factor is in close association with the viral

replicase as evidenced by the precipitation of replicase protein by the host factor antibody (Dasgupta,1983b). This relationship between host factor and viral replicase and the fact that exogenously added ATP stimulated host factor dependent RNA initiation (Morrow et al,1984) led these researchers to investigate a function requiring ATP associated with the host factor protein or the replicase protein. Studies with gamma-[^{32}P]ATP have identified the host factor [67Kd] as a protein kinase which phosphorylates itself (Morrow et al,1985). It also appears to possess a host factor associated protein kinase which phosphorylates the eucaryotic initiation factor 2 (eIF-2). eIF-2 is the key factor required for the formation of the ternary complex eIF 2:GTP:met-tRNA in the initiation step of mRNA translation. Phosphorylation of this protein inhibits protein translation (Morrow et al,1985). Therefore, host factor may have a role in regulation of protein synthesis in the normal HeLa cell. In addition, host factor may regulate virus replication. Phosphorylation of the host protein is stimulated by low concentrations of dsRNA within the infected cell but inhibited with higher concentrations. Apparantly, this property has complicated the study of viral RNA synthesis in vitro because of the build-up of dsRNA in the reaction mixture (Morrow et al, 1985).

In contrast to the above research, Andrews and Baltimore,(1986) find no kinase activity associated with the host facter but instead have reported the presence of a terminal uridyltransferase activity that is identical to the host factor. This activity has been shown to add uridine residues to the 3' poly(A) end of virion RNA and that these anneal back to the poly(A) and form a hairpin primer for polymerase longation.The genome-linked protein VPg has been a suspected primer in the initiation RNA synthesis by virtue of its 5'covalent linkage on intracellular plus and minus strand RNA and on nascent plus strands (Nomoto et al,1977; Flanegan et al,1977; Petersson et al,1978; Wu et al,1978). No free VPg has been found in poliovirus infected cells. This may be because it is extremely labile (Flanegan et al,1977; Lee et al,1977) or because it is covalently attached to newly synthesized RNA as a precursor protein, pre-VPg (Morrow et al,1984b). As a result of this property, antibodies to VPg have been produced by immunizing with synthetic polypeptides for which the amino acid sequence was deduced from the genome sequence of the virus (Morrow and Dasgupta,1983; Semler et al,1982). These anti-VPg antibodies inhibit the initiation at the 3'terminus of the poliovirus RNA in the replicase assay, but do not show any effect in the poly(U) assay (Morrow and Dasgupta,1983). Furthermore, RNA

synthesized *in vitro* was precipitated by these anti-VPg antibodies and were analyzed to reveal the presence of pUMP-VPg precursor proteins covalently linked to nascent RNA (Morrow et al 1984b). Consequently, these results directly implicate that VPg precursor proteins, pre-VPg, are contaminants in the purified replicase fraction. Indeed, a 49Kda protein has been detected in PAGE profiles of the purified replicase fraction and which also precipitates with the anti-VPg antibody (Morrow et al,1984b).

Andrews and Baltimore (1986b) again disagree with Morrow et al (1984b) concerning the evidence for VPg-priming during poliovirus RNA synthesis in the *in vitro* replicase reaction. They believe that the anti-VPg immunoprecipitable RNA labeled in vitro during a poliovirus polymerase reaction was formed by the elongation of VPg-containing template fragments rather than by initiation with VPg. Their reaction was dependent on host factor terminal uridylyltransferase and the incorporation of labeled UTP could be detected with only host factor present. Young et al (1986), have evidence that there is a covalent linkage present between the template RNA and the negative strand RNA synthesized in these in vitro replicase reactions and that the anti-VPg antibody precipitation of product RNA is mediated by VPg on the poliovirion RNA tem-

plate.

Lastly, infectious poliovirus RNA has been synthesized *in vitro* using full length negative strand RNA as template in the replicase reaction. This full length negative template was synthesized *in vitro* by transcription of poliovirus cDNA with bacteriophage SP6 DNA-dependent RNA polymerase (Kaplan et al, 1985). This technology allows further studies of negative strand synthesis in vitro and the use of synthetic positive strand RNA. This also verifies that only one RNA-dependent RNA polymerase is active in vitro to synthesize the RI RNA.

In summary, there are two theories for the initiation of RNA synthesis. In each theory the host factor protein, HF, acts as a catalyst for the reaction either through an ATP-requiring function or by a terminal uridyl-transferase activity. In the former approach pre-VPg is believed to act as a primer to which phosphates are added by the ATP-requiring function of the host factor protein. In the latter approach the primer activity *in vitro* is attributed to the formation of a hairpin loop by a string of UUU`s synthesized by the host factor using a terminal uridyl-transferase activity (Andrews and Baltimore,1986; Young et al, 1986).

Virus Morphogenesis

This area of study must bring together the spatial arrangements within the cytoplasm of the site of synthesis of capsid precursor proteins on the rough endoplasmic reti culum membranes and the synthesis of nascent RNA on the smooth endoplasmic reticulum in such a way as to allow energetically favorable assembly of the virion. During the course of the infection nascent RNA must find a pathway from its attachment on the smooth vesicles described by Bienz et al (1983) to the polyribosomes to become message. However, late in infection this nascent RNA is assembled into the capsid shell to form the virion structure while it is still attached to a smooth membrane (Caliguiri and Compans,1973).

The capsid shell is formed from 60 identical units of the P1-1a (NCVP1a) precursor protein (Figure 3). The NCVP1a is cleaved from the large polyprotein NCVP00 before the ribosomes fall off the mRNA and folds into a configur-ation to give each individual unit a sedimentation coeffi-cient of 5S. These 5S structures located on the virus induced membrane vesicles associate with four other 5S units to form a pentameric structure sedimenting at 13S. A 14S structure was isolated from infected cytoplasmic extracts which could self-assemble into an empty capsid (fig.3-a; Phillips et al, 1968). However, these empty

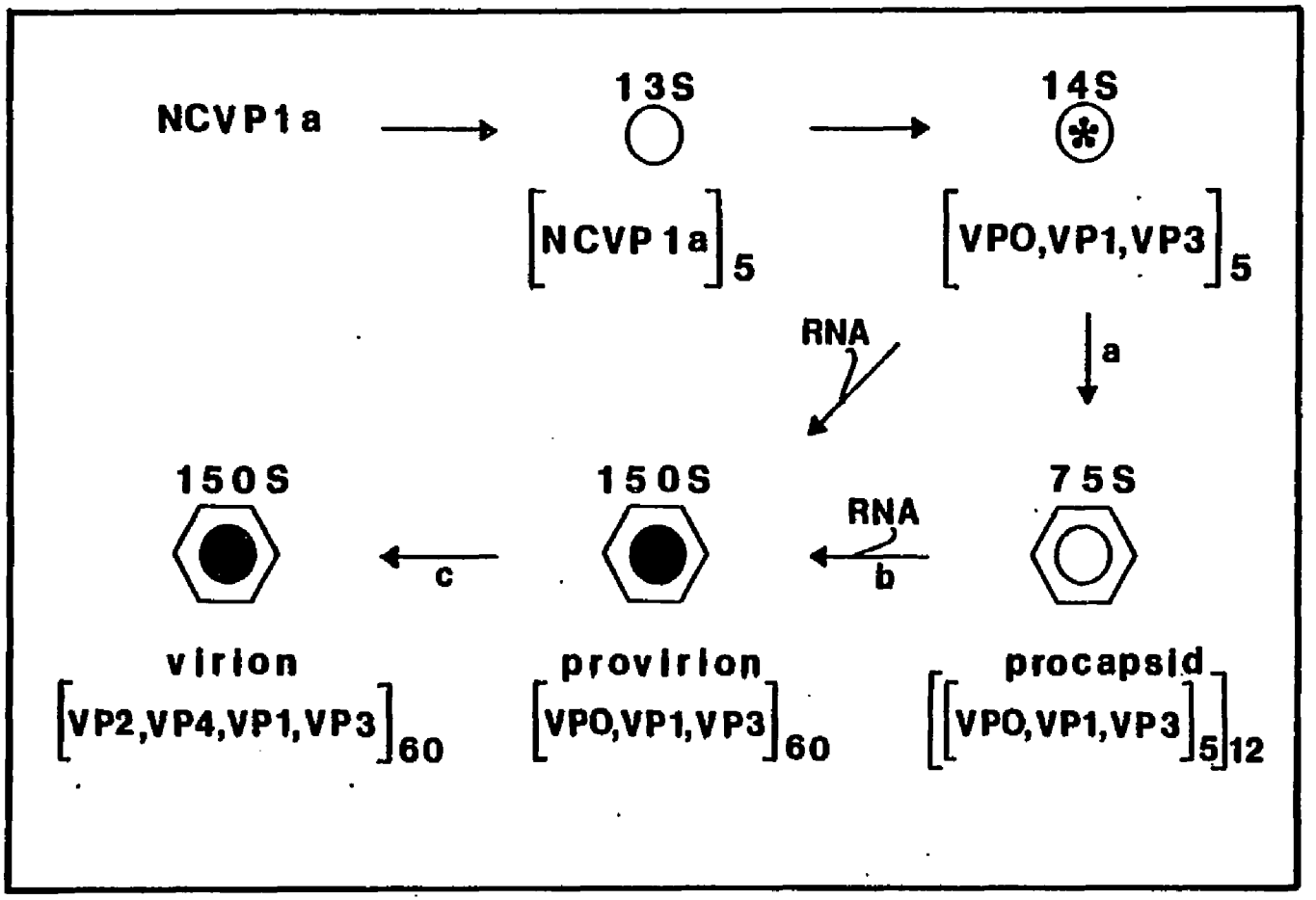

Figure 3. Virus Morphogenesis.

capsids (EC) differ conformationally from naturally occuring empty capsids (Onodera et al, 1986). Close analysis of the proteins in this 14S sedimenting structure shows that the NCVP1a polyprotein is proteolytically cleaved into the viral capsid proteins VP0, VP3, and VP1. A virus specific morphopoietic factor must be present for twelve 14S structures to assemble into a conformationally complete procapsid (75S; Putnak and Phillips, 1981 & 1982; fig.3-a).

vRNA becomes tenuously associated with the procapsid structure possibly through some recognition mechanism of the VPg protein which is associated with the virus induced membrane vesicles (Fernando-Munoz and Lavi, 1977; Lee et al, 1977; Bienz et al, 1983). There are two concepts conceivable for the initiation of interaction of the vRNA with capsid proteins: The assembly of capsid subunits about an RNA core, or the insertion of vRNA into a more or less completed capsid during ongoing synthesis of vRNA (Koch and Koch, 1985). When the viral RNA associates with the procapsid it becomes the provirion which sediments at 150S (Figure 3-b).

Finally, the virion RNA is encapsidated into the capsid shell by a specific morhpogenetic cleavage of VP0 into VP2 and VP4 (Figure 3-c). This final cleavage renders the assembley reaction irreversible and the complete virion

(150S) is formed (Koch and Koch, 1985). [A few VP0 (1 or 2) have been observed in extract preparations. It is unknown what possible role these may have in viral infection or the uncoating of the virus.] The identity and localization of the responsible protease (asn-ser amino acid pair) is unknown. The smallest cleavage protein, VP4, becomes associated internally with vRNA and may act as an uncoating "plug" to release the vRNA into the cytoplasm of the newly infected cell. It has been proposed that encapsidation of the viral RNA is accompanied by a gross conformational change of the protein shell (Carthew and Martin, 1974; Rueckert, 1976). Weigers and Dernick (1986), have evidence for the conformational changes of poliovirus precursor particles during virus morphogenesis through the use of monospecific antisera raised against capsid polyproteins.

Cytopathology

Poliovirus infection in HeLa cells causes host cell protein synthesis shut-off within 1.5 and 2 hours post-infection. Investigators agree that this inhibition is the result of a virus-specified protein (Baltimore and Franklin,1963; Penman and Summers,1965; Holland,1964; Bablanian et al,1965). More recent studies have shown that the protein responsible may act to modify an initiation factor necessary for the translation of capped mRNA's (Rose

et al,1978; Helentjaris et al,1979). Individual initiation factors were purified from uninfected cells and were added to a protein synthesizing extract of a ribosomal salt wash to determine restoration activity to VSV (vesicular stomatitis virus) translation in a poliovirus extract (Rose et al,1978). In this experiment the protein initiation factor eIF-4b restored the VSV translation activity in the poliovirus infected cell extract. In a second experiment individual initiation factors were purified from both the uninfected Hela cells and the poliovirus infected HeLa cells. The ribosomal salt wash from infected HeLa cells contained no functional eIF-3 activity (Helentjaris et al,1979). This discrepancy was explained after the discovery of a cap binding protein (CBP) which is necessary for ribosomal recognition of the eucaryotic capped mRNAs (Sonenberg et al, 1978). The cap binding protein co-purifies with both the eIF-4b and eIF-3 initiation factors (Trachsel et al,1980). Further studies with the cap recognition complex, which contains a 24kd CBP and some eucaryotic initiation factors, shows that it has the ability to restore translation of capped VSV mRNA in an extract from poliovirus infected cells (Tahara et al,1981). Finally, immunoblot analysis with an anti-eIF3 antiserum indicates the presence of a 220 kd polypeptide present in uninfected cells which becomes proteolytically degraded in

the poliovirus infected cell to a ca. 130 kd polypeptide and a ca. 100 kd polypeptide (Etchison et al,1982). Etchison et al (1982) postulates that this 220 kd polypeptide is an essential component of the cap recognition complex and that its degradation in poliovirus infected cells results in the inhibition of host cell translation. Lee et al,(1985a) agree with this data and hypothesis. They isolated the CBP complex (comprised of the 24K-CBP and the polypeptides of 50Kda(eIF-4a) and ca.220Kda) from uninfected and poliovirus infected HeLa cells by in vivo labelling and m^7 GDP affinity chromatography purification. The CBP complex from the poliovirus cells contains proteolytic cleavage fragments ranging from 110Kda to ca.130Kda. Lee et al (1985b), suspected that the poliovirus proteins P3-7c (3C: the gln-gly viral proteinase) or the P2-X (2C)protein might be involved in the proteolysis of this 220Kda large subunit of the CBP complex. However, they were unable to inhibit proteolysis of this large subunit in an <u>in vitro</u> assay using antisera against the 3C and 2C proteins. These results suggest that at least these two proteins are not directly involved in host protein synthesis shut-off.

Host cell hnRNA synthesis decreases by 50% by 4 hours post-infection. However, the synthesis of ribosomal RNA continues throughout the infection although at a

reduced rate. The kinetics of host cell RNA synthesis inhibition lead investigators to believe that it is independent of the protein synthesis inhibition (Bienz,1978). Bienz et al (1983), detected the accumulation of newly synthesized poliovirus proteins in the host cell nucleus using electron microscopic autoradiography in *in vivo* experiments. In the same laboratory *in vitro* studies with isolated HEp-2 cell nuclei show that there is an accumulation of poliovirus proteins in uninfected cell nuclei when the nuclei are incubated with poliovirus infected cell extract (Bossart et al,1984). The accumulation of these proteins is accompanied by the preferential inhibition of RNA polymerase II activity within these same nuclei but does not affect the RNA polymerase I concentration in the nucleoli. A clear differential uptake of individual poliovirus proteins was observed including many precursor proteins.

Host cell DNA replication comes to a halt shortly after host cell protein synthesis inhibition. This phenomenon is probably a by-product of host cell protein synthesis induced by a virus specific protein. This prevents the new synthesis of DNA replication proteins even though the half-life of the host cell mRNA is not significantly altered.

Finally, the poliovirus infection is a cytolytic one. The virions are produced within 6 hours of infection and they are released into the medium or tissue by a total lysis of the cell membrane approximately 8 hours post-infection. This cytopathic effect occurs too early in the cell cycle and is too precise of an event to be a result of the virus inhibition of host macromolecular synthesis (Bablanian,1965).

Many investigators have demonstrated that the cytopathic effect is under the control of a virus-specific protein(s) (Wolff and Bubel,1964; Amako and Dales,1967). Bienz et al (1983) have associated the viral protein P2-5b with the function of vesicle proliferation in the poliovirus infected HEp-2 cell. They concluded that the cytolytic effect is caused by the presence of these vesicles but that this is a by-product of their actual function to separate the replication complexes from other RNAs in the host cell.

In summary, the following functions have been assigned to virus proteins. The structural proteins, VP0-VP4, act in capsid formation. The P3-4b protein is the replicase (p63). The P3-7c protein is a protease for the cleavage of gln-gly sites. The VPg protein linked at the 5'terminus may act as primer for RNA replication or it may be necessary for vRNA encapsidation. Finally, the P2-5b protein has been implicated as the inducer of vesicle

proliferation in the infected cell to supply membrane sites for the replication complexes. Other viral functions dependent on virus-specific proteins are indicated but the exact protein responsible for these functions have not been identified. These are: a. The inhibition of host cell protein synthesis. b. The cytolytic function for virus release from the infected cell. c. And the morphopoietic factor in virus maturation. The proteins responsible for these functions could also be in the polyproteins which are precursors to the currently known mature proteins of the virus.

Genetics of Poliovirus

There has been the hope that the isolation and characterization of conditional lethal mutants of poliovirus would assist in defining the specific proteins responsible for the functions described above. Complementation between conditional lethal mutants has been a valuable tool in the identification of gene product function and gene mapping in biological organisms, specifically in bacteria and in yeast. Complementation between ts mutants would be ideal for the poliovirus system if mutants could be isolated which showed a deficiency in a specific recognizable function, e.g., host cell protein synthesis is not turned off during the infection.

Complementation occurs between two mutants when a co-infection is carried out at the restrictive temperature and the yield of one or both the parental viruses is enhanced while their phenotype remains unchanged (Fenner et al,1974). Temperature sensitive mutants with point mutations that affect different proteins may complement each other in a co-infection at the restrictive temperature by cooperative interaction of the unaffected proteins. Complementation groups define genetic loci by excluding any mutant from the group which can complement those mutants within the group (Burge and Pfefferkorn,1966b).

Cooper (1964) has characterized conditional lethal mutants of poliovirus. Temperature sensitive mutants are the only class of conditional lethal mutants occuring in animal viruses. A summary of temperature sensitive mutants (ts) mutants in poliovirus is listed in table 3 (Koch and Koch, 1985). Temperature sensitive mutants replicate efficiently at the physiological temperature but a shift toward 39°C/40°C causes an interruption of replication. Some properties affected in ts mutants are: The resistence or dependence upon inhibitory substances during replication (g^r and g^s). The sensitivity or resistence to inactivation by a variety of agents (ho,bo,dex,m,d,Hy,S-7,ox). The surface properties of the virion is reflected in an altered physical or antigenic behavior (MS,IC,/\H(T),EC).

Table 3. *Genetic markers of poliovirus*

Universal markers	
rct, ts	sensitivity of replication to elevated temperatures
Structural markers P-1 proteins	
ΔH (T)	thermal lability, heat sensitivity
IC	changes in antigenicity
psr	repression of host protein synthesis
bo	sensitivity to inhibitors in bovine serum
S-7	sensitivity to inhibition by ethyl-2-methylthio-4-methyl-5-pyrimidine carboxylate
ox	sensitivity to inhibition by 2-(3-chloro-p-tolyl)-5-ethyl-1, 3, 4-oxadiazole
Hy	sensitivity to inhibition by 5-methyl-5-3, 4-dichlorophenylhydantoin
dex	sensitivity to dextran sulfate
m	sensitivity to inhibitors in agar overlay
d	sensitivity to acid overlay (growth capacity at low bicarbonate concentrations)
ho	sensitivity to inhibitors in horse serum
MS	growth capacity in a stable monkey kidney cell line
n	neurovirulence
EC_{50}	adsorption capacity
Non structural markers P-2 proteins	
cy	cysteine dependence
g	sensitivity to inhibition by guanidine
Non structural markers P-3 proteins	
ssRNA	capacity to synthesize ssRNA
dsRNA	capacity to synthesize dsRNA
pti	repression of host DNA synthesis
chr	damage to cell chromatin
tb	damage to cell membrane (permeability to trypane blue)

The pathogenicity of the virion --neurovirulence,n. The capacity to interfere with host cell metabolism (psr,pti,chr,tb). AND the capacity to direct the synthesis of the viral RNAs in the infected cell (ssRNA, dsRNA,g, rct, and ts). Cooper determined the extent of genetic recombination between these mutants by three factor crosses (Cooper,1968). The total recombination frequency between the most distant markers averages 2.2%. This corresponds to 1% per 1250 nucleotide pairs of double stranded form (Cooper, 1975). The guanidine resistant mutant vs guanidine sensitive mutant was used as a marker in these crosses between temperature sensitive mutants. These experiments were difficult to standardize and were somewhat ambiguous due to the nature of the mutants (covariance) and that of the poliovirus genome (monocistronic; Cooper,1969). However, Cooper did arrive at a linear genetic map which distinguished that the structural proteins are clumped in the 5' region of the genome and the replication proteins are located in the 3'region. Cooper also demonstrated a low efficiency of complementation between a pair of temperature sensitive mutants (ts-5 and ts-19) of poliovirus by plaque assay (Cooper,1965). Only one strain appeared in the yield. This in indicative of asymmetric rescue rather than reciprocal complementation. Covariance in these mutants is indicated when some of these mutant

exhibit overlapping characteristics i.e., response to thymidine uptake and guanidine resistance. This kind of covariance of phenotype indcates pleiotropism. Pleitropism occurs when specific mutations along the single polyprotein, i.e. NCVP00, may affect its conformational folding. This change could inhibit specific cleavage sites of the polyprotein causing errors in the function of more than one of the derived proteins. These pleiotropic mutants are expected because of the monocistronic nature of the genome.

The recombinant DNA technology has opened up the study of poliovirus mutants. Agol et al (1985) constructed an intertypic recombinants from cloned poliovirus plasmids between type 1 (virulent) and type 3 (attenuated) to determine the region of virulence. Pincus et al (1986) has demonstrated that the g^r locus lies in the NCVP-2 (2C) protein of the P2 region using DNA recombinant cloned poliovirus plasmids. Now it is possible to construct deliberate mutations in specific regions of the genome and then study the resultant mutants in tissue culture. One difficulty is apparent: not all constructed virus are infectious and many are not viable possibly due to interactions of the proteins or the RNA or both (Stanway et al,1986).

The Nature of this Research

We have observed that our collection of temperature sensitive mutants fail to produce mature 150S virion when the intracellular viral extract is sedimented through a linear sucrose density gradient when the infection is carried out at the restrictive temperature (39°C). We have taken advantage of this property to define complementation in this system as the production of 150S virion particles seen in a gradient when two mutants simultaneously infected a HeLa cell preparation at the restrictive temperature.

CHAPTER 2

MATERIALS AND METHODS

Cell and virus growth.

Hela S3 cells were grown in suspension in Joklick modified minimum essential media (Gibco) supplemented with 5% horse serum (HS). Poliovirus type 1 (Mahoney) was used as the wild-type stock grown in 10X concentrated suspension culture (37°C) at a multiplicity of infection (M.O.I.) of 20 as described previously (Hewlett et al, 1982). Mutant stocks were grown in the same manner but at 33°C, the permissive temperature.

Plaque Assay.

Plaque assays were carried out as previously described by Cole et al (1971). Briefly, 0.2 ml of virus dilution was added to 4.0 X 106 cells in 0.6 ml MEM plus 1% HS. Top agar (0.6 ml) was mixed with the infected cells and poured onto a 60 mm petri dish containing 10 ml of bottom agar. These plates were incubated at 33.5°C, 37°C, and/or 38.5°C where appropriate.

Mutagenesis.

A portion of the wild-type poliovirus stock was mutagenized by mixing one volume of stock in MEM with 4

volumes 2.0 M hydroxylamine-H_2SO_4 in 4 M NaCl/0.8 M Tris (pH7.5) at room temperature. Mutagenesis was arrested by diluting a portion of the mixture 1:100 with cold MEM containing 10% acetone at selected time intervals. Survival curves were determined by plaque assay.

Isolation of temperature-sensitive mutants.

The mutagenized stock was diluted to allow for ca. 40 plaques in an agar overlay of a 100 mm petri dish at 33°C . All these plaques were transfered to replica plates and incubated at 38.5°C and 33.5°C. Mutants which showed no growth on plates at 39°C were isolated at a frequency of 1 plaque in 200 from the plates at 33.5°C and were used to prepare virus stocks after plaque purification twice.

Determination of viral RNA synthesis.

HeLa cells (8.0 X 10^6 cells) were infected with each mutant and also the wild-type. After virus adsorption (30 min.) cells were stirred at 39°C in 2 ml of MEM containing 5% HS. At 60 min. PI, 10 ug of actinomycin D was added to the 2 ml of infected cell suspension. ^{3}H-uridine (2 uCi/ml) was added to the infected cells at 90 min. PI. From the time of labeling, 100 ul samples were withdrawn every 30 min. into prepared test tubes containing 1 ml of 10% TCA. The precipitated material was collected on nitrocellulose millipore filters and assayed for radioactivity

in ACS aqueous scintillation fluid.

Determination of particle stability.

A 100 ul sample was removed at various times from a 1 ml virus infection (the individual mutant stocks or the wt stock) at the restrictive temperature of 39°C then diluted into 1 ml cold PBS and frozen as above. The yield of infectious virus remaining during this infection at 39°C was determined for all time points on a single day by plaque assay at 33°C for each mutant stock and the wild-type.

Gel electrophoresis of RNA and proteins.

Intracellular RNA was obtained from infected cells labeled with 3H-uridine and harvested 4 hours post-infection (PI). After phenol extraction of the cell pellet and ethanol precipitation of the aqueous phase, a sample of RNA was electrophoresed through a 1% agarose/TNE (10 mM Tris pH 7.5, 100mM NaCl, 1mM EDTA) verical slab gel. The gel was electrophoresed at 70 V constant voltage until the bromophenol blue dye was at the bottom of the gel. The gel was fixed in 10% acetic acid, enhanced with 10% PPO (in glacial acetic acid), then dehydrated before exposure to Kodak XAR-5 autoradiographic film.

Viral protein samples were taken from infected cells labeled with 35S-methionine in methionine free media containing 5% dialyzed horse serum. Infected cells at the

restrictive temperature were labeled for 30 min. starting at 200 min. PI. Infected cells at the permissive temperature were also labeled for 30 min. but labeling began 230 min. PI. Protein samples were taken from an intracellular extract in TNE + 0.5% SDS, then diluted with a 2X sample buffer consisting of 125mM Tris-HCl pH 6.8 , 4% SDS, 40% glycerol, 0.14 M mercaptoethanol, 0.002% bromophenol blue then boiled for 3 min. The samples were electrophoresed through a 10% polyacrylamide (Laemmli, 1970) verticle slab gel. After electrophoresis the gel was fixed and enhanced as above.

Determination of particle formation.

HeLa cells (2.0 X 10^7 cells) were infected with individual mutants at a multiplicity of infection of 10. After adsorption (30 minutes) the cells were resuspended in 5 ml of MEM and then stirred at 39°C. The infection was treated with 25ug of actinomycin D (60 min. PI) and labeled with 50 uCi of ^{3}H-uridine (90 min. PI). Infected cells in media were frozen after 10 hours then thawed and frozen 3 times. After clarification of the supernatent, the virus was pelleted from the supernatant in an SW41 rotor, at 35,000 rpm for 90 minutes at room temperature. This virus pellet was resuspended in 1 ml TNE+0.5%SDS, and 0.5 ml was layered onto a 15%-30% linear sucrose/TNE+0.5% SDS

gradient. Total gradient fractions were collected by bottom puncture and radioactivity assayed in ACS aqueous scintillation fluid.

Complementation experiments.

HeLa S3 cells (2.0 X 10^7) were harvested and washed with Earle's saline, and the cell pellet was infected with a mixture of two mutant viruses at a total multiplicity of 10 (5 moi for each virus stock). After the virus was adsorbed, the cells were resuspended in 5 ml of MEM plus 5% HS and stirred at 39°C as described above. TCA insoluble material was monitored throughout the infection by the removal of a 2 ml portion of the infection after addition of 25 ug actinomycin D at 60 minutes PI and then 10 uCi ^{3}H-uridine were added 90 min. PI. At 10 hours PI the entire infection was frozen at -70°C followed by three cycles of freeze-thaw to release the virus from the cells. Virus was pelleted from the entire supernatant in the Beckman SW41 rotor at 35,000 rpm for 90 min. at room temperature. The virus pellet was resuspended in 1 ml TNE+0.5% SDS overnight. Half of the resuspension (0.5 ml) was layered on top of a linear 15-30% linear sucrose/TNE + 0.5% SDS gradient in SW41 polyallomer tubes. Virion particles migrated approximately midway in the gradient when centrifuged at 35,000 rpm for 90 min. at room temperature. Gradient fractions were collected and radioactivity was assayed

in ACS aqueous scintillation fluid for 1 min.

Backcross experiments.

These experiments were carried out as in the complementation experiments. However, the co-infections were composed of the progeny virus from a complementing infection and one of each of the original mutants (parents).

CHAPTER 3

RESULTS

Mutagenesis.

I mutagenized poliovirus type 1 (Mahoney) with the intention of isolating mutants deficient in viral protein functions to assist in the study of RNA replication in poliovirus. The poliovirus type 1 stock was treated with 2.0 M hydroxylamine. A sample of the mutagenized solution was removed at 10', 15', 25', and 35' and the reaction halted by the addition of acetone as described previously by Hewlett et al (1982) and in Materials and Methods. Maximum viral inactivation (90%) occurred after exposure for 25' to the mutagen. However, when mutagenized stocks of virus were being screened, the stock treated for 35 min. produced 1 ts plaque in 200 randomly selected survivor plaques from the permissive temperature (33°C) while that stock treated for 25 min. produced fewer than 1 mutant per 800 randomly selected plaques. Therefore, I continued to isolate mutants from the stock mutagenized for 35 min.

Mutant selection.

I selected for ts mutants by the replica plate technique. The mutagenized stock was grown on a master

plate at 33.5°C. Each plaque was transferred from the master plate to replica plates, then each plate was incubated at either 33.5°C or 38.5°C. Those plaques showing growth at 33.5°C but not at 38.5°C were selected and plaque purified twice. Only those mutants with an efficiency of plating (e.o.p.) difference of -2 to -3 log units or a greater distance were retained for further study. Those mutants retained with a -2 log difference in e.o.p., e.g.ts10 and tsB18, have demonstrated a stable e.o.p. after several passages and retain other mutant characteristics of slow growth, and reduced RNA synthesis to justify their retention. Table 4 summarizes the information obtained for each of the twelve mutants in this collection. The relative efficiency of plating at permissive and restrictive temperatures range from 10^{-2} to 10^{-4} log units. The RNA phenotype is designated either plus(+) or minus(-) and the virion is either stable at 45°C (Y=yes) or unstable at 45°C (N= no).

Mutant characterization.

I characterized these mutants into RNA plus (RNA+) and RNA negative (RNA-) phenotypes by following the kinetics of viral RNA synthesis and testing for the thermolability of the virion structure. Further characterization by gel electrophoresis of visible RNA and protein species was done to support the information obtained from the

TABLE 4

Characteristics of *ts* Mutants

Virus	e.o.p.[a]	RNA[b] Synthesis	Heat[c] Stability
wild-type	1.0	+	Y
*ts*2	4.0 x 10-4	-	Y
*ts*D37	2.7 x 10-2	-	Y
*ts*B9	1.6 x 10-4	-	Y
*ts*F14	3.6 x 10-2	-	Y
*ts*10	1.0 x 10-2	-	Y
*ts*F23	3.0 x 10-4	-	Y
*ts*D	1.0 x 10-4	-	Y
*ts*G8	2.5 x 10-2	-	Y
*ts*E	3.3 x 10-2	+	N
*ts*8	1.0 x 10-3	+	N
*ts*B18	1.0 x 10-2	+	N
*ts*F10	2.8 x 10-3	+	N

a. e.o.p.= efficiency of plating; [titer at 39°C]/[titer at 33°C].

b. RNA synthesis, measured as incorporation of [3H]uridine into TCA insoluble material in the presence of actinomycin D during infection at 39°C.

c. Heat stability, measured by survival of infectivity after incubation at 45°C.

kinetic and virion studies. The RNA electrophoretic profiles should illustrate major species of RNA synthesized at the permissive and the restrictive temperatures to determine what type of mutation would be present in an RNA- type mutant. The protein electrophoretic profiles were to show possible electrophoretic changes in specific proteins synthesized at the permissive v.s. restrictive temperatures.

Synthesis of viral RNA.

Every mutant stock and the wild-type was monitored for the kinetics of viral RNA synthesis at both the permissive and restrictive temperatures. Mutants were characterized for viral RNA synthesis during an infection as described in Materials and Methods. RNA synthesis during the infection was determined by the accumulation of ^{3}H-uridine incorporation into TCA insoluble material (Figure 4). The mutants have been phenotyped as RNA(+) if they incorporate label into RNA in amounts similar to the wild-type incorporation and RNA(-) if incorporation is 25% or less of the amount of wild-type incorporation. The mutants tsE, tsB18, and ts8 have been designated RNA(+) and the mutants tsD and ts10 are RNA(-;table 2).

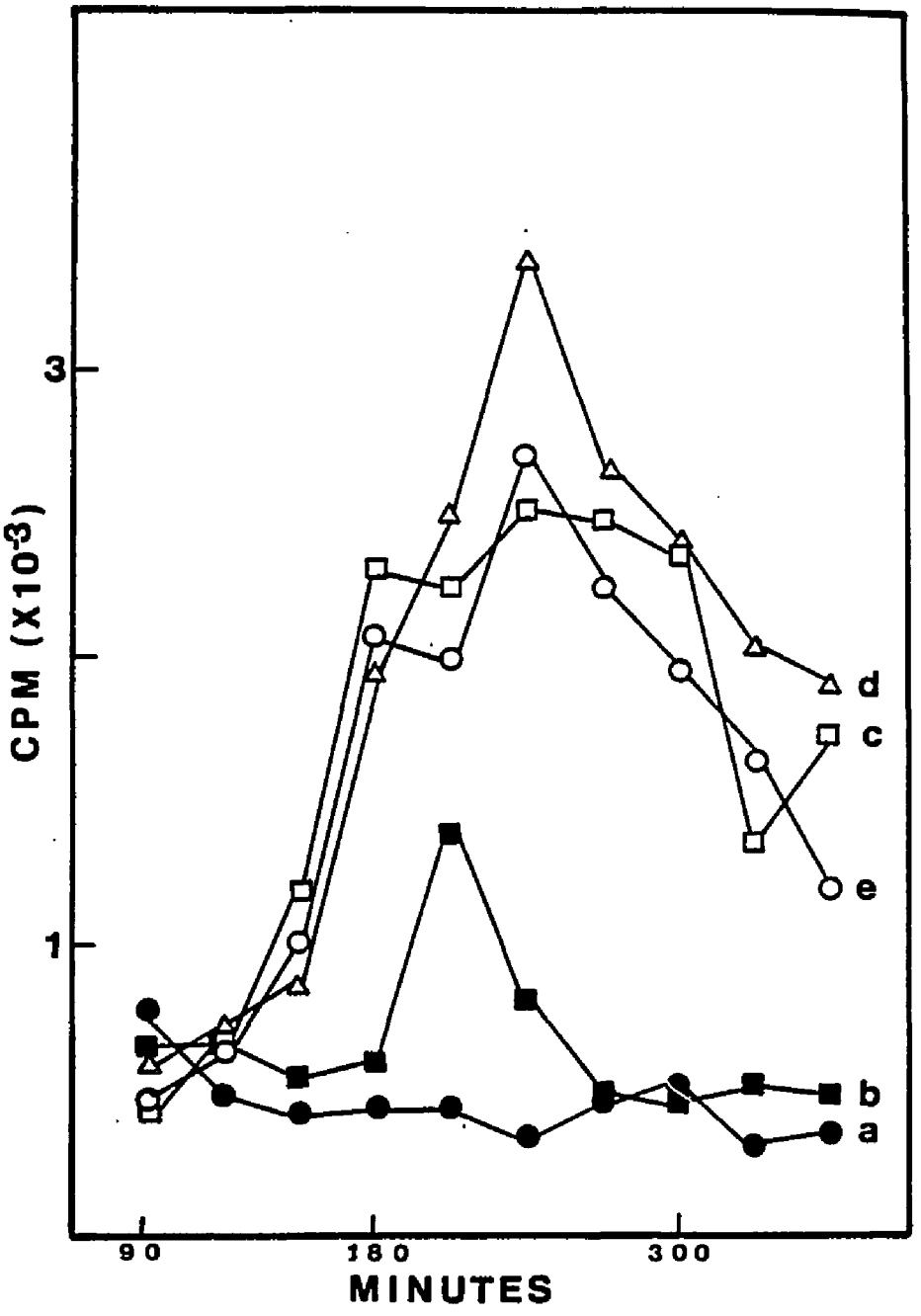

Figure 4. Time course of the incorporation of 3H-uridine into TCA insoluble material in HeLa cells infected with selected mutants and the wildtype.

a. ts10 b. tsD C. tsE d. tsB18 e. PV1

Thermolability of the virion.

The thermolability of each mutant and the wild-type virus was determined by the ratio of the number of plaque forming units produced at 33.5°C after incubation of the virion at 45°C to the number of plaque forming units before incubation (Figure 5). The RNA(-) mutants tsD, ts10, and tsB9 are examples of the temperature stable phenotype as is the wildtype. The mutants tsE,tsB18, and ts8, which happen to be RNA(+) mutants, are phenotyped temperature unstable. [RNA+ mutants have been found to be temperature unstable in otner studies (Burge and Pfefferkorn, 1966a).]

The thermolability of these RNA(+) mutants might be caused by either the loss of the virion structure or by the loss of infectivity of the intact virion. To test these possibilities one RNA(-) mutant, ts10, and two RNA(+) mutants, tsE and ts8, were labeled during growth at the permissive temperature. The labeled virus was split into two fractions, one exposed at 45°C for 15 minutes and the other left at room temperature for 15 minutes. The two samples were then analyzed separately for the 150S virion particle on a linear 15%-30% sucrose gradient. These data are summarized in Table 5. The RNA(-) mutant ts10 shows little difference between the amount of labeled virion before and after heating at 45°C and the fraction of labeled virion remaining is 0.96. The RNA(+) mutant tsE

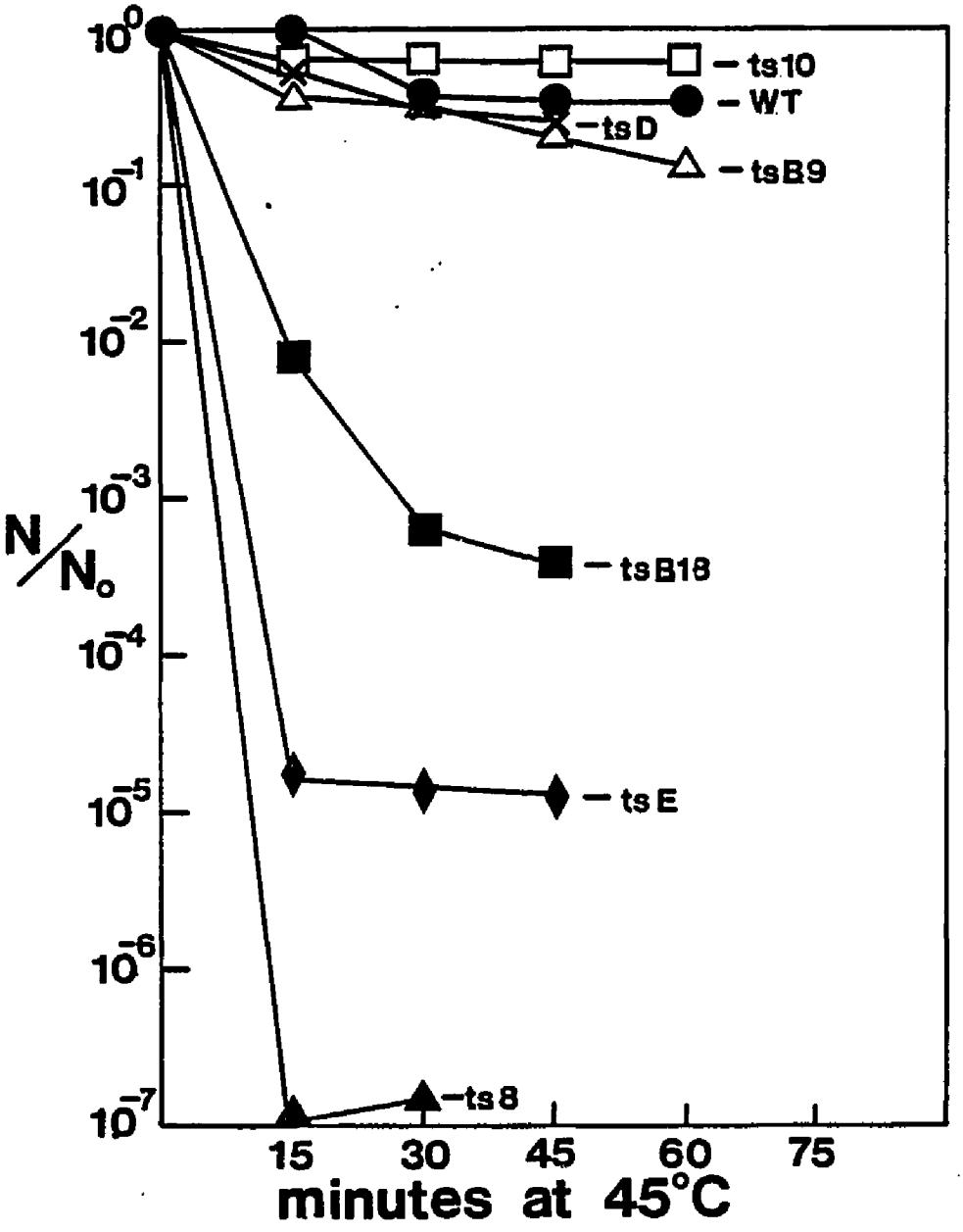

Figure 5. Time course of the amount of surviving infectivity after treatment at 45°C.

N/N_o is the ratio of the number of infectious virus remaining after treatment at 45°C to the number of infectious units before treatment. The mutants tsB9, tsD, and ts10, are temperature stable. The mutants tsB18, tsE, and ts8 are temperature unstable.

TABLE 5

Effect of Heating on Sedimentation Profile of *ts*Mutant Viral Particles[a]

Mutant	RNA (+/-)	Time at 45°C (min)	150S Particles (cpm)	Fraction Remaining
*ts*10	-	0	17,212	---
		15	16,525	0.960
*ts*E	+	0	14,743	---
		15	14,165	0.961
*ts*8	+	0	28,253	---
		15	7,191	0.254

a.Virions of the indicated ts mutants, containing 3H-labeled RNA, were incubated at 45°C and then sedimented through 15-30% sucrose gradients as described in Materials and Methods. Total radioactivity in the 150S peak was determined. The fraction remaining was calculated as [cpm in 150S at 15 minutes]/[cpm in 150S at 0 minutes].

retains the same virion sedimentation profile after heating at 45°C as before heating. Therefore, the fraction remaining of radioactivity beneath the peak is very close at 0.961. However, the RNA(+) mutant ts8 has lost approximately 75% of labeled virion particles after heating at 45°C resulting in a fraction remaining profile which is 0.254. In both cases of the RNA(+) mutants the infectivity detectable by plaque assay decreases sharply after incubation at 45°C for 15 minutes (Table 4). The virion of the mutant tsE appears to lose infectivity without loss of the virion structure. On the other hand it appears that the decrease in infectivity of the mutant ts8 is a result of the loss of the virion structure.

RNA electrophoresis.

Further characterization of these mutants was done by the electrophoresis of the radiolabeled intracellular extracts of poliovirus infected cells. Poliovirus typically contains three types of RNA: the single strand viral RNA (SS), the double strand RNA (ds), and the replicative intermediate RNA (RF). This studies pursued the presence of different quantities or types of RNAs present at the permissive and the restrictive temperatures to determine a possible mutant deficiency in RNA production.

Mutant virus stocks and the wild-type were analyzed by gel electrophoresis for the viral RNA (1% agarose/TNE)

species during the course of infection. Hela cells infected with the mutant virus or the wildtype virus were labeled with ^{3}H-uridine to obtain an intracellular RNA extract. The infected cells were harvested after 4 hours p.i. at 39^{o}C. The cellular pellet was resuspended in RSB/ NP-40 then phenol extracted and precipitated with ethanol.

The labeled intracellular extracts of the mutants ts8, tsE, tsB9 (RNA+) and tsD, ts10 (RNA- ; see table 4) and the wildtype, PV1, were electrophoreses though a 1% nondenaturing agarose gel (Figure 6). The mutants tsD and ts10 demonstrate a very marked decrease in the amount of ^{3}H-uridine label incorporated into double-stranded (ds-) and single-stranded (ss-) RNA species. However, the mutants ts8,tsE, and tsB9 have incorporated label into the ds-species of RNA and also into the ss-species. In conclusion, tsD and ts10 follow a pattern of RNA- mutants while tsB9, ts8, and tsE appear to follow the RNA+ pattern of RNA synthesis. The smear of radioactivity observed in this gel is caused by the presence of unlabeled cellular RNA which is overloading the gel. It was extremely difficult to isolate intracellular RNA species from infected cells at the permissive temperature (33^{o}C) due to the decrease in the cellular metabolism. As the cellular metabolism slows at the lower temperature the kinetics of viral RNA synthesis during an infection is also slowed.

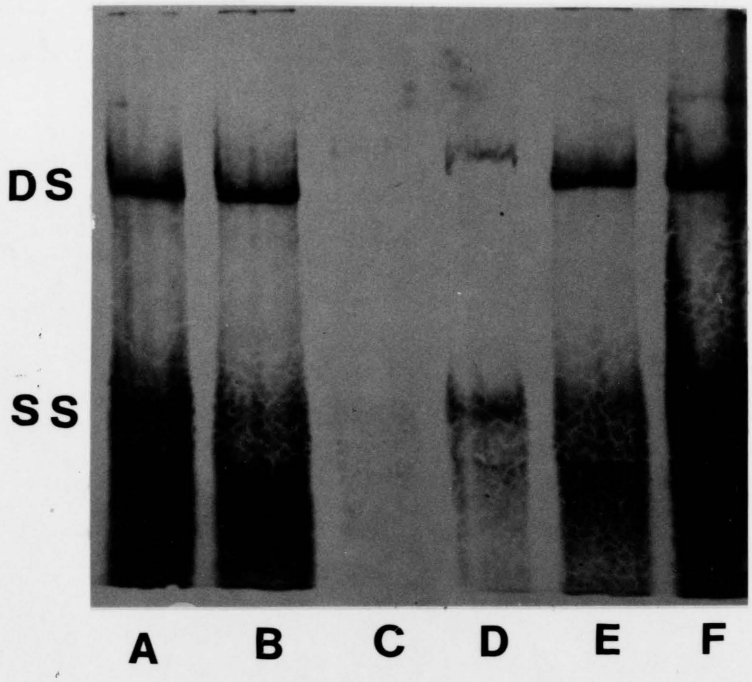

Figure 6. Autoradiogram of 3H-uridine labeled RNA during an infection at 39°C of selected mutant viruses and the wildtype virus, PV1.

An equal amount of HeLa cells were infected at an MOI of 10. The RNA species synthesized was visualized by label with 3H-uridine for 4 hours at 39°C. An intracellular RNA extract was prepared after cell lysis, phenol extraction and ethanol precipitation. These cell extracts were layered onto a 1% non-denaturing agarose gel. The faster migrating material represents single strand RNA(SS), the slower migrating material represents double strand RNA(DS). A. ts8 B. tsE C. tsD D. ts10 E. tsB9 F. PV1.

Protein Electrophoresis.

The protein profiles of wild-type virus stock and mutant virus stocks were compared by selective labeling of viral proteins with ^{35}S-methionine after host cell protein synthesis was inhibited as a natural course of the poliovirus infection. Intracellular extracts of a wildtype infection at 33°C and 39°C, and several mutant infections of tsB9, ts10, and tsD were electrophoresed through a 10% SDS-polyacrylamide vertical gel (Laemmli, 1970; Figure 7). The protein species produced at 39°C contain less of the ^{35}S-methionine than do the samples taken at 33°C. This may be a result of the higher temperature (39°C; heat shock) on the protein metabolism of the Hela cells. In figure 7 the viral proteins of the mutants have the same migration rate as the wildtype viral proteins at both 33°C and 39°C. Although I experimented with two-dimensional gel electrophoresis of the mutant tsD it was not pursued to determine of there might be small isoelectrophoretic differences between the virus proteins produced by some of these mutants.

Complementation experiments.

I began the study of complementation between these temperature sensitive mutants to assist in the characterization of these mutants into comprehensible groups. If these mutants were classified into

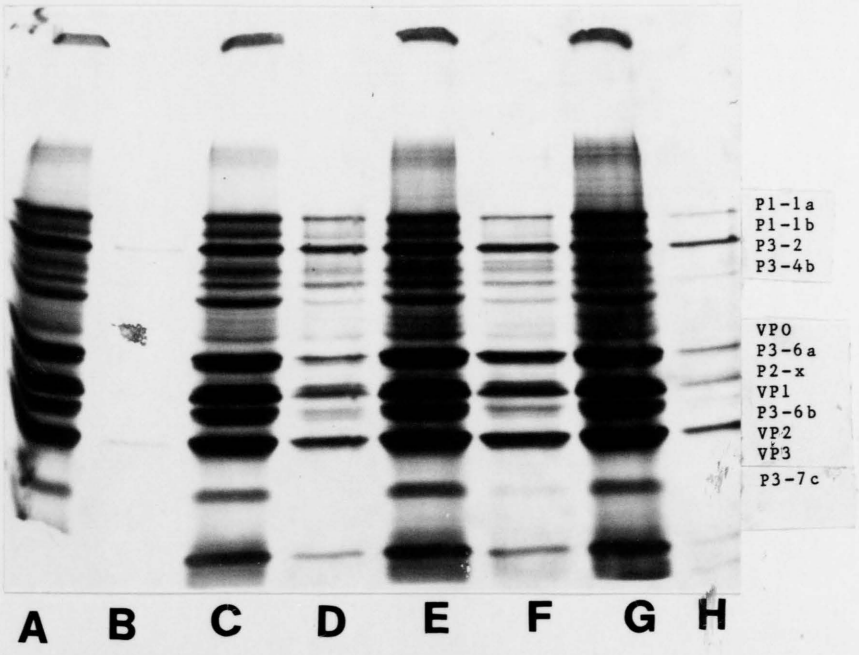

Figure 7. Autoradiogram of 35S-methionine labeled proteins of selected mutant viruses and the wildtype virus at 33°C and 39°C.

HeLa cells were infected at a MOI of 10 with the mutants tsB9, ts10, tsD, and the wildtype,PV1. Each infection was resuspended in methionine free MEM containing 5% dialyzed horse serum. The infections were split equally and placed at 33°C and 39°C. 35S-methionine was added to the infected cells at 39°C after 200 minutes and to the infected cells at 33°C after 230 minutes. Intracellular extracts were prepared after 30 minutes of labeling. Equal amounts of each intracellular extract were electrophoresed through a 10% SDS polyacrilamide verticle gel.
A. PV1, 33°C B. PV1,39°C C. tsB9,33°C D. tsB9,39°C
E. ts10,33°C F. ts10,39°C G. tsD, 33°C H. tsD,39°C.

complementation groups then, different protein functions along the length of the genome could be defined. Specifically, I was interested in the functional proteins in the 3' region of the genome which are responsible for replication.

Complementation between two mutant virus stocks was measured by quantitation of labeled 150S virion particles sedimented through a sucrose density gradient. When HeLa cells are infected with a mutant virus stock at the restrictive temperature (39^{o}C) under the conditions described in Materials and Methods very little, if any, 150S virion particles can be detected after sedimenting the cell extracts in a linear sucrose gradient (Figures 8E,8F,8G, 8H). Also, in the majority of co-infections (52 of 66) between several of these mutants at the restrictive temperature there is no significant increase in the amount of 150S virion particles (Figure 8B). However, in 14 of the 66 co-infections the amount of 150S virion particles are observed to be significantly above the background level. Complementing pairs occurred most frequently in co-infections between two RNA(-) mutants (9 of 14). For example, in the co-infection between two RNA(-) mutants, tsD and tsB9, complementation is observed in the sedimentation profile as an 8 fold increase of virion particles in the co-infection (Figure 8D) over the

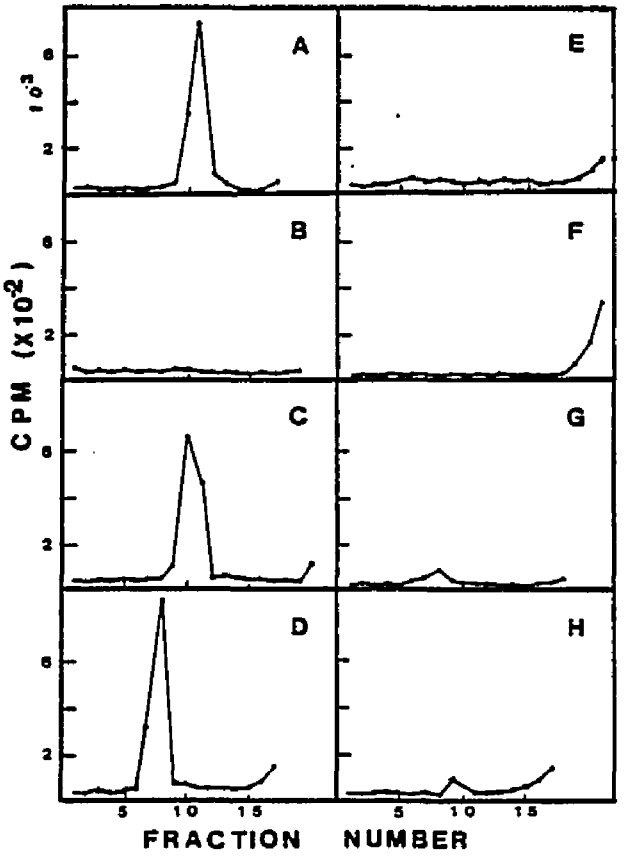

Figure 8. Sucrose gradient sedimentation profiles of selected mutant virus infections and co-infections at the restrictive temperatures.

a. PV1 control to show the amount of label incorporated and the region of 150S virion particle sedimentation. b. Co-infection of tsD and ts10. c. Co-infection of tsE and ts10. d. co-infection of tsD and tsB9. e. single infection of tsD. f. single infection of ts10 g. single infection of tsE. h. single infection of tsB9.

individual mutant infections (Figure 8E,and 8H).
The remainder of complementing pairs (5 of 14) occurred between mutants of opposite RNA phenotype. For example,150S virion particles were observed in the co-infection between the RNA(+) mutant tsE and the RNA(-) ts10 (Figure 8C). The sedimentation profiles of the individual mutant infections of both tsE and ts10 show very little background radioactivity in this region (Figure 8G and 8F). The production of 150S virion particles in the co-infection (tsE X ts10) is four fold greater than the sum of the particles produced in the single mutant infections (Figure 8G and 8F).

Finally, no complementation was ever observed in co-infection pairs between two RNA(+) mutants (0 of 66). The yield of virions in the mutant complementation experiments was approximately 10 fold less than the wild-type yield. These numbers have been observed for other viruses which complement (NDV, Sindbis). However, these results could be due to the the small sample number of RNA+ mutants which are available for complementation studies. Or that the complementing proteins in the capsid are not easily tested with this assay.

Complementation matrix.

A complementation matrix has been arranged to summarize the results of all pair-wise co-infections between

each of the mutants from Table 4.(Figure 9) These mutants have been organized into RNA(-) and RNA(+) regions in the complement matrix to easily visualize the frequency of complementation between the two different RNA phenotypes and also between the individual RNA phenotypes. Complementation occurs between two RNA(-) mutants and between an RNA(-) and an RNA(+) mutant but not between two RNA(+) mutants. Complementation groups are not well defined in this study. However, from the complement matrix it is observed that no one mutant complements all the other mutants. For example, ts 10 complements all but three mutants. Also, each mutant is complemented by at least one other mutant. For example, ts2,tsD37, and tsE complement only one other mutant. The mutant tsF10 is an exception as it is not complemented by any other mutant. This mutant may be a double mutant.

Nature of the complementation.

I chose to study the nature of the progeny virion particles in the complementing co-infections of ts mutants by backcross experiments. I thought these experiments would define whether the peak I observed in the sucrose gradient were mutant variants, wildtype or wildtype revertants. I define backcross as the co-infection of the resulting virion (progeny) of a complementing co-infection with each of the

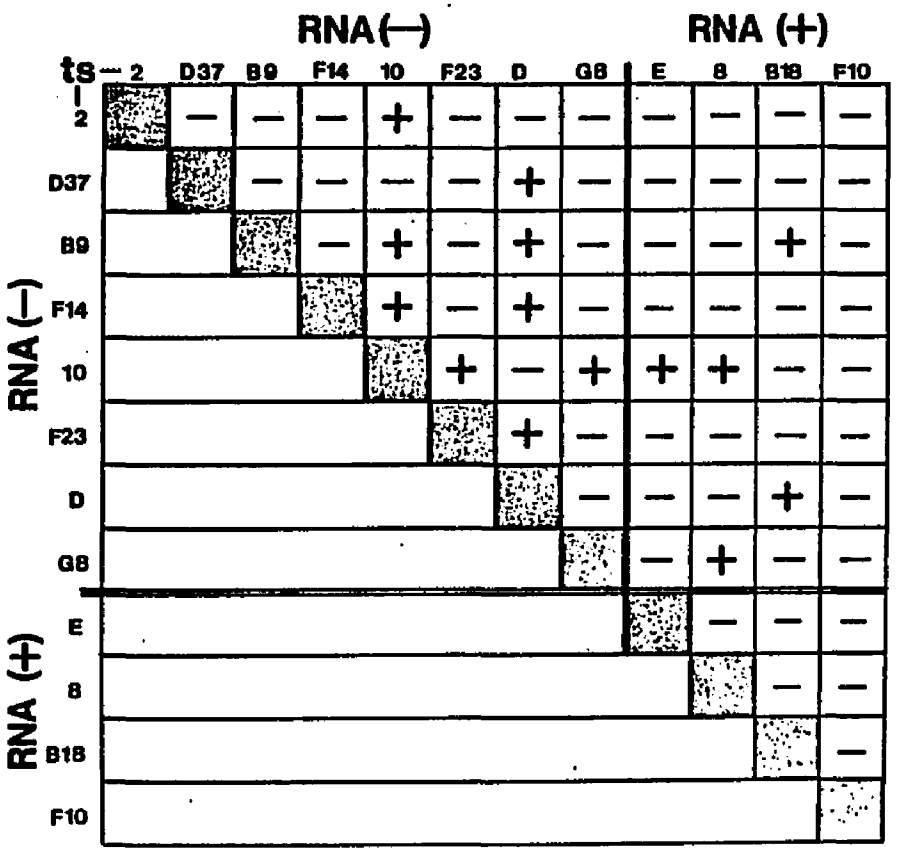

Figure 9. Complementation matrix

This matrix illustrates three possible regions of complementation. a. between two RNA minus mutants. b. between two RNA plus mutants. and c. between and RNA minus and an RNA plus mutant. In only fourteen of the sixty-six co-infections was the amount of 150S virion particles above background. There is no complementation detected between two RNA plus mutants. Five out of fourteen of the RNA plus and RNA minus complement. Nine out of fourteen of the co-infections between two RNA minus mutants complement.

two original mutant virus species (parent) in two different co-infections. I have observed that an increase in the multiplicity of the mutant virus in an infection does not increase the small amount of virion particles sometimes observed in the individual mutant infections. I expect that the virion peak produced in the backcross infections should decrease if it is composed of only particles from the same type of mutant as the parent. However, if the virion peak increases in the backcross experiment this may be an indication that a second round of complementation is occurring. Therefore, the virion particles present in the progeny peak might be composed of the parent not used in that co-infection. In the event that the progeny were wildtype then the peak representing virion concentration would approach the size of that peak observed for the wildtype virus because this virus will rapidly overgrow in the infection.

These experiments were done with several different complementing pairs, ts10 X tsE (E10) and tsB9 X tsD (DB9). In figure 10A the progeny virion peak E10 decreases in the amount of virion particles when it is co-infected with ts10 (Figure 10B). But, this E10 peak also decreases when it is co-infected with tsE (Figure 10C). This result does not fit the pattern predicted above unless the progeny virion contains approximately equal amounts of tsE and ts10.

In the second example (Figure 10D), the progeny

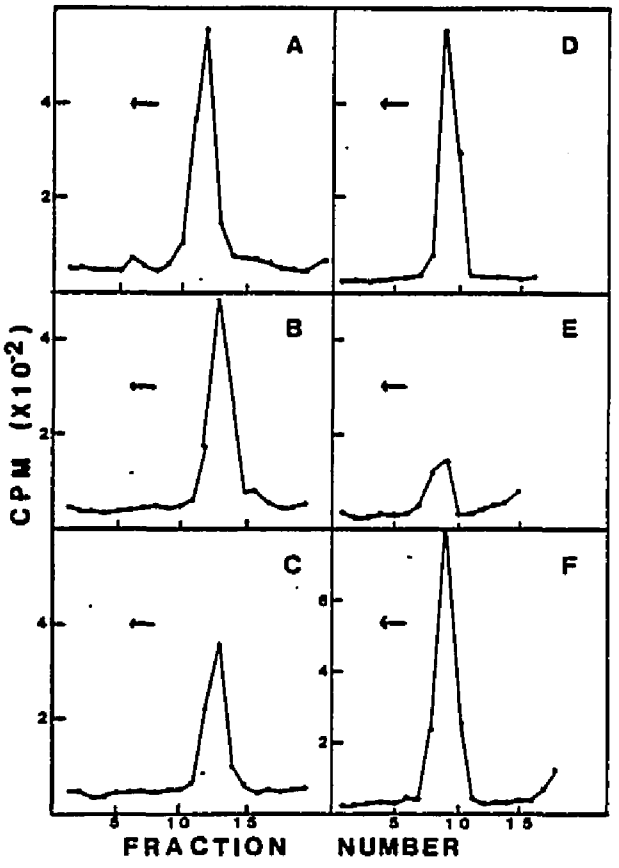

Figure 10. Sucrose gradient 150S profiles of co-infections between the progeny of a co-infection and each of the original mutant viruses.

a. An infection with the virus resulting from the co-infection between tsE and ts10. b. E10 virus co-infected with ts10 virus. c. E10 coinfected with tsE. d. An infection using the virus resulting from a co-infection between tsD and tsB9 (DB9). e. DB9 virus coinfected with tsB9. f. DB9 virus coinfected with tsD.

peak DB9, of the complement infection between tsD and tsB9, shows an increase in virion particles when co-infected with tsD (Figure 10F), but a decrease when co-infected with tsB9 (Figure 10E). Therefore, the progeny particles might consist of a majority of tsB9 virion and small amount of tsD virion. These experiments do not assist in the discussion of why the progeny peak is so large even upon passage at the restrictive temperature. I questioned whether these virion particles were revertants to wildtype or wildtype recombinants. The following experiment tests the virion stability of these progeny particles at 45°C in order to indirectly determine the nature of these particles.

Thermal stability of the progeny virion.

I hoped to take advantage of the differences in thermal stability between the RNA(+) and RNA(-) mutants to further evaluate the nature of the progeny particles present between the complementing pairs. This can be done only with the complementing pairs between an RNA(+) and an RNA(-) mutant. The progeny of these pairs was assayed for inactivation of the virus after complementation. If there is no reciprocal complementation occurring between these two distinct mutants then the inactivation curve would be more or less similar to that of one of the individual mutants. If reciprocal complementation does occur in a 50/50 ratio then the inactivation curve would be only a

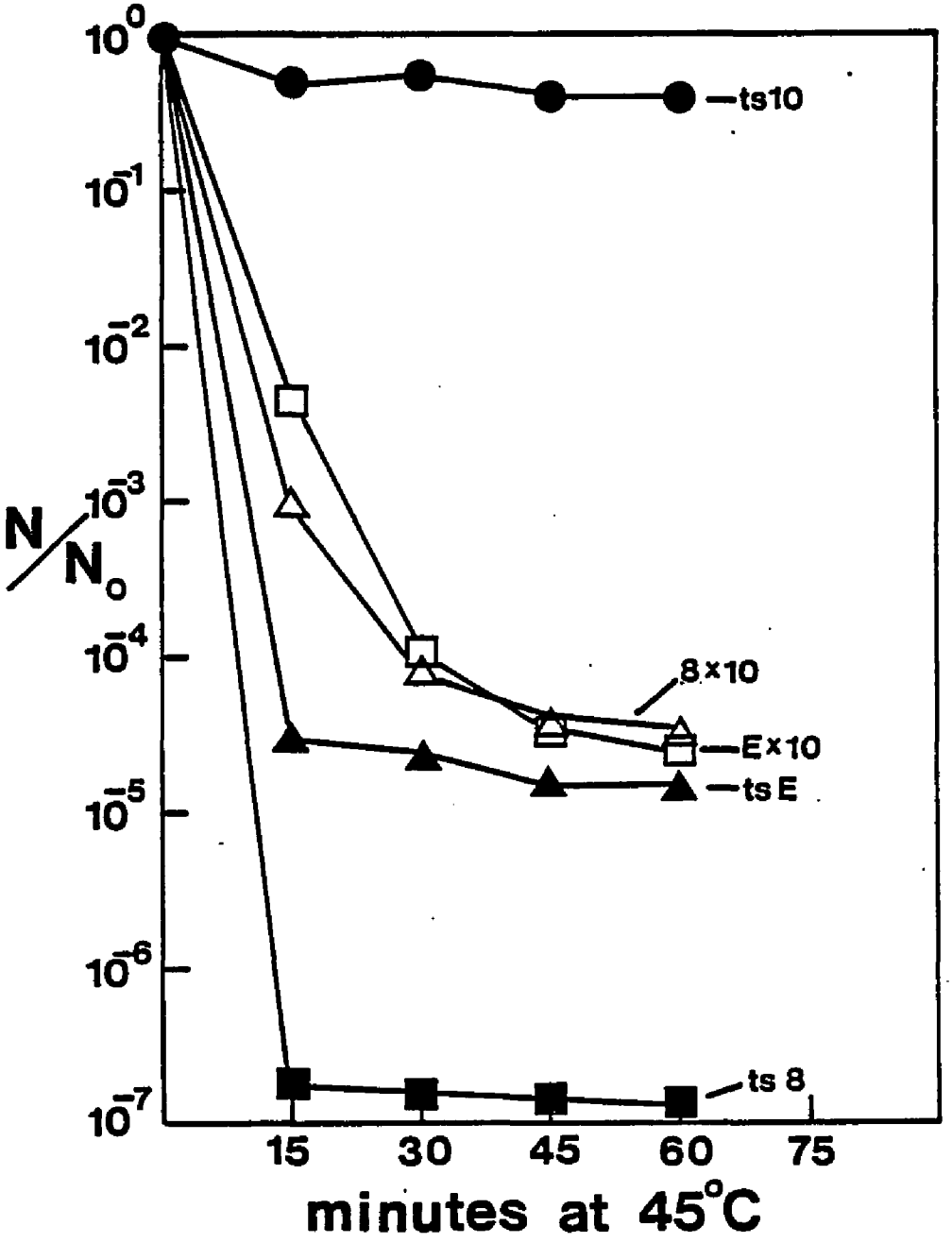

Figure 11. Thermal stability of progeny virions.

The progeny of co-infections between ts8 and ts10, and tsE and ts10 were tested for thermal stability by the ratio of surviving infectious units after treatment at 45°C to determine the nature of the progeny particles.

half log less stable than the inactivation observed for the stable RNA(-) mutant. Figure 11 shows that the complement pairs of tsE X ts10 and ts8 X ts10 have an intermediate virion stability. Following the mathematics of plaque forming units (pfu), the progeny peak contains a majority of the RNA(+) mutants. This information supports my belief that the particles in the progeny infections are not wild-type revertants, contaminants, nor recombinants because the wild-type phenotype is strictly thermal stable.

CHAPTER 4

DISCUSSION

Isolation of poliovirus type I mutants.

Conditional lethal mutants of Poliovirus type 1 were induced with the mild mutagen hydroxylamine-H_2SO_4. Mutants from this mutagenized stock were selected for temperature sensitivity between the broad range of 33.5°C (permissive) and 38.5°C (restrictive) temperatures. This is an increase of the range (37.5°C, permissive; and 39°C, restrictive) used in the early experiments by Cooper (1964). The increase in the temperature range between permissive and non-permissive temperature conditions of these mutants has decreased the percentage of mutant stocks which revert to a wild-type phenotype within a few passages after isolation.

Mutant Characterization.

The monitoring of RNA synthesis by the accumulation of radioactivity into trichloroacetic acid insoluble material has been reproducible for each mutant and is indicative of the kinetics of viral RNA synthesis during an infection labeled with [^{3}H]uridine in the presence of actinomycin D (Baltimore,1969). The mutants have been

classified from this information as RNA negative-(- ; less than 25% of the wild-type RNA synthesis) and RNA positive-(+ ; RNA synthesis of the same scale as wild-type; Hewlett et al, 1982).

Furthermore, temperature stability of the mutant virion was measured after treatment of the virus stocks at 45°C. The RNA(-) mutants have all demonstrated a thermostable virion while the RNA(+) mutants which are typically thermal labile vary in their degree of instability. This information agrees with the data reported for Sindbis virus (Burge and Pfefferkorn,1966). The virion stability of the RNA(-) mutants at 45°C for up to 60 minutes might indicate the presence of a mutation expressed early in the infection. These could be mutations in the functional region of the genome affecting the RNA replicase or the pre-VPg proteins. On the other hand, the RNA(+) mutant virions are generally unstable when exposed to 45°C. These mutant viruses may carry mutations which affect the proteins in the structural region of the genome. These proteins form the capsid structure of the virus. Aberrant proteins in the capsid might denature at the higher temperature causing the virion structure to fall apart or the protein structure may affect a protein involved in antigenic recognition of the cell surface receptor causing a decrease in the infectivity of the

virion but no loss of virion structure. These two possibilities have been examined in this thesis and both possibilities appear to occur between two different RNA(+) mutants, tsE and ts8 (Table 3).

The majority of the temperature sensitive mutants in this laboratory are of the RNA(-) phenotype. These mutants could be important in the study of RNA mechanisms of replication in poliovirus if specific functions could be tested and visualized during an infection or in in vitro systems. For example, an in vitro replicase assay or poly(U) assay which uses purified components from the RNA(-) mutant infection at 33°C and 39°C could indicate if the replicase protein is functional and if it is, further studies of the primer function and elongation functions could be investigated. Some preliminary experiments conducted in collaboration with Dr. A. Dasgupta indicate that the virus ts10 is a mutant which carries a temperature sensitive replicase protein. Dr. Bert Flanegan also reports that the ts10 virus has an unstable replicase protein at 39°C but not at 33°C. Two-dimensional gel electrophoresis might indicate if any of the proteins of the mutant stocks are produced with different isoelectric mobilities. Then the purification of this protein could be pursued and reconstitution experiments done in an in vitro assay.

Biochemical characterization.

Biochemical characterization of these mutants by gel electrophoresis in 1% agarose (RNA species) and in 10% SDS-polyacrylamide (protein species) has not shown any significant differences of the types of RNA or proteins synthesized between the ts mutants and the wild-type. The decreased concentrations of each individual protein synthesized at the restrictive temperature can be explained because the mutant viruses typically synthesizes approximately one tenth of the protein of the wildtype at this temperature. The RNA profiles are less intense because there is less protein in the cell to carry out the synthesis of the RNA, i.e., replicase. Thus, the information obtained from the biochemical evidence confirms a mutant phenotype but was not neatly definitive in pinpointing where a mutation took place.

Complementation.

I was interested in complementation between these ts mutants to determine if the RNA replicase from an RNA(+) mutant is able to act in trans within a co-infected cell to allow the replication of the RNA(-) mutant within the same cell. In these experiments complementation has been observed between an RNA(-) vs. an RNA(+) mutant and an RNA(-) vs. an RNA(-) mutant, but not between an RNA(+) vs. an RNA(+) mutant. In 2 cases, ts10 X tsE and ts10 X ts8,

complementation appears to be a rescue phenomenon. The ts 10 mutant rescues the RNA(+) mutant but there is no evidence that the ts10 mutant is present among the progeny in greater than the input multiplicity. This has been demonstrated by the thermal stability of progeny virion after treatment at 45°C (Figure 12).

Research in the area of genome structure, translation and replication of poliovirus and recombinant DNA technology can assist in postulating how poliovirus mutants may complement each other within the framework of this knowledge.

First, no complementation is observed between two RNA(+) mutants. This could be an effect of the small sample size (of only 4 out of 12 mutants) or a result of the virion morphogenesis derived from the NCVP1a polyprotein. One possibility for complementation would be phenotypic mixing between the two types of mutant polyproteins to form the 13S pentamers which later undergo cleavage and assembly reactions to eventually become the 150S virion. Thus, of the 60 subunits composing the capsid each will be in one of two ways depending on where the mutation was in each of the parents. For example, a mutated nucleotide can code for mutant amino acids which have several possibilities: The folding of the entire NCVP1a protein may be affected such that the appropriate cleavage sites are dis-

guised or inaccessible to the virus-specific protease. Or the faulty amino acid might be one of the pairs in the cleavage site and inhibit the correct gln-gly cleavage site in favor of a second site or of no site. Lastly, the amino acid change might be chemically compatible with the wild-type amino acid which would not significantly change the chemistry of the protein nor would it significantly alter the function, but it could cause the protein to change configuration under conditional conditions, e.g.,temperature. At any rate, if the two defective NCVP1a polyproteins add together to form a complete capsid the result will be an aberrant capsid which is faulty especially with respect to temperature changes in the specific proteins affected. In this case 60 wrong subunits do not add to make one right capsid. There are very few types of mutations in this region which would allow two negative factors in the capsid to interact in such a way as to restore wild-type function. Therefore, complementation would rarely be observed between two RNA(+) mutants.

In the cases of the complementation co-infections between an RNA(+) mutant and an RNA(-) mutant I hypothesized that the replicase from the RNA(+) mutant would act in trans to allow replication of the RNA(-) mutant genome. This phenomenon was not observed. Experimentally the RNA(-) mutant seems to enhance the growth of

the RNA(+) mutant while being relatively unaffected in the infection (Figure 12). This result is difficult to explain on the molecular level. If these RNA(+) mutants are assembly mutants then there could be a factor produced by the RNA(-) mutant to catalyze the assembly of the RNA(+) mutant. However, these mutants are thermolabile indicating that the mutation is in the capsid structure itself and not in the ability of the capsid to assemble with the RNA during virion morphogenesis (Figure 3). If capsid rescue is occurring, e.g., the RNA(+) mutant scavenges the capsid produced by the RNA(-) mutant then we would observe the inactivation of the progeny virion at 45°C (Figure 12). -- Also, only a very small amount of capsid proteins would be available for assembly from the RNA(-) mutant because amplification can't occur without RNA synthesis. Could there be a defect in the cleavage of the capsid by a protease functional on the RNA(-) mutant? Perhaps VP3? In this case complementation between the RNA(-) mutant with the functional protease could restore wildtype capsid fuction to the RNA(+) mutant on the condition that the RNA(+) can restore RNA synthesis to the RNA(-) mutant. Thus, the capsid would be wildtype but possibly each of the two types of RNAs would be encapsidated. However, the more abundant RNA would overgrow as the major mutant observed in a second round of replication. This is a possibility observed in the backcross infections.

Although reciprocal complementation has been not been indicated in these experiments, there is still one possibility that reciprocal complementation might occur between two RNA(-) mutants. However, examination of this is beyond my scope because there is no selection method to determine the individual phenotypes or genotypes between this class of ts mutants. Out of curiosity, how could two RNA(-) mutants undergo reciprocal complementation within the framework of the current knowledge on picornavirus replication and some additional speculation? I propose a model to discuss this possibility.

It has been proposed that the NCVP1b polyprotein remains uncleaved until after the initiation event in replication (Dasgupta, 1983; Semler, 1982). This proposal is reasonable in the light of the hypothesis that the NCVP1a polyprotein folds into pentameric subunits before cleavage into individual mature proteins (Phillips,1971; Putnak,1981). Therefore, a similar mechanism may hold true for the 3' end of the genome. However, if the cleavage site between P2-3b and P3-1b is virus specific, specified by the P3-7c protein (Hanecak,1982) then the P2-3b poly protein must fold along with the P3-1b polyprotein. As the ribosome releases the P3-1b protein it folds immediately to prevent proteolysis by the host cell enzymes. Consider the possibility that a limited number of ribosomes may trans-

late an individual viral mRNA (Summers et al, 1967). Suppose that a maximum of 35 ribosomes are competent to translate an individual mRNA (viral), then approx. 34 P3-1b proteins would be free in the cytoplasm while the last P3-1b polyprotein synthesized by the last translating ribosome can remain on the 3'terminus to initiate replication of the complementary strand RNA. Initiation of RNA replication requires several processes before it can occur. The P3-7c protein is located between the pre-VPg protein and the replicase (p63) protein. There may be a mechanism by which the P3-7c protease recognizes its amino and carboxy termini and in the process brings the pre-VPg protein into the vicinity of the replicase protein. The host factor protein may carry an ATPase requiring activity (Morrow et al, 1985) which in associtation with the replicase protein could covalently link the pre-VPg protein to a pUp nucleotide forming the primer necessary for initiation of RNA replication. Then the protease can autocatalytically cleave itself from between the replicase and the pre-VPg protein. The replicase is then free to elongate the initiated RNA along the plus RNA template and the P3-7c protein can continue to recognize gln-gly cleavage sites and produce mature proteins (Semler et al,1982). A ds RNA (RF) molecule is observed as a result of the <u>in vitro</u> replicase assays. Apparently, the necessary energy producing and/or

energy requiring steps are not available in this in vitro system to continue synthesis of the complementary RNA into the replicative intermediate form. I postulate that the presence of the polyprotein P3-2 in this system might restore the ability to produce the replicative intermediate. Needless to say this polyprotein will have to be cloned and expressed in an expression vector before it can be purified in sufficient quantity to add to in vitro assays. It may be reasonable to believe that multiple initiations of plus strand RNA synthesis can occur on the 3' end of the newly synthesized complementary RNA in the presence of the NCVP1b polyprotein. This forms the replicative intermediate. The nascent RNAs become message and each message produces one complementary RNA when translation of that message is "turned off". In this manner there is a cycle of amplification. If a limited amount (35; Summers et al,1967) of ribosomes translate each incoming message then the RNA will always be destined to produce a sufficiently large amount of capsid proteins and the same amount of P3-2 protein to initiate replicative intermediates while the single translating RNA will be the only method of producing the single complementary RNA.

With this model in mind how could reciprocal complementation occur between two RNA(-) mutants? The P3-2 polyprotein (which contains the replicase and proteinase)

could have trans activity especially in the formation of the replicative intermediate. If a replicase protein is unable to elongate the RNA but can still initiate RNA replication then the replicase from the second mutant can conceivably act in trans because the replicase is found to be stable within the cytoplasm of the infected cell (Flanegan and Baltimore,1977). In the above model there would not be complementation if the pre-VPg were non-functional in one of the mutants and could not be energetically attached to a pUp to act as primer or if the replicase failed to interact with the host protein which might supply the ATP requiring function to phosphorylate a primer or possibly to add uridine residues onto the VPg protein. In a second case complementation would not occur if the replicase were abberrant and elongation of the initiated RNA strand failed to occur. Other complementing functions may depend of recognition sites on these proteins by the host factor. In this case the polyprotein P3-1b from the second mutant was used to initiate the genomic RNA of the first mutant. Other complementing functions may depend on recognition sites on these proteins by the host factor which is necessary in initiation of replication. In this case the polyprotein P3-2 from the functional mutant may act to initiate RNA replication on the genome of the non-functional mutant.

Complementation studies in poliovirus had been

limited due to the lack of adequate techniques to visualize the molecular differences which are occurring in the *in vivo* experiments. Biochemical *in vitro* data combined with recombinant DNA technology and are promising tools for complementation between constructed mutants.

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