1984

Genome Organization And Expression Of Selected Potexviruses

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GENOME ORGANIZATION AND EXPRESSION OF SELECTED POTEXVIRUSES

By

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Submitted in partial fulfillment

of the requirements for degree of

Doctor of Philosophy

Faculty of Graduate Studies

The University of Western Ontario

London, Ontario

1984

TO THE MEMORY OF MY FATHER

WILLIAM BENDENA

ABSTRACT

Plant viruses categorized within the potexvirus group represent a collection of nonrigid filamentous viruses of lengths between 470 nm and 570 nm. Although much is known about the assembly and physical properties of potexviruses, very little is known about the physical and functional properties of their genomic RNAs. We have used *in vitro* translation to analyze the protein products produced from the genomic RNAs of three definitive members of the potexvirus group, namely papaya mosaic virus (PMV), clover yellow mosaic virus (CYMV), and viola mottle virus (VMV). We have also examined two tentative members whose structures conform to that of definitive potexviruses, namely, barrel cactus virus (BCV), and foxtail mosaic virus (FTMV). Polyacrylamide gel electrophoresis of polypeptides made *in vitro* from the genomic RNAs of these viruses in either wheat germ extract or rabbit reticulocyte lysate indicated that only PMV and CYMV RNA directed the synthesis of polypeptides with properties similar to those of their respective coat proteins. They have the same molecular weight as their respective coat proteins and are precipitated by antibodies raised against their respective virions. All viral RNAs tested *in vitro* produced high molecular weight non-structural polypeptides. Peptide mapping of the high molecular weight polypeptides directed by PMV and CYMV

RNAs indicated that they and many of the minor products of translation are related to each other but not to their respective coat proteins. The ability of partially-encapsidated PMV ribonucleoprotein particles to direct protein synthesis had also been assessed. As the extent of encapsidation increased, the relative synthesis of the high molecular products decreased markedly. In contrast, the synthesis of the in vitro coat protein not only persisted but increased up to four fold relative to the untreated RNA until a substantial fraction of the PMV RNA is completely encapsidated. In view of the polarity of assembly in vitro this finding indicated that the cistron for PMV coat protein is localized towards the 3' end of the RNA. The coat protein cistron for CYMV was also found to have a 3' terminal location using the same methodology. That coat protein synthesis was directed by the genomic RNAs of only two members of the potexvirus group suggested that morphological relatedness was not a reliable indicator of the functional behavior of their corresponding RNA's. This led us to examine the relationship of these viral genomes at the level of nucleotide sequence. Using RNA-cDNA hybridization we found that no homology exists between all members of the potexvirus group tested, nor with unrelated members. As a further means of characterizing the genomes of these

viruses, we have constructed double-stranded cDNA copies of PMV and CYMV RNA and have subsequently cloned them into E. coli using the plasmid pBR322 as a vector.

ACKNOWLEDGEMENTS

I am grateful to my supervisor, Dr. George A. Mackie for his patience (perserverence), guidance and encouragement throughout the course of this work and during the preparation of this thesis.

I wish to thank Drs. John Bancroft, John Erickson, Ted Lo and other members of the Biochemistry department for their advice and support.

Also, I would like to thank Ron Layden and Mark Peters for helping to smooth over the rough edges through provision of friendship, food , shelter and muscle.

I would like to thank Mrs. Ruth Weber for typing this thesis while maintaining a cheerful disposition through preparation of the tables, and Gay Parsons for assistance with the figures.

Finally, I would like to thank my mother and brother for their encouragement and my wife and children for their support and coping with my absence.

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NOMENCLATURE

AMV	avian myeloblastosis virus
ATP	adenosine triphosphate
BCV	barrel cactus virus
CYMV	clover yellow mosaic virus
DNA	deoxyribonucleic acid
cDNA	complementary DNA
dNTP	deoxyribonucleoside triphosphate
DTT	dithiothreitol
EDTA	(ethylenedinitrilo) tetraacetic acid
EGTA	ethyleneglycol-bis-(β-amino ethyl ether) N, N'-tetraacetic acid
FTMV	foxtail mosaic virus
GTP	guanosine triphosphate
Hepes	(N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)
LMC	low molecular weight component
MES	(2[N-Morpholino]ethane sulfonic acid)
MOPS	(3-[N-Morpholino]propane sulfonic acid)
PBS	phosphate buffered saline
Pipes	(Piperazine-N,N'-bis[2-ethane sulfonic acid])
POL-1A	klenow fragment of Escherichia coli DNA Polymerase I
PMV	papaya mosaic virus
PVX	potato virus X

SDS	sodium dodecyl sulfate
SSC	standard (saline) citrate
TCA	trichloroacetic acid
Tris	tris(hydroxymethyl)aminomethane
TMV	tobacco mosaic virus
tRNA	transfer ribonucleic acid
VMV	viola mottle virus

CHAPTER 1 - INTRODUCTION

Plant viruses have long been regarded as "model systems" in which to study the basic principles of virus self-assembly, protein-protein and protein-nucleic acid interactions. Their availability, stability and simplicity in composition has no doubt contributed to their use. The constituent components of most simple plant viruses are a sole molecule of single-stranded RNA and a single type of protein subunit which is polymerized along the length of the RNA to form a protein shell. Although much research has been devoted to understanding the physicochemical properties and interactions between these two components (for review see Butler and Durham, 1977), information regarding the "life cycle" and in particular the organization and expression of the nucleic acid component has only recently become available. A contributing factor to recent progress has been the development of translation systems derived from wheat embryo (Marcus, 1970), wheat germ (Marcu and Dudock, 1974; Roberts and Patterson, 1973) or mammalian cells such as rabbit reticulocytes (Lockard and Lingrel, 1969; Pelham and Jackson, 1976) or mouse ascites cells (Mathews and Korner, 1970; Aviv et. al., 1971). Bacterial extracts have also been programmed by plant viral RNAs to direct translation. The efficiency and fidelity with which products are made in bacterial extracts is, however, very

low (Van Ravenswaay Classen et. al., 1967; Stubbs and Kaesberg, 1967; Klein et. al., 1972; Rice and Fraenkel-Conrat, 1973).

1.1 In vitro Translation as a Tool in Plant Virology

There are several reasons for applying in vitro translation to the study of plant viral genomes. The first is to determine what proteins are encoded by the viral genome since other than coat protein the nature and function of other products is unclear. Presumably, non-structural proteins would be required for replication, alteration of host cell metabolism and/or cell to cell movement within the host. Secondly, in vitro translation can provide information regarding which regions of the genome encode each protein. Finally, in vitro translation can provide insight into the translational strategy or how the information within the RNA is converted into discrete proteins. In this regard the study of plant viral translation and replication in vivo is complicated because the number of cells infected initially by inoculation is extremely small and because the stage of infection in individual cells becomes random as infection spreads (Takabe, 1975). Moreover, many plant tissues are difficult to handle for biochemical experiments due to the presence of a tough cell wall and inhibitory substances (e.g., polyphenols). Plant protoplasts offer a viable alternative; however, at present only a few

combinations of plant and virus have been tested (Takabe, 1975). For many plant viruses, therefore, in vitro translation is the only feasible avenue for the investigation of their life cycle.

It is now appreciated that the choice of cell-free system is important if biologically valid results are to be obtained from in vitro translation experiments. The initial failure of attempts to reduce endogenous globin incorporation in rabbit reticulocytes made the use of other cell-free extracts popular. Wheat embryo and wheat germ extracts, in addition to having low endogenous incorporation, are also relatively easy to prepare (Efstratiadis and Kafatos, 1976a). These systems as well as the mouse ascites cell-free system, however, suffer from poor re-initiation frequency and translational fidelity as compared to the reticulocyte system (Brawerman, 1974; Efstratiadis and Kafatos, 1976a). For example, the translation products directed by tobacco mosaic virus RNA in wheat germ extracts are heterogeneous in size ranging from 10,000 to 110,000 (reported as 130,000 to 140,000 daltons) (Marcu and Dudock, 1974; Hunter et. al., 1977). In contrast, addition of TMV RNA to reticulocyte lysates rendered dependent on exogenous RNA by treatment with the calcium-dependent micrococcal nuclease (Pelham and Jackson, 1976) resulted in the synthesis of only two proteins of 165,000 and 110,000 daltons. The heterogeneity of products directed in the

wheat germ extract probably arises from premature termination, probably because of nucleolytic damage to the RNA (Hunter et. al., 1977; Pelham, 1979)

1.2 Translational Strategies Employed by Plant Virus Genomes

There are several general mechanisms for conversion of the genomic information of RNA viruses into discrete proteins. These are not necessarily mutually exclusive, as will be seen.

First, the genome may consist of two or more discrete RNAs which may be packaged within single or multiple virions. Brome mosaic virus, for example, is a multicomponent virus which contains four separate messengers (one of which is a sub-genomic RNA) packaged within three virions. The independent translation of these messages results in the synthesis of four proteins, the yields of which are regulated by the translational efficiency of the individual message (Shih and Kaesberg, 1976) (Figure 1a). Viruses with monocomponent genomes may, however, also behave functionally as if they possessed multicomponent genomes by generating a sub-genomic or low molecular weight component RNA during the replication process. For example, in the best studied plant virus, tobacco mosaic virus (TMV) (Tobamovirus group), the coat protein cistron localized near the 3' end of the viral RNA is not expressed in vitro (Hunter

Figure 1 Translational strategies employed by plant virus genomes.

For each virus the abbreviated virus name is followed by the approximate number of nucleotides which comprise the RNA molecule(s).

a) brome mosaic virus

b) tobacco mosaic virus

c) turnip yellow mosaic virus

d) southern bean mosaic and turnip rosette virus

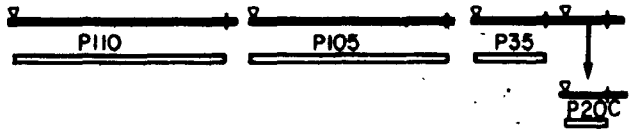
e) carnation mottle virus

RNA molecules (——) have the following features indicated: ∇ , translational initiation site; A, presence of an amber codon; a vertical line represents the translation termination site.

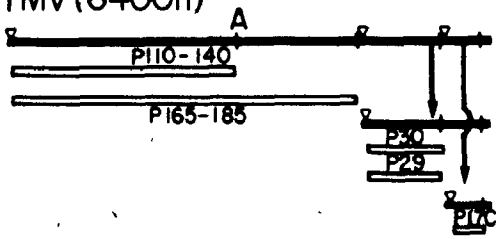
The polypeptides (═══) resulting from the translation of each genome are positioned to represent the region of the genome from which they are derived. Protein molecular weights are in kilodaltons. Pr represents the sites of protease cleavage and C represents the coat protein.

Details of each translational strategy are found in the text.

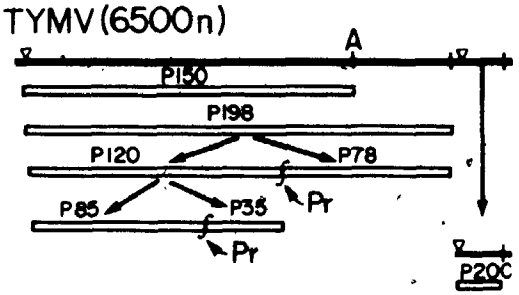
A. BMV (3300, 3100, 2120n)



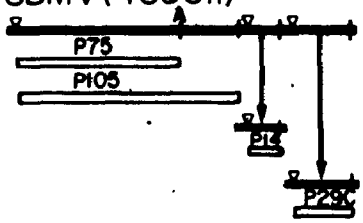
B. TMV (6400n)



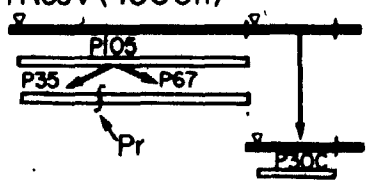
C. TYMV (6500n)



D. SBMV (4500n)



TRosV (4500n)



E. CarMV (4300n)

et al., 1976): Partial transcription of the full length RNA in vivo generates a low molecular weight component RNA (LMC RNA) which codes for coat protein in the wheat germ cell-free system (Hunter et al., 1976; Bruening et al., 1976; Whitfield and Higgins, 1976; Higgins et al., 1976). Similarly a TMV-specified 30,000 dalton protein is also produced from a separate subgenomic message (Beachy and Zaitlin, 1977) (Figure 1b).

In a second mechanism, two proteins can originate from the same region of the genome by translational readthrough of one or more nonsense or stop codons. The in vitro translation of the genomic RNA of TMV results in the synthesis of two proteins of 165,000 and 110,000 daltons both of which initiate at the same site at the 5' end of the RNA. The 165,000 dalton protein is generated by partial readthrough of an amber (UAG) termination codon (Pelham, 1978) (Figure 1b). Other viruses such as turnip crinkle virus (ungrouped) (Dougherty and Kaesberg, 1981), southern bean mosaic virus (Sobemovirus group) (Rutgers et al., 1980; Salerno-Rife, et al., 1980), alfalfa mosaic virus (Alfalfa mosaic virus group) (Gerlinger et al., 1977; van Tol and van Vloten-Doting, 1979), tobacco rattle virus (Tobravirus group) (Pelham, 1979a) and turnip yellow mosaic virus (Tymovirus group) (Pleij, et al., 1976; Benicourt et al., 1978) also use the same two mechanisms as TMV (i.e. LMC RNAs and readthrough).

In a third translational strategy, the genomic RNA may act as a monocistronic messenger which encodes a precursor protein which is then processed by proteolytic cleavage. Turnip yellow mosaic virus utilizes proteolytic processing (Morch and Benicourt, 1980) in addition to the mechanism described for tobacco mosaic virus (Figure 1c). Proteolytic processing has been found to occur in turnip rosette virus (Sobemovirus group) (Morris-Krsinich and Hull, 1981), tobacco etch virus (Potyvirus group) (Dougherty and Hiebert, 1980) and cowpea mosaic virus (Comovirus group) (Pelham 1979b). All of the proteases appear to be virally encoded. Parenthetically, it is of interest to note that turnip rosette virus and southern bean mosaic virus, although both classified in the Sobemovirus group, employ different translational strategies (Figure 1d).

Fourth, the genomic RNA may act as a polycistronic messenger whose secondary structure and ribosome binding sequence(s) serve to regulate the amount of each of the encoded proteins. Such a proposal is perhaps the most controversial for reasons discussed below. Internal initiation of translation has been suggested for tobacco necrosis virus (Salvato and Fraenkel-Conrat, 1977), southern bean mosaic virus (Salerno-Rife et al., 1980), cowpea mosaic virus RNA (Pelham, 1979b), and carnation mottle virus (Salomon et al., 1978) (Figure 1e).

The scanning or Kozak model (Kozak, 1978) was proposed to explain how eukaryotic ribosomes initiate protein synthesis. The model postulates that the 40S ribosomal subunit together with the appropriate initiation factors and Met-tRNA$_i$ bind to the 5' end of the messenger RNA, then migrate towards the interior of the message. At the first AUG initiation codon, migration stops, the 60S ribosomal subunit joins and peptide bond formation is initiated. Evidence supporting this model has been the subject of numerous reviews (Kozak, 1978, 1980 and 1981). Internal initiation would be in violation of the scanning model. Kozak (1978) has argued that products which appear to arise by internal initiation *in vitro* are actually the result of cleavage of the messenger RNA under the conditions of *in vitro* translation. Sub-genomic fragments having an AUG near the 5' end of the RNA could then initiate translation according to the rules of the model. That translation can occur from cryptic sites activated by the fragmentation of RNA has been demonstrated by Pelham (1979) and by Lawrence (1980).

There are, however, exceptions where initiation is not limited to the first AUG triplet of intact RNA. For example, the 16S late messenger RNA of SV40 encodes two proteins, the agnoprotein (Jay *et. al.*, 1981) and the VP1 capsid protein. The agnoprotein is initiated at the first AUG codon (position 303) whereas the VP1 capsid protein

is initiated at the third AUG (position 1417) (Kozak, 1981) within the interior of the message. This implies that there are exceptions to the Kozak model and certainly it is not unrealistic that such exceptions should exist.

A possible fifth mechanism, translation in more than one reading frame has been suggested to occur in two plant viruses, southern bean mosaic (Ghosh et al., 1981) and the cowpea strain of tobacco mosaic virus (Meshii et al., 1981).

The net effect of all these mechanisms and their combinations is to amplify the amount of information contained within each genome and possibly provide a means of regulation for each product. With the exception of the fourth and fifth mechanisms all others are consistent with the scanning model for initiation of protein synthesis.

1.3 Structure of the TMV Genome

The techniques of recombinant DNA have opened another avenue through which the organization of the genomic RNA component of plant viruses can be analyzed. Recently, the complete nucleotide sequence of the common strain of TMV was determined (Goelet et al., 1982) using cloned complementary DNA copies of the RNA as substrates. Quite surprisingly very little new information was gained from the analysis of the sequence.

The sequence simply verified the conclusions and predictions drawn from the in vitro translation studies with TMV RNA and mapped in a more precise manner the boundaries of the three open reading frames. The first AUG codon specifying the initiator methionine resides at nucleotides 69 to 71 of TMV RNA and is followed by an open reading frame for a protein of 125,941 daltons. The reading frame for this protein terminates at an amber codon at nucleotide residue 3,417. An inphase reading frame extends beyond this amber codon for 1,497 nucleotides. The readthrough protein is therefore 183,153 daltons. The sizes of the proteins predicted by the nucleotide sequence are in fairly close agreement with the sizes of products determined by in vitro translation, 130,000 (110,000) and 165,000 daltons. The presence of an amber codon had also been predicted since the inclusion of amber suppressor tRNAs during translation converted the 130,000 dalton polypeptide into one of 165,000 daltons (Pelham, 1978). This 183,253 dalton protein overlaps a second open reading frame by five codons. This reading frame encodes a protein of 29,987 daltons in agreement with a 30,000 dalton protein previously found in translation experiments (Beachy and Zaitlin, 1977; Pelham, 1979). The coding sequence for the 29,987 protein terminates two nucleotides before the initiator codon of the coat protein which is contained within the terminal 692 residues of the genomic RNA.

One advantage to this method, aside from its accuracy, is that cDNA clones from any particular region of the genome can be used as probes to study the nature of intermediates during the replication of the virus. Such probes also provide a means of directing mutagenesis so that sequences involved in initiating protein assembly or replication can be defined. Directed mutagenesis may also aid in ascribing functions to the non-structural proteins directed by this genome.

1.4 What is a Potexvirus?

The Potexvirus group is a collection of morphologically related viruses, some of which are economically important due to their potential to reduce crop yield (Pratt, 1967; Shepard and Clafin, 1975). As an example, up to 90% of all cassava plants in Kenya are infected with the potexvirus, cassava mosaic virus reducing the yield of this essential crop by 75% (Kurstak, 1981). The transmission of potexviruses appears to be primarily mechanical and the host range for individual viruses is narrow. Potato virus X, however, infects at least 260 species in 16 plant families, although over half of these species are in the family Solanaceae. By comparison, tobacco rattle virus (Tobravirus group) is considered to have a wide host range infecting 400 species of plants in over 50 families (Purcifull and Edwardson, 1981).

TABLE 1 Properties of Selected Rod-Shaped Viruses

Virus	Particle Length (n.m.)	Number of Genome Components	Sedimentation Coefficient (s)	Molecular Weight of RNA	Molecular Weight of Protein	Transmission
Potato Virus X (Rotexvirus)	515	1	118	2.1×10^6	2.6×10^4	Mechanical
White Clover Mosaic Virus (Potexvirus)	480	1	119	2.4×10^6	$1.4 - 2.0 \times 10^4$	Mechanical
Clover Yellow Mosaic Virus (Potexvirus)	539	1	121	2.2×10^6	2.0×10^4	Mechanical
Cactus Virus X (Potexvirus)	520	1		2.2×10^6	2.35×10^4	Mechanical
Papaya Mosaic Virus (Potexvirus)	530	1	118.7	2.2×10^6	2.2×10^4	
Foxtail Mosaic Virus (Potexvirus)	508	1	122	2.24×10^6	3.0×10^4	Unknown
Viola Mottle Virus (Potexvirus)	480	1	-	2.2×10^6	2.1×10^4	Mechanical
Barley Stripe Mosaic Virus (Hordeivirus Group)	128	3	185 178 200	1.4×10^6 1.25×10^6 1.15×10^6	2.15×10^4	Seed transmitted
Tobacco Rattle Virus (Tobravirus Group)	190+ 45-115	2	300+ 155-243	2.3×10^6 + $0.6 - 1.3 \times 10^6$	2.4×10^4	Nematodes
Tobacco Mosaic Virus (Tobamovirus Group)	300	1	194	2.05×10^6	1.75×10^4	Mechanical
Poplar Mosaic Virus (Carlavirus Group)	685	1	165	2.3×10^6	3.4×10^4	Vector Unknown
Beet Yellow Virus (Closterovirus)	1270	1	110	4.3×10^6	2.35×10^4	Aphids

Morphologically these viruses are non-rigid filamentous rods with lengths ranging between 470 and 580 n.m. and widths of 11 to 13 n.m. (Harrison et. al., 1971; Koenig and Lesemann, 1978). Their morphology is therefore distinct from those of rigid rod viruses such as tobacco mosaic virus. Table 1 lists the physicochemical characteristics of a few potexviruses which have been analyzed as well as those of rod-shaped viruses in six other groups for comparison. Potexviruses as far as it is known contain a single molecule of infectious RNA whose molecular weight ranges from 2.1×10^6 to 2.6×10^6 daltons and which constitutes approximately 5-7% of the particle weight (Koenig and Leseman, 1978). The RNAs of papaya mosaic virus (Abouhaidar and Bancroft, 1978), clover yellow mosaic virus (Abouhaidar, 1981), and potato virus X (Sonenberg et. al., 1978) have all been shown to contain 7-methylguanosine (cap structure) at their 5'-termini.

Virions of members of the potexviruses contain a single type of protein subunit which ranges in molecular weight from 1.4×10^4 to 2.6×10^4 daltons depending on the virus (Koenig and Lesemann, 1978). All potexviruses examined thus far are helically constructed with a pitch of 3.3 to 3.6 n.m. and appear to have approximately the same number (nine) protein subunits per turn (Richardson et. al., 1981; Tollin et. al., 1979, 1980 and 1981).

Although much is known about the composition and architecture of select members of this group very little is known with regard to the mechanism of replication of these viruses. Generally, replication takes place in the cytoplasm. Clover yellow mosaic virus replication may however, be initiated in the nucleus (De Zoeten and Schlegel, 1967).

1.5 The Assembly of Potexviruses

The reconstitution of potexviruses from their constituent components has only been attempted for three members; potato virus X (PVX) (Goodman, 1975 and 1976), papaya mosaic virus (PMV) (Erickson and Bancroft, 1978a and b; Erickson et al., 1978; Abouhaidar and Bancroft, 1978) and clover yellow mosaic virus (CYMV) (Bancroft, et al., 1979; Abouhaidar, 1981). Of these, the reconstitution of PMV has been most extensively characterized. Under optimal ionic conditions, at a coat protein to RNA ratio of 20:1, PMV protein assembles with its respective RNA to produce particles which are indistinguishable from the native virus in both morphology and sedimentation properties (Erickson and Bancroft, 1978a). Reconstituted particles are also infectious. The assembly process occurs in two energetically and kinetically distinct steps, a rapid initiation phase followed by a slower elongation phase (Erickson and Bancroft, 1978b). During the rapid

initiation phase, PMV protein recognizes and binds to an adenylate-rich region at or within 100 nucleotides of the 5'-terminus of the RNA (Abouhaidar and Bancroft, 1978). This was determined by initiating assembly using less than stoichiometric amounts of coat protein in the assembly reaction. Due to the kinetics of the reaction partially assembled particles accumulated. These particles were treated with T1 RNAse and the protected fragment isolated and analyzed for the presence of a 7-methylguanosine cap structure (Abouhaidar and Bancroft, 1978). Indeed, the 5'-terminal cap structure was protected against nuclease attack. Therefore, the assembly process is occurring in the 5' to 3' direction along the length of the RNA. This same specificity for the 5' end has been demonstrated for CYMV RNA and CYMV coat protein (Abouhaidar, 1981). Conditions of specific assembly have not yet been demonstrated for PVX (Goodman, 1975; Goodman, 1976).

1.6 Goals of This Research

Although much is known about the assembly and physical properties of potexviruses, nothing was known at the time that this project was initiated about the physical and functional properties of their genomic RNAs. We therefore used *in vitro* translation to analyze the protein products produced from the genomic RNAs of three definitive members of the potexvirus group, namely papaya

mosaic virus (Purcifull and Hiebert, 1971), clover yellow mosaic virus (Bos, 1973) and viola mottle virus (Lisa and Dellavalle, 1977). We also examined two tentative members whose structures conform to that of definitive potexviruses, namely, barrel cactus virus (Attathom et al., 1978) and foxtail mosaic virus (Paulson and Niblett, 1977).

During the course of this work, the translation in vitro of potato virus X (Ricciardi et al., 1978; Wodnar-Filipowitz et al., 1980) was reported. These author's results implied that the translational strategy of PVX resembled that of tobacco mosaic virus in that coat protein was not produced in vitro from the genomic RNA. We have undertaken a more definitive study of the molecular biology of the potexvirus group with the objective of determining whether morphological relatedness is in fact a reliable indication of the functional behaviour of the corresponding viral RNAs.

CHAPTER 2 - TRANSLATION OF POTEXVIRAL RNA IN VITRO

2.1 Introduction

Potato virus X (PVX) the type member of the potexvirus group contains a single genomic RNA which fails to direct the synthesis of coat protein upon translation in vitro (Ricciardi et. al., 1978; Wodnar-Filipowitz et. al., 1980). In this regard, it resembles the behaviour of TMV RNA in vitro (Hunter et. al., 1976). To determine whether this observation is characteristic of all members of the potexvirus group, we have subjected to in vitro translation the RNAs extracted from three definitive potexviral members, namely, papaya mosaic (PMV), clover yellow mosaic (CYMV) and viola mottle viruses (VMV) and two tentative members, namely, barrel cactus (BCV) and foxtail mosaic (FMV) viruses. This chapter presents evidence that of the RNAs tested only PMV and CYMV are capable of directing the synthesis of a protein in vitro which is similar if not identical to native coat protein.

2.2 Materials and Methods

2.2.1 Chemicals

All chemicals were of reagent grade. Components of the in vitro translation systems such as creatine phosphate, creatine phosphokinase and

micrococcal nuclease were obtained from Boehringer-Mannheim (Canada). Human placental RNAse inhibitor was from Biotec Inc. Staphylococcus aureus V8 protease was obtained from Sigma and trypsin (TPCK-treated) was from Worthington. Avian myeloblastosis virus reverse transcriptase was a kind gift of Dr. James Beard. ^{35}S-methionine was New England Nuclear's "translation grade" as other grades and sources were less consistent and occasionally inhibitory. All electrophoretic reagents were from Bio-Rad except that methylmercury (II) hydroxide (1 M in water) was from Alfa Chemicals, Inc.

2.2.2 Purification of Viruses

Papaya mosaic virus was propagated in greenhouse-grown papaya trees (Carica papaya L.) and purified by a modification (Dr. J. Erickson, personal communication) of the procedure of Erickson and Bancroft, 1978. Virus-infected leaf tissue was homogenized at 4°C in a Waring ® blender in 2 volumes of 0.05 M sodium phosphate, 0.01 M EDTA, pH 8.0. Ten percent Triton X-100 was then slowly added to a final concentration of 1% (v/v) and homogenization continued for 2 minutes. To clarify the tissue homogenate, one-half volume of butanol: chloroform (1:1) was added, then homogenization continued for an additional 2 minutes. Subsequently, the homogenate was centrifuged at 10,000 rpm for 10 minutes at 4°C in a Sorvall ® GSA rotor. The

resultant supernatant was again centrifuged at 17,000 rpm for 30 minutes at 4°C in a Sorvall ® SS34 rotor. The virus was then precipitated from this clarified tissue homogenate by addition of solid polyethylene glycol 6000 to 1% (w/v), with slow stirring at 4°C for 2 hours. The viral precipitate was collected by centrifugation in a Sorvall ® SS34 rotor at 5000 rpm for 10 minutes at 4°C, and resuspended in 5 mM Tris-HCl, 5 mM EDTA, pH 8.0. Further purification was achieved by differential centrifugation (Erickson and Bancroft, 1978). The yield of PMV was estimated assuming $E^{0.1\%}_{260\ nm} = 2.85$ (Hiebert, 1970).

Clover yellow mosaic virus (CYMV) was grown in broad bean (Vicia faba) for 14 days prior to purification from infected leaf tissue exactly as described (Bancroft et. al., 1979), using 0.02 M Borate, pH 8.2 containing 0.5% (w/v) Na_2SO_3 and 0.01 M ascorbate as the homogenization buffer. CYMV was quantitated assuming $E^{0.1\%}_{260\ nm} = 3.1$ (Purcifull and Shephard, 1964). Samples of viola mottle virus, barrel cactus virus and foxtail mosaic virus were kindly provided by Dr. J. Bancroft.

The coat proteins of PMV and CYMV were extracted using 67% (v/v) acetic acid (Erickson and Bancroft, 1978) and 2 M LiCl, pH 8.0 (Bancroft et. al.,

1979) respectively as described. Protein purity in each case was monitored by SDS-polyacrylamide gel electrophoresis. (Refer to Section 2.2.6).

2.2.3 Purification and Analysis of Viral RNA

Viral RNA was extracted from all viruses used in this study with 2.5 M guanidine-HCl, 5 x 10^{-4}M EDTA, pH 8.0 at 4°C (Erickson and Bancroft, 1978; Reichmann and Stace-Smith, 1959) and quantitated assuming $E^{0.1\%}_{260\ nm} = 25.0$.

The integrity of the extracted RNA was judged by electrophoresis in 1% (w/v) agarose gels containing 10 mM methylmercury hydroxide (Bailey and Davidson, 1976) as a denaturing agent. Electrophoresis was performed at 0.75 V/cm for 17 hours at 25°C. To detect the RNA, the gel was first washed three times with 5 mM β-mercaptoethanol (20 minutes each) to remove the methylmercury from the gel, then stained with 1 μg/ml ethidium bromide in 5 mM β-mercaptoethanol for 20 minutes (Setzer et al., 1980) prior to visualization under U.V. light at 302 n.m.

Alternatively, some RNA samples were denatured by incubation for 60 minutes at 50°C in 50% DMSO, 1 M deionized glyoxal and 10 mM sodium phosphate, pH 7.0 (McMaster and Carmichael, 1977), prior to electrophoresis in 1% (w/v) agarose gels under conditions described by Thomas (1980). In this case, RNA

was detected by staining the gel with 30 μg/ml acridine orange in 10 mM sodium phosphate, pH 7.0 (McMaster and Carmichael, 1977) followed by destaining with 1×10^{-3} M EDTA, pH 8.0 prior to visualization under U.V. light at 267 n.m.

RNA samples which showed a major single band of molecular weight equal to 2.2×10^6 daltons with minimal smearing as judged against molecular size markers were deemed sufficiently pure to use in these studies.

2.2.4 Preparation of wheat germ cell-free extracts

Commercial untreated wheat germ was obtained from General Mills, Inc., Vallejo, California, and stored at 4°C *in vacuo*. An S30 extract was prepared with modifications of the protocol of Roberts and Patterson (1973) as follows: 12 g of wheat germ was ground to a fine powder with 6 g of sterile sea sand in a chilled (4°C) mortar. Grinding was then continued after addition of 28 ml of 1 mM magnesium acetate, 2 mM calcium chloride and 90 mM potassium acetate, until none of the extract stuck to the pestle. The extract was then centrifuged in a Sorvall ® SS34 rotor at 30,000 x g for 10 minutes at 4°C. The supernatant was collected and adjusted to 20 mM in Hepes-KOH (pH 7.6), 3.5 mM in magnesium acetate and 2 mM in dithiothreitol and again centrifuged at 30,000 x g as above.

The resultant supernatant was supplemented with ATP to 1 mM, GTP to 0.02 mM, creatine phosphate to 40 µg/ml, phosphocreatine to 8 mM and dithiothreitol to 2 mM then incubated at 30°C for 10 minutes. Following incubation, the extract was passed through a (1.5 cm x 30 cm) column of Sephadex ® G-25 (medium) equilibrated in 20 mM Hepes-KOH, pH 7.6, 120 mM potassium acetate, 5 mM magnesium acetate and 2 mM dithiothreitol at 4°C. Only the first half of the turbid fractions was collected (Efstratiadis and Kafatos, 1976a), pooled and stored in aliquots at -70°C until use. Such extracts had an A280/A260 of 0.6

Cell-free protein synthesis was carried out in a volume of 0.05 ml with the following components: 15-20 µl of wheat germ S30, 20 mM Hepes-KOH, pH 7.6, 2 mM dithiothreitol, 1 mM ATP, 0.1 mM GTP, 8 mM phosphocreatine, 40 µg/ml creatine phosphokinase, 30 µM of each of the 19 unlabelled amino acids and one labelled amino acid, either ^{14}C-leucine or ^{35}S-methionine at 5-10 µM. The concentrations of potassium acetate and polyamines spermine and spermidine which provided maximal stimulation of incorporation were 135 mM, 6 µM and 100 µM respectively. Samples were incubated for 90 minutes at 25°C. Incorporation of labelled amino acid into protein was monitored by precipitating aliquots of the reaction with 10% (w/v) trichloroacetic acid in the presence of carrier bovine

serum albumin. Subsequently, precipitates were heated to 100°C for 15 minutes to hydrolyze charged tRNA molecules. The precipitates were collected by filtration on glass fiber filters soaked in 5% (w/v) trichloroacetic acid and 0.1% (w/v) casamino acids. These filters were washed extensively under gentle vacuum with 5% (w/v) trichloroacetic acid containing 0.1% casamino acids followed by a quick wash with acetone. Dried filters were counted in 3 mls of 0.4% (w/v) Omnifluor (NEN) in toluene.

2.2.5 Preparation of the rabbit reticulocyte lysates and use in in vitro protein synthesis

3 Kg New Zealand white rabbits were made anaemic by five daily subcutaneous injections with 2.5 mls of freshly prepared 1.25% (w/v) acetylphenylhydrazine (Hunt and Jackson, 1974). To quantitate the percentage of reticulocytes, a sample of blood was taken from the ear vein on the eighth or nineth day post-primary injection and stained with New methylene blue (Singh Ranu and London, 1979). Routinely, by the eighth day, reticulocytes comprised 70-80% of the total stainable blood cells as judged by light microscopy under an oil immersion objective. Blood cells were collected by cardiac puncture and washed

extensively with 140 mM NaCl, 5 mM KCl, and 1.5 mM magnesium acetate at 4°C prior to lysis in water as described by Villa-Komaroff et al., 1974.

Lysates were rendered dependent on added mRNA by treatment with micrococcal nuclease for 10 minutes at 20°C in the presence of 1 mM $CaCl_2$. Subsequently, the nuclease activity was inhibited by the addition of EGTA to 2 mM (Pelham and Jackson, 1976). The amount of nuclease used was determined for each preparation and was the amount which gave maximal stimulation of incorporation over background dependent on exogenous RNA.

Cell-free protein synthesis was carried out at 30°C for 60 minutes in volumes of 0.05 ml containing the following components: 0.03 ml of micrococcal nuclease-treated reticulocyte lysate, 20 mM Hepes-KOH, pH 7.6, 2 mM dithiothreitol, 25 μM hemin, 30 μM of each of 19 unlabelled amino acids, 10-25 μCi of ^{35}S-methionine, 8 mM phosphocreatine, 10 μg/ml creatine phosphokinase, 110 mM potassium acetate and magnesium acetate as described in the text. Incorporation of radioactivity into acid precipitable material was monitored as described (Pelham and Jackson, 1976).

2.2.6 Electrophoresis of Proteins

Samples from in vitro translation reactions intended for electrophoretic analysis were diluted threefold in sterile water and precipitated at -20°C with 5 volumes of acetone. The precipitated protein was collected by centrifugation, dried, and suspended in 0.05 ml (or less) SDS sample buffer (Laemmli and Favre, 1973). Samples were boiled for 2 minutes, cooled, and loaded onto SDS-polyacrylamide slab gels which were prepared and run as described (Laemmli and Favre, 1973). At the end of the run, gels were processed for fluorography (Bonner and Laskey, 1974), dried, and exposed with Dupont Cronex 4 ® or Kodak X-Omat AR ® X-ray film at -70°C for the detection of radioactive products.

Protein standards used in molecular weight determination were iodinated using the chloramine-T method of Hunter and Greenwood (1962). Iodinated protein markers had identical electrophoretic mobilities to those of their unmodified counterparts.

2.2.7 Preparation of Antisera to Viral Coat Protein

Antibodies against PMV were raised by injecting rabbits intramuscularly with 8 mg of PMV emulsified in Freund's incomplete adjuvant. At weekly intervals thereafter, 1 mg of PMV was injected intravenously. Collection of blood by cardiac puncture

and preparation of antiserum took place 6 weeks after the primary immunization. Monospecific antisera were prepared by affinity chromatography. 5 ml of crude antisera was incubated for 12 hours at 4°C within a 10 ml column of Ultragel ® AcA22 to which PMV coat protein (2 mg protein/ml gel support) had been attached with glutaraldehyde (Ternynck and Avrameas, 1976). Unbound material was washed from the column with phosphate buffered saline until the eluant had an A_{280} < 0.02. Subsequently the antibody fraction bound to PMV coat protein was eluted with 0.2 M glycine-HCl, pH 2.2, then neutralized immediately with 1 M K_2HPO_4. After elution the fractionated antiserum was dialyzed against phosphate buffered saline (PBS) and stored at 4°C. From 5 ml of crude antisera, 2 mg of monospecific antibody was obtained. The specificity of each antiserum or antibody fraction was tested by the Ouchterlony double diffusion method against several viral antigens as well as cell-free extracts. Immunoprecipitin lines were observed only when PMV or PMV coat protein were used as antigens.

2.2.8 Growth and Preparation of Staphylococcus aureus Immunoadsorbant

Staphylococcus aureus, strain Cowan I (ATCC 12598) was grown at 37°C in the Woodin modification of CCY media (Arvidson et al., 1971) composed of the

following components per liter of solution: 10 g yeast extract, 40 g casamino acids, 20 g β-glycerophosphate, 8.3 mls of 60% sodium lactate, 0.8 g Na_2HPO_4, 0.4 g KH_2PO_4, 1.0 g $(NH_4)_2SO_4$, 0.08 g l-tryptophan, 0.10 g l-cystine, 0.002 g thiamine, 0.004 g nicotinic acid, 0.02 g $MgSO_4 \cdot 7H_2O$, 0.0075 g $MnSO_4 \cdot H_2O$, 0.006 g $FeSO_4 \cdot 7H_2O$ and 0.006 g citric acid.

Fixation and inactivation of Staphylococcus aureus for use as an immunoadsorbant followed Kessler's (1975) procedure. Overnight cultures of cells were harvested and washed twice in PBS-azide (150 mM NaCl, 40 mM sodium phosphate, pH 7.2, containing 0.05% (w/v) NaN_3) at 4°C in a Sorvall ® GSA rotor at 7000 rpm. Subsequently the cells were adjusted to a 10% (w/v) suspension in PBS-azide containing 1.5% (v/v) formaldehyde, then stirred at room temperature for 90 minutes. Cells were then washed twice in PBS-azide and readjusted to a 10% (w/v) suspension in PBS-azide prior to incubation at 80°C for 5 minutes followed by quick cooling in an ice water bath. Finally, cells were washed twice again in PBS-azide and stored as 10% (w/v) suspensions at -70°C in one ml aliquots.

Prior to use in immunoprecipitation reactions an aliquot of cells was thawed, diluted 10-fold in RIA buffer (0.1 M NaCl, 10 mM sodium phosphate, pH 7.4, 1% (v/v) Triton X-100, 0.5% (w/v) deoxycholate, and 0.1% (w/v) SDS (Philipson et al., 1978)) then incubated at

room temperature for 30 minutes followed by 4°C for 60 minutes. Cells were then recovered by centrifugation in a Sorvall ® SS34 rotor at 4000 rpm for 20 minutes. The cell pellet was then washed three times in RIA buffer with centrifugation as above. The final pellet was resuspended in RIA buffer to the original volume. These washes serve to remove any free protein A.

2.2.9 Immunoprecipitation

We used the procedure of Philipson et al. (1978) for indirect immunoprecipitation of products synthesized in vitro. 0.01 ml aliquot of the cell-free reaction mixture (from either the wheat germ or reticulocyte systems) was incubated first for 30 minutes at 37° C, then for 12-16 hours at 4° C with either 1 µl of crude antisera or 1 µg of affinity column purified antibody in 0.10 ml of RIA buffer. A 10% (w/v) suspension of Staphylococcus aureus (0.01 ml) was then added to the soluble antigen-antibody complexes and adsorption allowed to occur for one hour at 4° C. Complexes bound to Staphylococcus aureus cells were then recovered by centrifugation and washed three times in RIA buffer prior to resuspension in 0.05 ml of SDS-sample buffer (Laemmli and Favre, 1973). When using crude antisera, it was sometimes necessary to execute this procedure twice, first with pre-immune sera to rid

the reaction of components which bound non-specifically to the Staphylococcus adsorbant, followed by addition of the immune antisera to be tested.

2.2.10 Tryptic Peptide Maps

In vitro products of interest were cut from dried, fluorographed gels using an exposed x-ray film as a guide. Gel slices were swollen in water, then sequentially treated with 60 minute dimethylsulfoxide washes (3 times), followed by two 30 minute methanol washes, then two 30 minute washes with methanol: water (1:1). Gel slices were finally resuspended in 1% (w/v) ammonium bicarbonate containing 150 µg of trypsin (Worthington, TPCK-treated trypsin). Digestion proceeded for 24 hours at 37° C. The aqueous material was saved and the gel slices re-digested in fresh 1% (w/v) ammonium bicarbonate containing 75 µg of trypsin. Digestion was allowed to proceed for an additional 18 hours. The digested eluates were then pooled and lyophilized. Peptides so recovered were performic acid oxidized (Weber et al., 1972) for one hour at 4° C, then diluted with water and re-lyophilized. Peptides were then resuspended in water and applied in bands 2 cm wide and 15 cm from the bottom of a sheet of Whatman 3 MM® paper. The paper was then subjected to

electrophoresis in pyridine: acetate pH 3.6 for 3000 V hours (approximately 30 V/cm). After electrophoresis the paper was dried and autoradiographed.

2.2.11 Peptide Mapping Using Partial Proteolysis

Partial proteolytic digestion of products synthesized in the cell-free systems was performed using Staphylococcus aureus V8 protease as described by Cleveland et. al. (1977).

2.2.12 Extraction and Analysis of RNA from Virus Infected Plants

Total RNA was extracted 6 days after infection (mid-exponential phase) of broad bean plants. Leaves were washed and ground to a fine powder under liquid nitrogen prior to extraction at 65° C with buffer-saturated phenol as described by Hunter et al. (1976). 200 µg of total extractable RNA was heated at 70° C for 3 minutes and quick cooled in an ice-water bath prior to fractionation on 5-20% sucrose gradients in 10 mM Tris-HCl pH 7.4, 1 mM EDTA, and 0.5% SDS. Gradients were centrifuged at 97,500 x g for 18 hours at 24° C in an SW 40 rotor. Gradient fractions were ethanol precipitated and the precipitates washed extensively prior to final resuspension in water.

2.2.13 Synthesis of ^{32}P-DNA probes complementary to viral RNA sequences

A representative population of ^{32}P-labelled cDNA molecules with molecular size of approximately 400 bases was transcribed from viral RNA templates using purified avian myeloblastosis virus (AMV) reverse transcriptase in the presence of oligo-deoxyribonucleotide primers of random nucleotide sequence generated by DNAse digestion of calf thymus DNA (Taylor et al., 1976). The reverse transcription reaction was carried out at 37° C for 120 minutes in a reaction volume of 0.05 ml containing the following components: 50 mM Tris-HCl, pH 8.3, 8 mM $MgCl_2$, 8 mM DTT, 100 µg/ml Actinomycin D, 0.67 mM each of dATP, dTTP, and dGTP, α-^{32}P-dCTP (0.1 mM), 60 µg calf thymus DNA primer, 2 µg heat denatured viral RNA and AMV reverse transcriptase at 234 U/ml. To terminate the reaction and hydrolyze the RNA template, the reaction was made 0.6 M in NaOH, then incubated at 37° C for 3 hours. After this time, the reaction was neutralized and loaded onto a Bio-gel ® A 1.5 m column (0.9 x 30 cm) equilibrated in 10 mM Tris-HCl, 1 mM EDTA, pH 7.5. Fractions of approximately 300 µl were collected and radioactivity detected by Cerenkov radiation. Fractions comprising the void volume were pooled, made 0.2 M in NaCl then precipitated with 2.5 volumes of 95% (v/v) ethanol at -20° C.

2.2.14 Northern Transfer and Hybridization

RNA was separated by electrophoresis in submerged 1% agarose gels containing 10 mM methylmercury hydroxide (Bailey and Davidson, 1976). Gels were run at room temperature for 17 hours at 40 V. After electrophoresis gels were processed according to Setzer et al. (1980) and RNA transferred to Ultra-Blot ® (Collaborative Research) using procedures described by Alwine et al. (1979). Conditions of hybridization with CYMV cDNA and processing for autoradiography were as described (Coulter-Mackie et al., 1980).

2.2.15 RNA-cDNA Hybridization

RNA-cDNA hybrids were formed essentially as described by Varmus et al. (1973). Reaction mixtures of 50 µl were prepared on ice in siliconized Eppendorf micro test-tubes and contained 50 mM Tris-HCl, pH 7.4, 2 mM EDTA, 10 µg/ml of each RNA sample, 1 mg/ml yeast RNA, approximately 10,000 cpm ^{32}P-labelled cDNA and 0.6 M or 0.2 M NaCl as described in the results (section 2.3.8). Once assembled, reactions were heated to 100° C for 3 minutes then quick-cooled in an ice water bath. Each reaction was overlayed with mineral oil then incubated for 22 hrs at 68° C. Samples were hybridized to a Rot of 13.5 mole. sec/l as calculated by the following formula:

$$\text{Rot} = \gamma \times \text{0.D. of 1 } \mu g \text{ RNA} \times \frac{\text{mass RNA } \mu g}{\text{volume of reaction (ml)}} \times \frac{\text{time (hrs)}}{2} = \text{mole. sec/liter.}$$

γ = relative reassociation constant which equals 4.9 at 0.6 M NaCl and 1.4 at 0.2 M NaCl (Britten et al. 1974).

At the end of this time each reaction was added to 2 ml of S1 buffer containing 30 mM sodium acetate, pH 4.5, 0.3 M NaCl, 3 mM $ZnCl_2$ and 10 μg/ml sheared, denatured calf thymus DNA. The reactions were then divided into two equal portions. To one was added 500 units of S1 nuclease and to the other an equivalent volume of S1 nuclease dilution buffer. Both samples were incubated at 50° C for 2 hours prior to TCA precipitation and collection of precipitate as glass fiber filters.

The estimated percentage sequence homology was calculated as described by Palukaitis and Symons (1980) using the following formula which corrects for the S1 nuclease resistance of the cDNA alone. The corrected percentage S1 nuclease resistance

$$= 100 \times \frac{\text{S1 nuclease resistance of hybrid (\%)} - \text{S1 nuclease resistance of cDNA (\%)}}{100 - \text{S1 nuclease resistance of cDNA (\%)}}$$

The estimated percentage sequence homology was then calculated by dividing corrected % S1 nuclease resistance of a heterologous hybrid by the corrected % S1 nuclease resistance of the homologous hybrid and multiplying by 100. (Palukaitis and Symons, 1980).

2.2.16 Preparation of ^{125}I-labelled RNA and Use in Hybridization

CYMV RNA was iodinated using modifications of the procedure of Efstratiadis et al., 1975. Briefly, a 0.1 ml reaction was assembled on ice and contained the following components added in the order given: 340 µCi $Na^{125}I$ (NEN, NEZ 033A, 100 mCi/ml), 2.8×10^{-3} N HCl, 0.1 M NaOAc, pH 5.0, 6.24×10^{-5} M KI, 1 µg CYMV RNA and 2.3×10^{-3} M $TlCl_3$. The assembled reaction was then incubated at 60° C for 20 minutes, then transferred to an eppendorf micro test-tube containing 0.5 ml of 0.54 M Pipes-NaOH, pH 6.8, 0.95 M NaCl and 3 µl of 14 M β-mercaptoethanol. The sample was then incubated at 60° C for a further 30 minutes. The iodinated RNA was then separated from free isotope and salts on a Bio-Gel ® A150 m column (0.9 x 25 cm) equilibrated with water. 0.4 ml fractions were collected and the radioactivity in each determined by counting a 0.01 ml aliquot in 3 mls of ACS ® (Amersham). The fractions corresponding to the iodinated RNA were pooled, made 0.2 M NaCl then precipitated with ethanol.

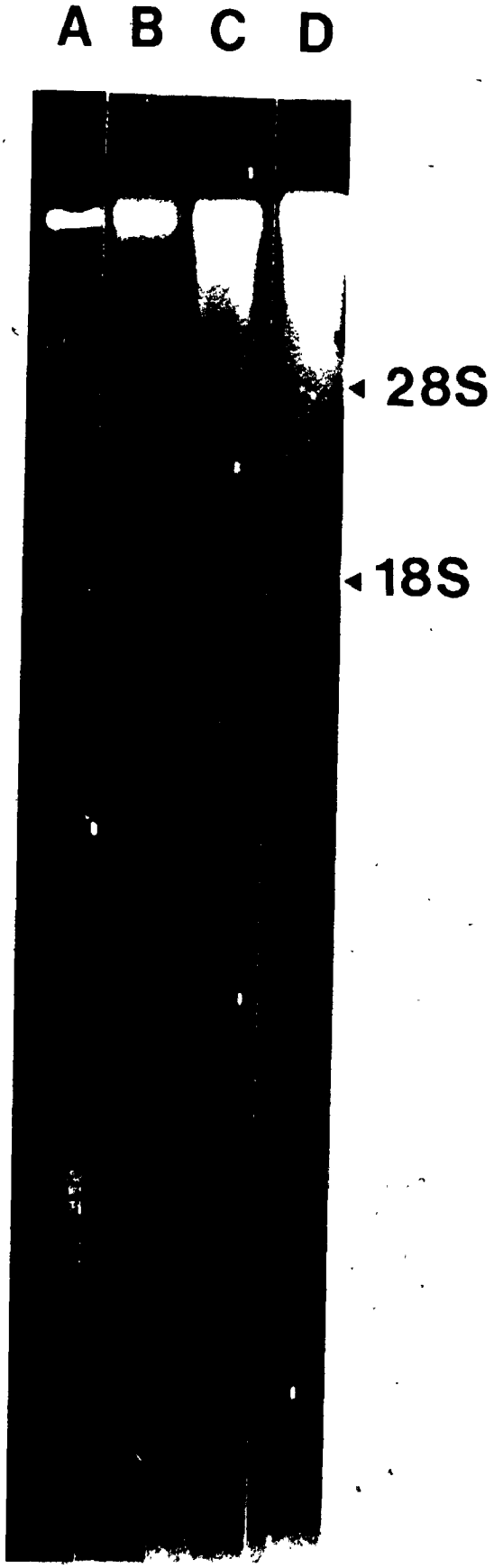
^{125}I-CYMV RNA (4000 cpm) was added to 0.05 ml reactions containing serial dilutions of unlabelled cDNA (in excess) which was prepared by reverse transcription in the presence of calf thymus DNA primers using the same RNA template that was used in the preparation of the ^{125}I-RNA. Hybridization was performed using the conditions described in Section 2.2.15. After formation of hybrids, the hybridization reaction was diluted into 0.5 ml of S1 buffer containing 20 µg/ml yeast RNA rather than calf thymus DNA. The reaction mixture was then divided into two equal reactions. Duplicate reactions were then incubated with and without 10 units of ribonuclease T1 and 2 µg ribonuclease A. Samples were incubated at 37° C for 1 hour. Samples were then TCA precipitated, filtered, counted and the percent hybrid remaining calculated from a comparison of the duplicates.

2.3 Results

2.3.1 Potexviral RNA consists of a single component

The genomic RNA of potexviruses used in this study has previously been reported to consist of a single molecule of approximately 2.2 x 10^6 molecular weight (refer to Table 1) corresponding to 6800 nucleotides (Purcifull and Hiebert, 1971; Abouhaidar and Bancroft, 1978). To test whether these genomes contained

Figure 2 Electrophoretic analysis of Potexvirus RNA under denaturing conditions. 10 µg of each potexviral RNA was subjected to electrophoresis on 1% (w/v) agarose gels containing 10 mM methylmercury hydroxide as described in Materials and Methods, Section 2.2.3. Lanes correspond to a) CYMV RNA, b) PMV RNA, c) VMV RNA and d) BCV RNA.


A B C D
28S
18S

LMC RNAs as has been reported in other viral systems (Klein et al., 1976; Hunter et al. 1976), we have analyzed purified RNA extracted from virions (Materials and Methods, Section 2.2.3) on 1% (w/v) agarose gels containing 10 mM methylmercury hydroxide. Figure 2 illustrates that under denaturing conditions the RNAs extracted from CYMV (lane a), PMV (lane b), VMV (lane c) and BCV (lane d) consisted of only one component of approximately 2.2×10^6 daltons as visualized by staining with ethidium bromide. The sensitivity of this method is sufficient to permit detection of 0.1 moles of an LMC component per mole of full length RNA, assuming complete denaturation. This result was confirmed in the case of CYMV RNA by using a more sensitive means of detection referred to as Northern transfer (refer to Materials and Methods, Section 2.2.14 and Figure 11). Similarly, the high molecular weight RNA of PMV was the only component which could be ^{32}P-end labelled with T4-induced polynucleotide kinase (Efstratiadis et al., 1977) (data not shown). Furthermore, translation of high molecular weight PMV RNA which was purified on a 6 - 20% sucrose gradient after denaturation with 10 mM methylmercury hydroxide resulted in the same in vitro products (discussed in Section 2.3.2) as unfractionated RNA purified from virions (data not shown). Finally, an analysis of viral particles for all viruses tested in extracts of their respective host cells by electron

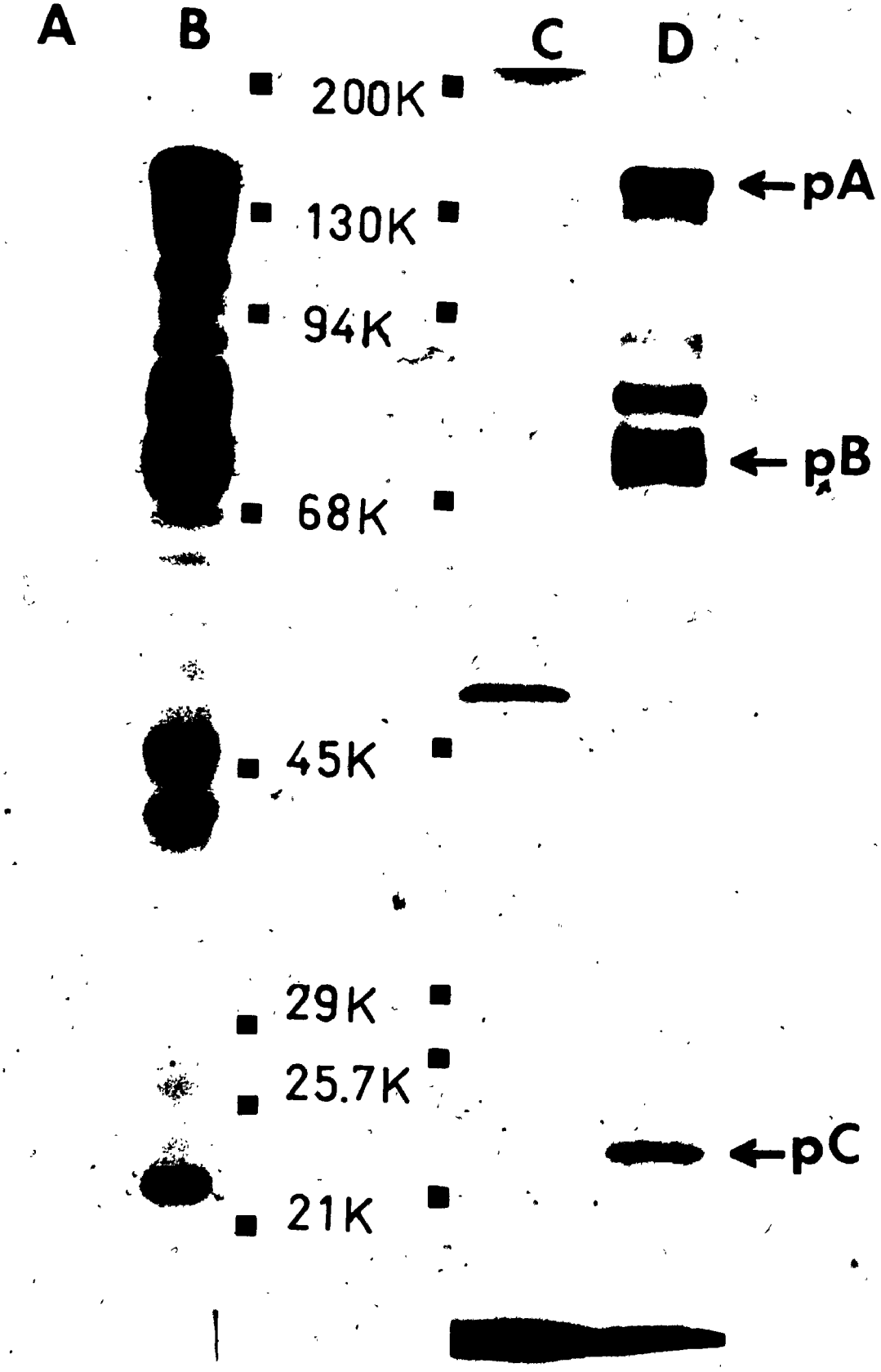
microscopy, comparable to the method successfully applied to tobacco mosaic virus (Whitfield and Higgins, 1976) has not uncovered short rod-like particles suggestive of encapsidated LMC RNAs (Dr. J.B. Bancroft, personal communication). Thus within the limits of detection employed, PMV, CYMV, BCV and VMV give no indication of containing multiple components.

2.3.2 Template Properties of PMV RNA

In either the wheat germ extract (Roberts and Patterson, 1973) or the rabbit reticulocyte lysate treated with micrococcal nuclease (Pelham and Jackson, 1976), (See also Section 2.2.5), PMV RNA directs the synthesis of a variety of products whose apparent molecular weights range from 22,000 to 155,000 daltons (Figures 3b and 3d). Three prominent products are reproducibly synthesized in both systems and exhibit electrophoretic mobilities relative to iodinated marker proteins corresponding to molecular weights of 155,000 (pA), 73,000 (pB), and 22,000 (pC). In addition, there are numerous minor products, particularly among the products synthesized in the wheat germ extract (Figure 3b). These minor products probably represent prematurely terminated polypeptides ("early quitters"). Multiple products seem to be a common feature of PMV RNA directed translation as well as that of related viral templates. Another viral RNA, cowpea chlorotic mottle virus RNA

Figure 3 Electrophoretic separation of products synthesized in vitro with PMV RNA as template.

A wheat germ extract (lanes a and b) was programmed with no added RNA (lane a) or with 5 μg purified PMV RNA under the optimal conditions described in the text and in Materials and Methods. Aliquots of the completed reaction were resolved by electrophoresis in a 10% SDS-polyacrylamide slab gel. Iodinated protein standards were included in adjacent lanes for molecular weight determination. These included myosin (200,000 molecular weight), β-galactosidase (130,000), phosphorylase a (90,000), bovine serum albumin (68,000), ovalbumin (45,000), carbonic anhydrase (29,000), α-chymotrypsinogen (25,700), and soybean trypsin inhibitor (21,000). Their positions are noted in the central margins, as they required different exposure time for visualization. Lanes c (no added RNA) and d (2 μg purified PMV RNA) illustrate products synthesized in the rabbit reticulocyte system and separated similarly. pA, pB, and pC are prominent products found reproducibly in both in vitro systems.


A
B
C
D
200K
130K
94K
68K
45K
29K
25.7K
21K
pA
pB
pC

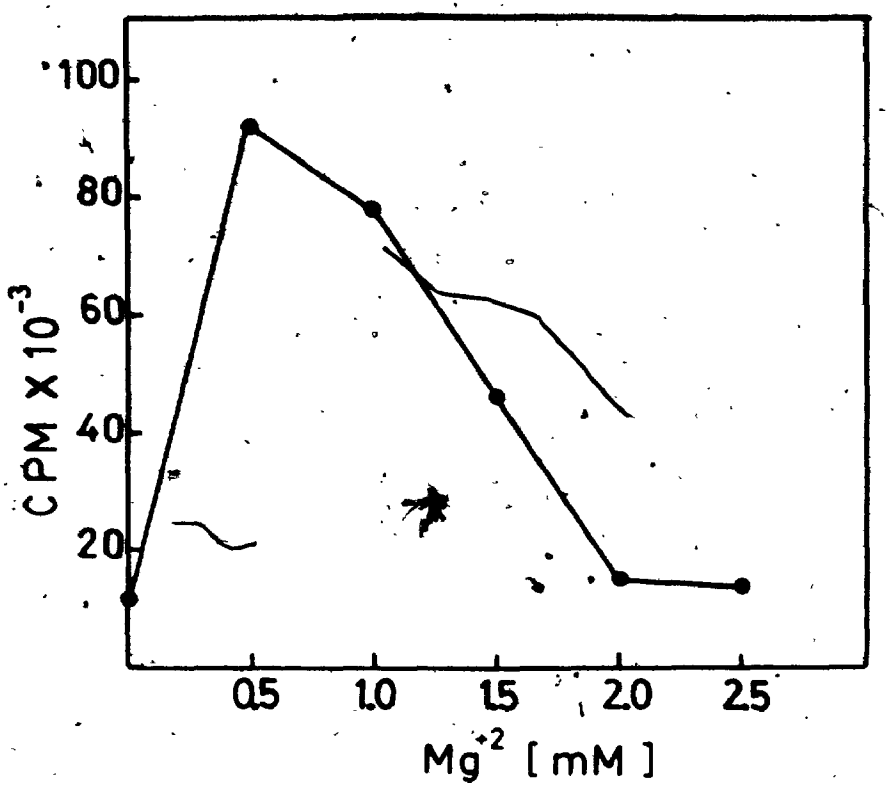
component 4 (0.32×10^6 daltons) directs the synthesis of a discrete product of apparent molecular weight 19,000 daltons in the same extracts used here, in agreement with Davies and Kaesberg (1974) (data not shown). Also, the inclusion of human placental ribonuclease inhibitor in either in vitro translation system did not alter the proportion or size of the PMV RNA directed products. Therefore, multiple products reflect a property of the PMV template and not the in vitro translation systems.

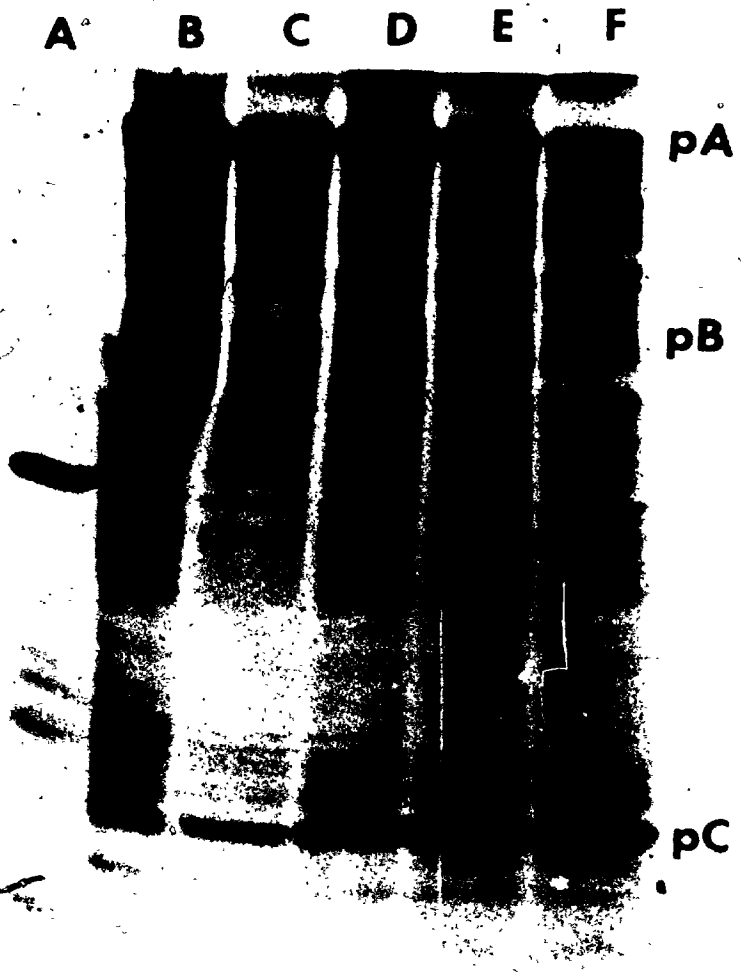
When all the components of the wheat germ system are optimized, the incorporation of ^{14}C-leucine into acid precipitable material was stimulated 90 - 120 times over the background by the addition of PMV RNA. This corresponds to almost 200 moles of leucine per mole of RNA. Assuming leucine to comprise 10% of the amino acid residues in the products, the equivalent of one complete round of translation of each template molecule occurs during a 90 minute incubation. This efficiency is comparable to that reported for TMV RNA (Roberts and Patterson, 1973; Marcu and Dudock, 1974; and our own measurements) and substantially greater than that of PVX RNA, the type number of the potexviruses (Ricciardi et al., 1978 and our own results).

Several parameters of the two in vitro translation systems were varied primarily to optimize the conditions of translation of PMV RNA and secondarily

Figure 4 Effect of Mg^{+2} concentration on overall incorporation and on the synthesis of individual products in a rabbit reticulocyte lysate programmed with PMV RNA.

The left hand panel illustrates the overall incorporation of ^{35}S-methionine into acid-precipitable material as a function of added Mg-acetate (final concentration in millimoles/1) in a reaction of 0.05 ml incubated 60 min at 30°. In the right panel, aliquots of the same reactions adjusted to contain nearly the same amounts of acid-precipitable radioactivity were resolved on a 12.5% SDS-polyacrylamide slab gel. The samples are: (a) no added RNA at 0.5 mM Mg-acetate final concentration: (b-f) 2.0 g PMV RNA at the following final concentrations of Mg-acetate: (b) 0.5 mM, (c) 1.0 mM, (d) 1.5 mM, (e) 2.0 mM, and (f) 2.5 mM.


100
80
60
40
20
CPM X 10^{-3}
0.5
1.0
1.5
2.0
2.5
Mg^{+2} [mM]


A
B
C
D
E
F
pA
pB
pC

to select conditions which would allow for the maximal synthesis of the putative viral coat protein, pC. In either system, gel electrophoresis revealed that manipulating the concentrations of potassium, spermine and spermidine caused no qualitative alterations in the spectrum of polypeptides synthesized.

A dramatic difference is found between the two cell-free systems in regard to the effect of magnesium ion concentration on translation. In the wheat germ extract the efficiency of synthesis is maximal at 2.5 mM magnesium acetate. At optimal and supraoptimal levels of magnesium ion the ratios of the radioactivity in products pA:pB:pC is relatively stable at 3:4:1, respectively. At suboptimal concentrations there is a slight increase in the synthesis of product pA but a twofold reduction in product pB resulting in a ratio of 3.5:2:1. In the reticulocyte lysate the pattern is altered dramatically with increasing magnesium ion. Optimal stimulation of incorporation is achieved in our system at 0.5 mM magnesium acetate (Figure 4a). At this concentration, the relative incorporation of label into product pA is maximum. As the magnesium ion concentration increases beyond the optimal level the synthesis of product pA decreases. Concommitantly, there is a striking enhancement of the incorporation of label into product pC (Figure 4b). The correct choice of divalent cation concentration therefore appears to be

critical for the complete synthesis of all PMV-directed polypeptides. This effect may be due to alteration of the RNA structure and/or accessibility of the RNA to various translational components.

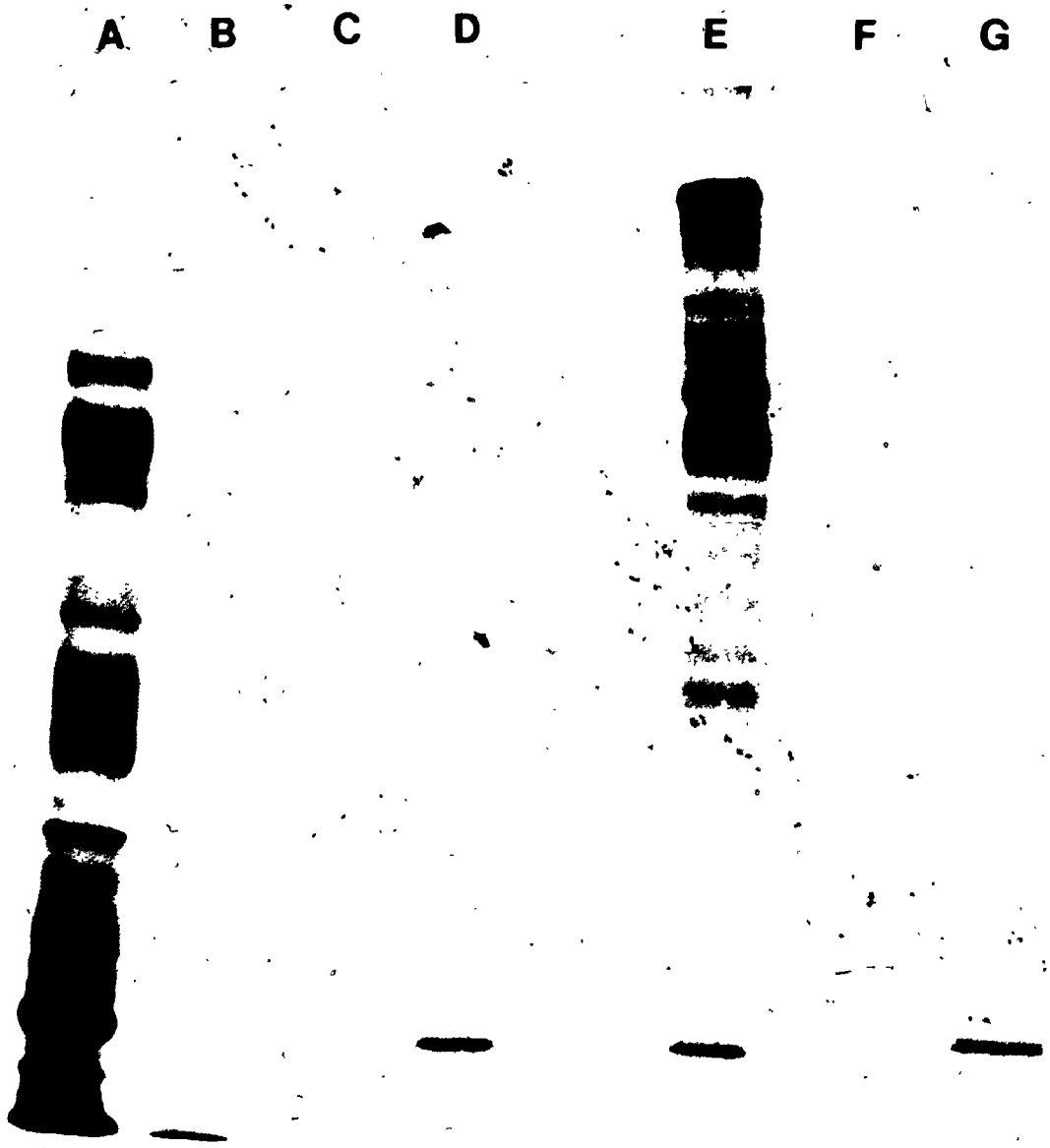
Once the ionic optima for the translation systems had been determined, the concentration of PMV RNA which stimulated maximal incorporation was assessed. In the wheat germ system, maximal incorporation of labelled amino acid into protein was achieved at 0.14 mg/ml. The efficiency of incorporation (moles amino acid incorporated/mole input RNA), however, was best at 0.10 mg/ml and this concentration was used subsequently. With the reticulocyte lysate, incorporation was maximal at 0.04 mg/ml. In both cell-free systems, the synthesis of minor products increased substantially at RNA concentrations beyond that which gave maximal efficiency of incorporation. Changing the RNA concentration did not alter the ionic optima.

2.3.3 Identification of the in vitro polypeptide pC as PMV coat protein

In general, the coat protein of plant viruses is the only protein in the virus particle and presumably, a major translation product in vivo. Unlike both TMV RNA (Hunter et al., 1976) and PVX RNA (Ricciardi et al., 1978), PMV RNA can direct the synthesis in vitro of a polypeptide, pC, whose

Figure 5 Indirect immunoprecipitation of products synthesized in two cell-free systems programmed with PMV RNA.

Either the wheat germ (lanes a - d) or rabbit reticulocyte (lanes e - g) cell-free systems were programmed with PMV RNA as described in the text. Aliquots of each reaction were incubated with one of the following: Preimmune serum (lanes b and f), unfractionated serum raised against PMV (lanes c and g) or with anti-PMV coat protein IgG purified by affinity chromatography (see Materials and Methods) in lane d. Lanes a and e show the unfractionated mixtures of products synthesized in the wheat germ and rabbit reticulocyte cell-free systems respectively. The conditions of incubation and the subsequent precipitation of the antibody-antigen complexes with S. aureus are described in Materials and Methods, Section 2.2.9. The immunoprecipitates were applied to a 10% SDS-polyacrylamide slab gel and were ultimately visualized by fluorography.


A
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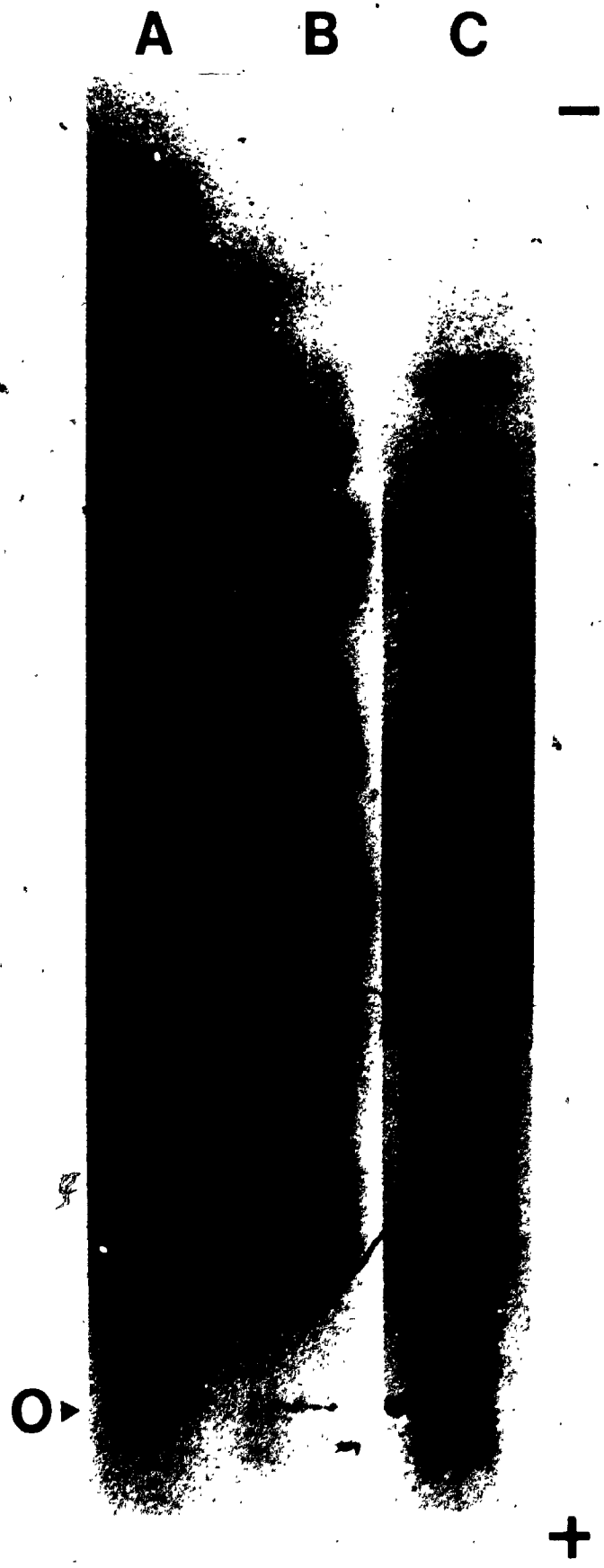
G

electrophoretic mobility (see Figure 3b and 3d) is the same as that of authentic coat protein. To substantiate this observation we assayed the in vitro products for coat protein antigenic determinants by indirect immunoprecipitation. Rabbits were immunized with purified virus and the resultant antiserum was either used directly or further purified on an affinity column (see Materials and Methods, Section 2.2.7) to yield a coat protein-specific IgG fraction. A limiting quantity of antiserum was added to an aliquot of the unfractionated products of in vitro translation without carrier antigen. The antibody-antigen complexes which formed were adsorbed to heat-inactivated Staphylococcus aureus and prepared for polyacrylamide gel analysis (see Materials and Methods, Section 2.2.9). Figure 5 illustrates a fluorogram of an SDS-polyacrylamide gel used to separate such immunoprecipitates. Lanes c, d and g show that regardless of whether column purified or crude PMV antisera were used, only product pC is precipitated. Precipitation with pre-immune serum (Figure 5, lanes b and f) indicated a negligible background. Similar results have been obtained several times, in both the wheat germ and the rabbit reticulocyte systems. The results of this experiment indicate first, that pC shares antigenic determinants

Figure 6 Tryptic Peptides of the Major Products Translated from PMV RNA in the wheat germ system.

Samples extracted from gel slices containing about 10^4 cpm each were concentrated, oxidized with performic acid and then digested with trypsin as described under Materials and Methods, Section 2.2.10. The products were separated by electrophoresis in pyridine acetic acid, pH 3.6. Electrophoresis was in the direction of the cathode. Lane a, Peptides of pA; Lane b, Peptides of pB; and Lane c, Peptides of pC.

The letter O denotes the origin where samples were initially applied.


A
B
C
−
O
+

with PMV coat protein and second, that none of the other polypeptides synthesized in vitro are antigenically related to PMV coat protein.

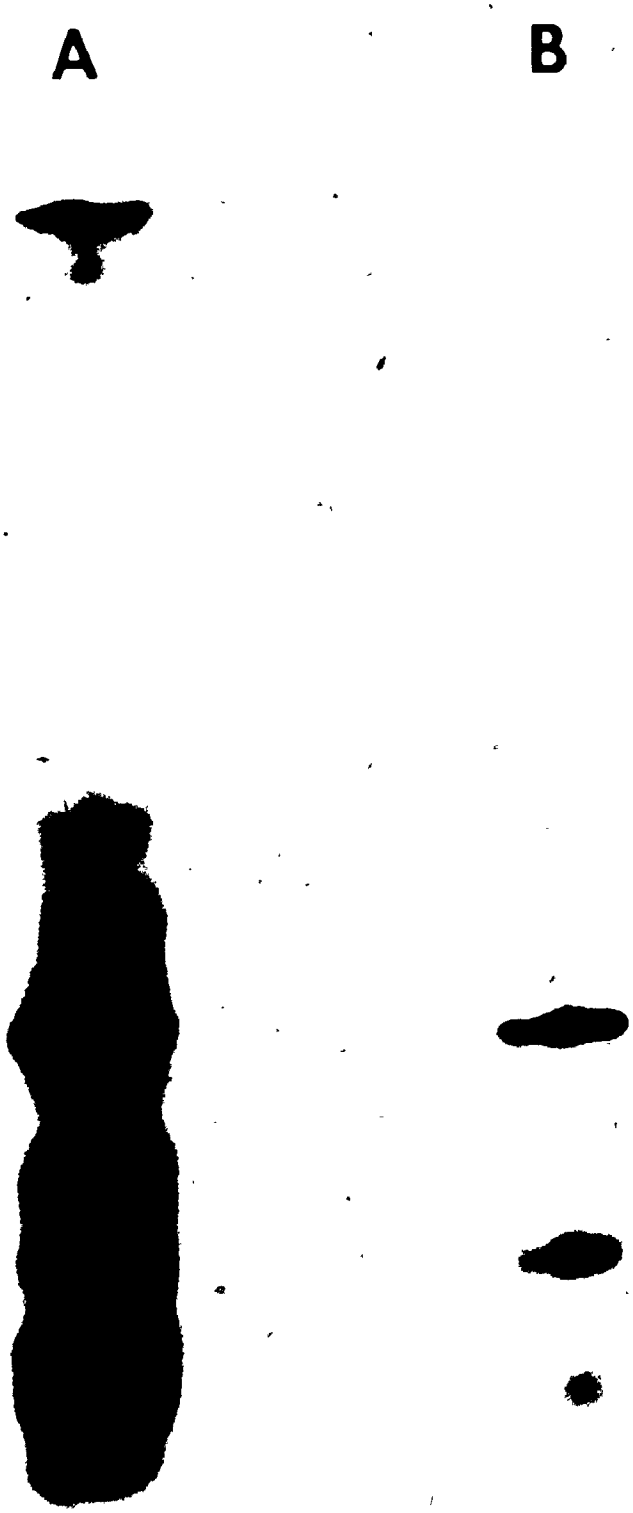
2.3.4 Comparison of In Vitro Translation Products by Peptide Mapping

The three major in vitro translation products pA, pB and pC, as well as most of minor products, were labelled with ^{35}S-methionine in the wheat germ cell-free system and separated by SDS gel electrophoresis. Products excised from gels were digested with trypsin and the peptides were analyzed by high voltage electrophoresis on Whatman ® 3 MM paper in pyridine-acetic acid, pH 3.6. Figure 6 illustrates an autoradiogram of the electrophoretically separated peptides. Lanes a and b demonstrate that products pA and pB have tryptic peptides with the same electrophoretic mobility at this pH. In addition, all minor products analyzed were found to have peptides whose electrophoretic mobilities coincided with either those of pA or pB (data not shown). However, the electrophoretic mobilities of tryptic peptides derived from pC (lane c) were distinct from those of pA or pB.

To confirm the relationship between pA and pB the ^{35}S-methionine-labelled polypeptides directed by PMV RNA in the reticulocyte lysate were resolved on a SDS-polyacrylamide gel. Slices of interest from this gel

Figure 7 Partial proteolysis of the High Molecular Weight Products Translated from PMV RNA in the Reticulocyte Lysate.

Products directed by PMV RNA in the reticulocyte lysate were resolved by electrophoresis on a 6 - 15% polyacrylamide gel, dried without further treatment then autoradiographed. The bands of interest were sliced from the gel using the autoradiogram as a guide, rehydrated and then placed in the wells of a second polyacrylamide gel composed of a 3% stacking gel and 15% separating gel. The gel slices were overlayed with 100 ng of Staphylococcus aureus V8 protease then electrophoresis was allowed to progress until the bromophenol blue tracking dye reached the center of the stacking gel. Electrophoresis was then stopped for 45 minutes. After completion of electrophoresis, the gel was treated for fluorography as described in Materials and Methods, Section 2.2.6. Lane a, Peptides of pA; Lane b, Peptides of pB.


A
B

were then subjected to partial proteolysis using Staphylococcus V8 protease (Materials and Methods, Section 2.2.11). The resolution of the peptides is illustrated in Figure 7. Again, the peptides generated by digestion of pA and pB have the same electrophoretic mobility.

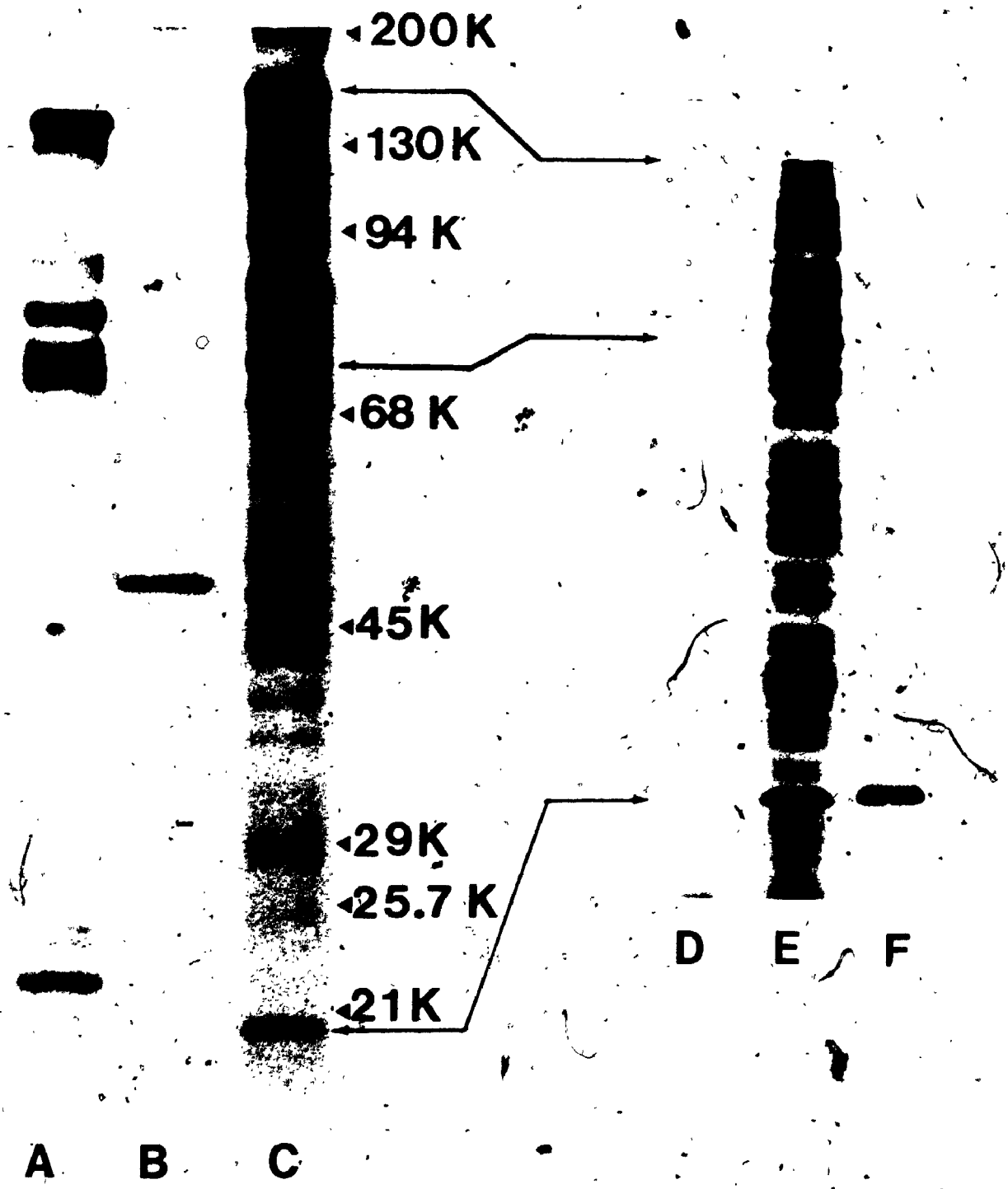
The conclusion that can be drawn from these peptide maps is that polypeptides pA and pB are clearly related. The sequences of pB are most likely nested within that of pA since pB contained no peptides distinct from that of pA. pC, however, has peptides which are distinct from those of pA and pB and therefore is a separate protein.

2.3.5 Analysis of In Vitro Translation Products of other Members of the Potexvirus group

The demonstration that papaya mosaic virus does, in fact, direct the synthesis in vitro of a polypeptide with properties identical to those of native PMV coat protein is in direct contrast to other members of the potexvirus group, namely potato virus X and plantagovirus which fail to direct the synthesis of their corresponding coat proteins in vitro (Ricciardi et al., 1978; Wodnar-Filipowitz et al., 1980, Atabekov and Morozov, 1979). To examine the generality of in vitro coat protein expression, we extended our observations to two other definitive members of the

Figure 8 Electrophoretic separation of Products Synthesized in Vitro with CYMV RNA as Template.

Rabbit reticulocyte lysates programmed with no added (lane b) or 2 µg purified CYMV RNA (lane c) were incubated for 60 minutes at 30° C. Aliquots of the completed reaction were resolved by electrophoresis on a 10% SDS-polyacrylamide slab gel. Iodinated protein standards were the same as those described in Figure 3 and are noted in the margins. The translation products directed by PMV run on the same gel (lane a) is included for size comparison. Lanes d (no added RNA) and e (2 µg CYMV RNA) illustrate products synthesized in wheat germ extracts and separated similarly. Lane f illustrates the position of migration of ^{125}I-CYMV coat protein. The major products made in each system are connected by arrows.


200K
130K
94 K
68 K
45K
29K
25.7 K
21K
A
B
C
D
E
F

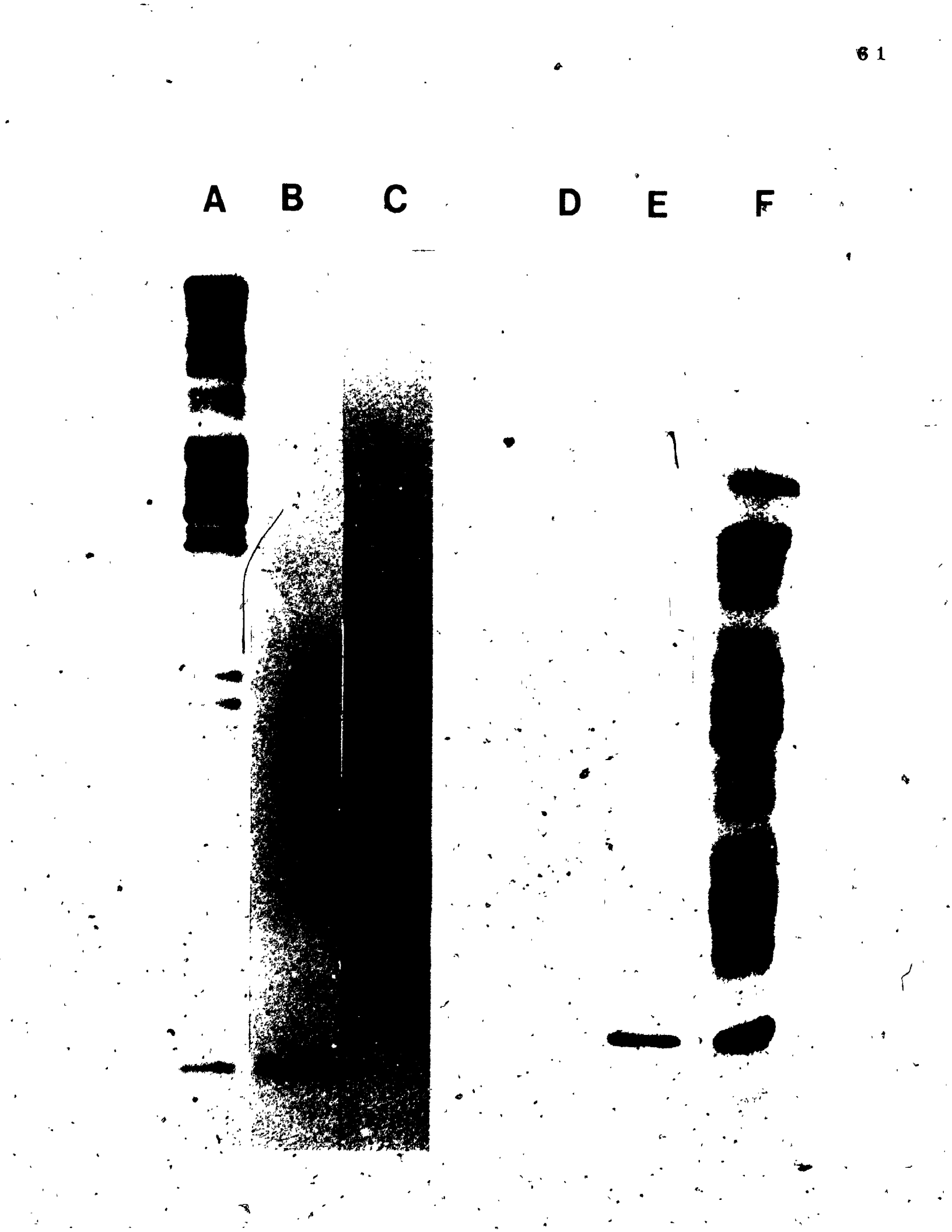
potexvirus group, namely clover yellow mosaic virus and viola mottle virus as well as two tentative members, namely barrel cactus virus and foxtail mosaic virus.

2.3.6 The In Vitro Translation Products of Clover Yellow Mosaic Virus

The ability of CYMV RNA to direct the synthesis of identifiable polypeptide products in either the wheat germ or rabbit reticulocyte cell-free systems was measured by electrophoretic analysis of the products on SDS-polyacrylamide gels. Figure 8 illustrates the spectrum of products synthesized in vitro in those systems. Both extracts direct the synthesis of products ranging in size from 182,000 to 20,000 daltons. Polypeptides of apparent molecular weights 182,000, 76,000 and 20,000 daltons are most prominent. The reticulocyte lysate is more efficient in directing the synthesis of high molecular weight products as a comparison of lanes e (wheat germ extract) and c (reticulocyte lysate) in Figure 8 reveals. The relative paucity of products in the intermediate molecular weight range (75,000 to 22,000 daltons) in the reticulocyte lysate programmed with CYMV RNA may reflect this greater efficiency. (i.e. fewer prematurely terminated products), or the absence of nuclease activity. The 182,000 dalton product was synthesized efficiently in wheat germ extracts only when these extracts were

Figure 9 Indirect Immunoprecipitation of Products Synthesized in two cell-free systems programmed with CYMV RNA.

Immunoprecipitates from CYMV-programmed rabbit reticulocyte lysate (lanes a - c) or wheat germ extract (lanes d - f) were analyzed on 10% polacrylamide gels. Aliquots of each reaction were incubated with one of the following: pre-immune serum (lanes c & d), CYMV antisera (lanes b and e). Lanes a and f show the unfractionated mixtures of products synthesized in the rabbit reticulocyte and the wheat germ cell-free systems respectively.


A
B
C
D
E
F

62

freshly prepared. In the absence of the 182,000 dalton product, the number and intensity of products in the 75,000 dalton to 22,000 dalton range increased.

Several lines of evidence suggest that the prominent 20,000 dalton product programmed by CYMV RNA in either extract is the CYMV coat protein. First, it comigrates with authentic CYMV coat protein in SDS-polyacrylamide gels (Figure 8, lane f). Secondly, it is precipitable by antisera raised against CYMV particles. These antisera were added to the in vitro translation reactions and antigen-antibody complexes were precipitated through the use of killed Staphylococcus aureus as an immunoadsorbant (Materials and Methods, Section 2.2.9). Such complexes were analyzed on SDS-polyacrylamide gels illustrated in Figure 9. Only the 20,000 dalton product would form a complex with anti-CYMV antisera (Figure 9, lanes b and e). None of the higher molecular weight products of the in vitro translation were precipitable. This specificity for the 20,000 dalton product is reinforced by the failure of the pre-immune serum to precipitate anything more than a negligible background under otherwise identical conditions (Figure 9, lanes c and d).

Thirdly, the peptide maps of ^{125}I-labelled coat protein and the ^{35}S-methionine labelled in vitro 20,000 dalton protein were compared. The electrophoretically resolved coat protein and 20,000-

Figure 10 Comparison of Peptides generated by Partial Proteolysis of *In Vitro* Products Directed by CYMV RNA.

Lanes a through c illustrate peptides separated on a 20% polyacrylamide gel and represent the 20,000 dalton *in vitro* product (Lane b) and the ^{125}I-labelled CYMV coat protein (Lane c). Conditions were the same as described in Figure 7 except that 1 µg of *Staphylococcus* V8 protease was used in lanes b and c and 0.1 µg in lane a. The arrow indicates the position native coat protein migrated as determined by staining. Electrophoresis was interrupted for one hour. Lanes d through f represent peptides separated on a 15% polyacrylamide gel. Conditions were the same as described in Figure 7 and 0.1 µg of *Staphylococcus* V8 protease was used.

Lane d represents peptides generated from the 182,000 dalton protein, Lane e those of the 76,000 dalton and lanes a and f those of a 30,000 dalton product which sometimes arose during translation.

A B C D E F

dalton product were sliced from dried SDS-polyacrylamide gel using an autoradiogram as a guide. The gel slices were rehydrated, placed on a second SDS-polyacrylamide gel and electrophoresed in the presence of Staphylococcus aureus V8 protease (see Materials and Methods, Section 2.2.11). Figure 10 illustrates the peptides generated under identical conditions of partial proteolysis. Comparison of the peptides derived from ^{125}I-labelled coat protein (lane c) with that of the ^{35}S-methionine labelled 20,000 dalton *in vitro* product (lane b) demonstrates that the one peptide generated from the 20,000 dalton product co-migrates with one of the peptides derived from the ^{125}I-labelled coat protein. The uppermost band in each case represents undigested protein. CYMV coat protein contains one methionine and six tyrosine residues (J.B. Bancroft, personal communication). That the ^{35}S-methionine labelled 20,000 dalton *in vitro* product generated only one peptide and the demonstration that this peptide co-migrates on SDS-polyacrylamide gels with a peptide from ^{125}I-labelled coat protein digested under identical conditions strongly suggest homology between the two.

The sum of the molecular weights of the products translated *in vitro* exceeds the apparent coding capacity of CYMV RNA (MW. 2.2×10^6) in a single reading frame. To determine whether the higher molecular weight *in vitro* products are related, peptide maps of

partially digested proteins were compared. Figure 10 (lanes d and e), demonstrates that the higher molecular weight products do share peptides with identical electrophoretic mobilities and as such are related.

A 30,000 dalton protein which sometimes appeared during the translation of CYMV RNA was also subjected to proteolysis and the peptides separated by electrophoresis (Figure 10, lanes a and f). The peptides of the 30,000 dalton protein co-migrated with those of the 182,000 dalton and the 76,000 dalton proteins. Its peptides are, however, distinct from those of the 20,000 dalton protein.

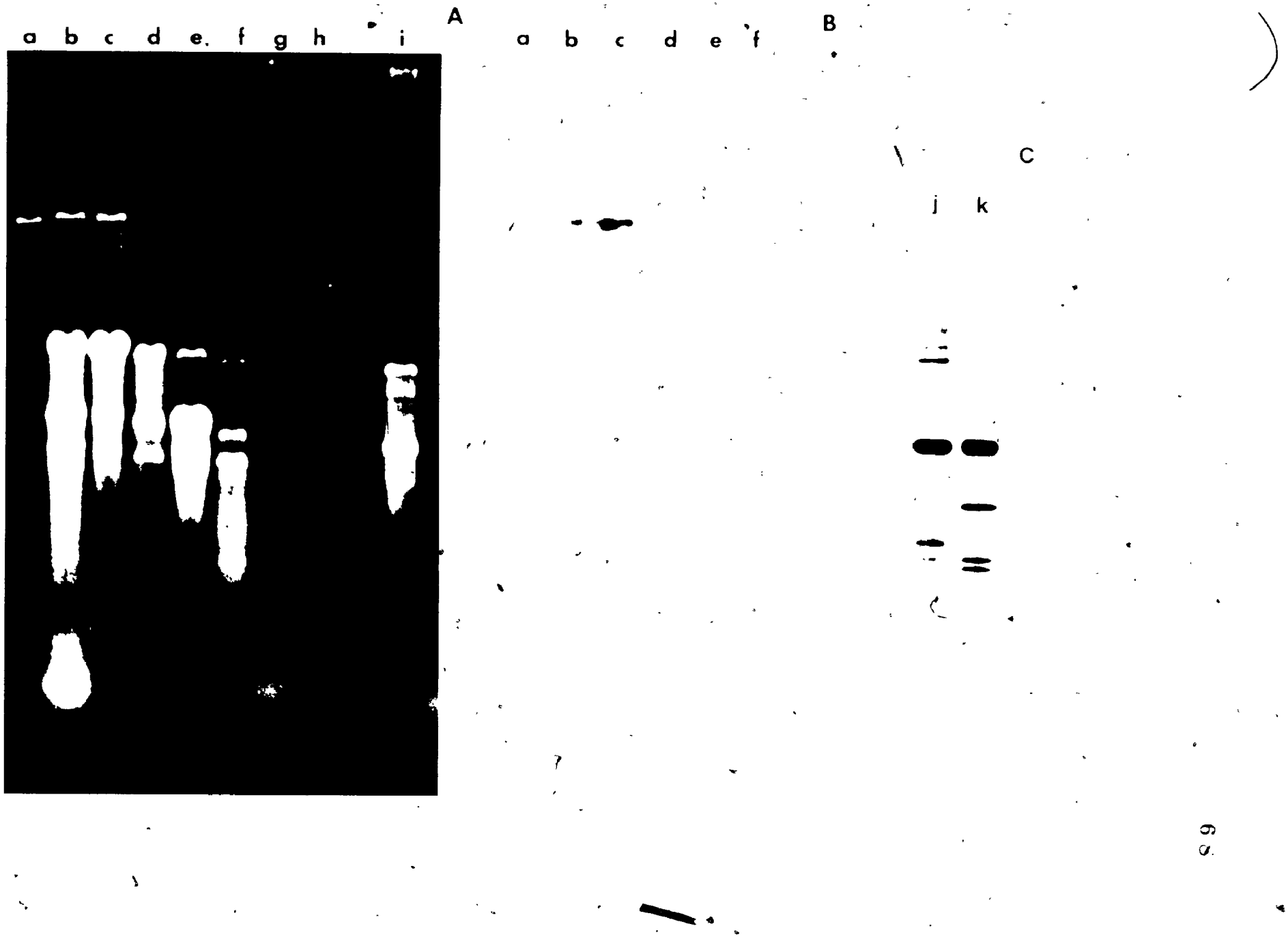
The results presented above suggest that the genomic RNA of CYMV contains a functional cistron for coat protein synthesis which is distinct from that of the non-structural proteins. In view of the wide occurrence of low molecular weight component RNAs in other plant virus groups (see Davies, 1979 for review) we analyzed RNA extracted from CYMV infected plants for the presence of a low molecular weight component RNA which might in fact encode the viral coat protein in vivo. Figure 11, panel A (lane b) illustrates the electrophoretic mobilities of RNA extracted from CYMV infected plants, separated on a 1% (w/v) agarose gel containing 10 mM methylmercury hydroxide. Similarly, lanes c through h illustrate the same RNA which was previously fractionated on a sucrose gradient to enrich

Figure 11 Analysis of RNA extracted from CYMV-Infected plants.

Panel A illustrates the separation on a 1% agarose gel containing 10 mM methylmercury hydroxide of genomic CYMV RNA (lane a), total RNA extracted from CYMV infected plants (lane b) and the RNA used in lane b fractionated on a 6 - 20% sucrose gradient (lanes c - h).
Markers were provided by total RNA extracted from Chinese hamster ovary (CHO) cells (lane i).

Panel B represents an autoradiograph of the same RNA in panel A transferred to Ultra-Blot ® paper and hybridized with ^{32}P-CYMV cDNA as described in Materials and Methods, Section 2.2.13 and 2.2.14.

Panel C (lane k) illustrates the translation products of the RNA represented in lane c of Panel A and those directed by genomic CYMV RNA (lane j) in the reticulocyte lysate. Products were separated on a 10% polyacrylamide gel. The position to which coat protein migrates is noted.


A
a b c d e f g h i
B
a b c d e f
C
j k

for sequences within each particular size class. After electrophoretic separation, the RNA from this same gel was transferred to Ultra-Blot ® paper (Collaborative Research), then probed for CYMV-specific positive strand sequences using a ^{32}P-labelled cDNA probe generated by reverse transcription of the CYMV RNA template in the presence of random DNA primers (see Materials and Methods, Section 2.2.13). Figure 11, panel B, lanes a through f demonstrate that CYMV RNA sequences are present only in RNA with the same electrophoretic mobility as CYMV RNA extracted from the native virus (lanes a of panel A and B). To determine whether coat protein was still produced from the high molecular weight RNA, the sucrose gradient fraction (i.e. lane b of Figure 11, panel A) was used to program in vitro protein synthesis in the reticulocyte lysate. Figure 11, panel C (lane k) illustrates that the gradient fraction containing high molecular weight RNA extracted from CYMV-infected plant does produce a polypeptide with an identical electrophoretic mobility to the 20,000 dalton in vitro translation product specified by the genomic RNA (lane b). The absence of the 182,000 dalton product in this experiment is most likely a function of the particular lysate used.

Figure 12 Effect of Mg^{+2} concentration on the synthesis of individual products in a rabbit reticulocyte lysate programmed with BCV RNA.

Aliquots of the reactions adjusted to contain nearly the same amounts of acid-precipitable radioactivity were resolved on a 10% SDS-polyacrylamide slab gel. The samples are: translation products programmed by 2 μg BCV RNA at the following concentrations of Mg-acetate (a) 0.5 mM, (b) 1.0 mM, (c) 1.5 mM, (d) 2.0 mM, (e) 2.5 mM, (f) 5.0 mM. Lane g illustrates the position of migration of ^{125}I-BCV coat protein. The arrow indicates the position to which intact native coat protein migrated.


A
B
C
D
E
F
G

2.3.7. The In Vitro Translation of the Genomic RNA's of Viola Mottle Virus, Barrel Cactus Virus and Foxtail Mosaic Virus

As before, the in vitro translation products directed by the genomic RNA's of barrel cactus virus (BCV), foxtail mosaic virus (FMV) and viola mottle virus (VMV) were analyzed by electrophoresis on SDS-polyacrylamide gels. Figure 12 illustrates the spectrum of ^{35}S-methionine labelled polypeptides directed by BCV in the reticulocyte lysate as a function of increasing magnesium ion concentration. At the optimal magnesium concentration, 0.5 mM, BCV RNA stimulated incorporation of ^{35}S-methionine into acid precipitable material 8-fold. PMV under identical conditions stimulated incorporation 13-fold. The resultant products directed by BCV RNA at this magnesium concentration range in molecular weights from 140,000 daltons to 25,000 daltons (Figure 12, lane a). The 140,000 dalton product was, however, the most prominent polypeptide. As the magnesium ion concentration was increased beyond the optimal level (lanes b through f) the synthesis of the 140,000 dalton product decreased. In contrast to PMV, however, none of the products synthesized at any magnesium level tested, co-migrated with the 22,000 dalton BCV coat protein (Lane g). The analysis of

2

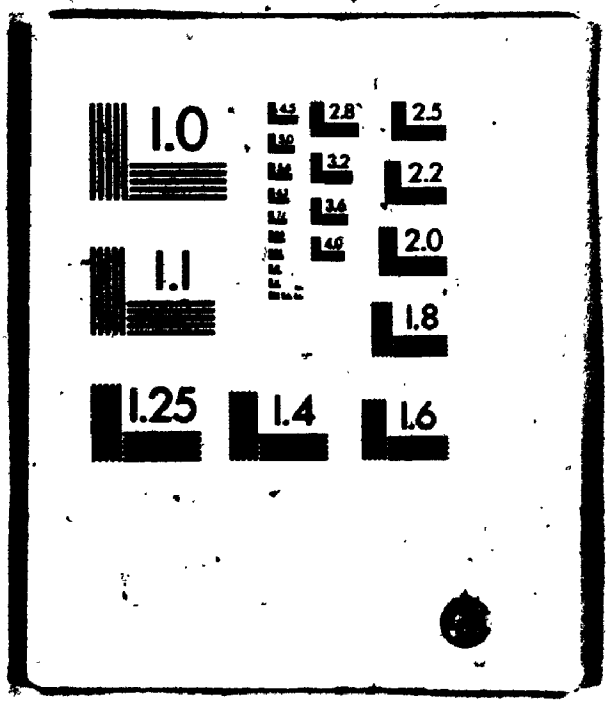
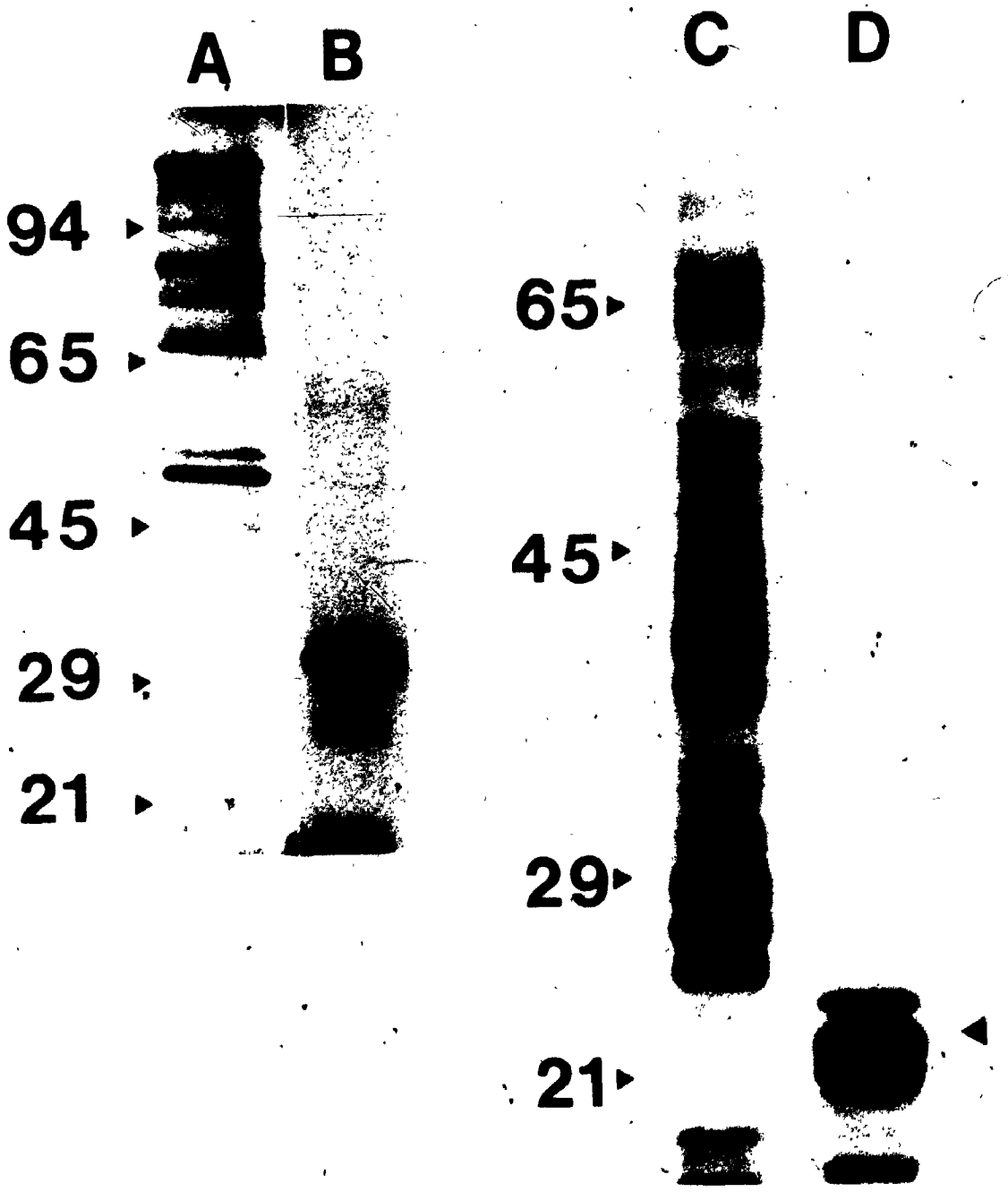


Figure 13 Electrophoretic separation of the *in vitro* translation products directed by FTMV and VMV RNAs.

FTMV RNA (1 μg) and VMV RNA (5 μg) were used to program *in vitro* protein synthesis in the rabbit reticulocyte and wheat germ cell-free systems respectively. Products were analysed on 10% SDS-polyacrylamide gels (Materials and Methods, Section 2.2.6). Lane a, products directed by FTMV RNA in the reticulocyte lysate; lane b, ^{125}I-labelled FTMV capsid protein; lane c, products directed by VMV RNA in the wheat germ extract; lane d, ^{125}I-labelled VMV capsid protein. The arrow indicates the position to which unlabelled VMV coat protein migrated. The positions to which molecular weight markers migrated after electrophoresis in the same gels are noted in the margins.


A
B
C
D
94
65
45
29
21
65
45
29
21

products directed by BCV RNA in wheat germ extracts also revealed that coat protein was not made (data not shown).

Similarly, none of the products directed by FMV in the reticulocyte lysate (Figure 13, lane a), and VMV in the wheat germ extract (Figure 13, lane c) co-migrated with their respective coat proteins (Figure 13, lanes a and d respectiyely).

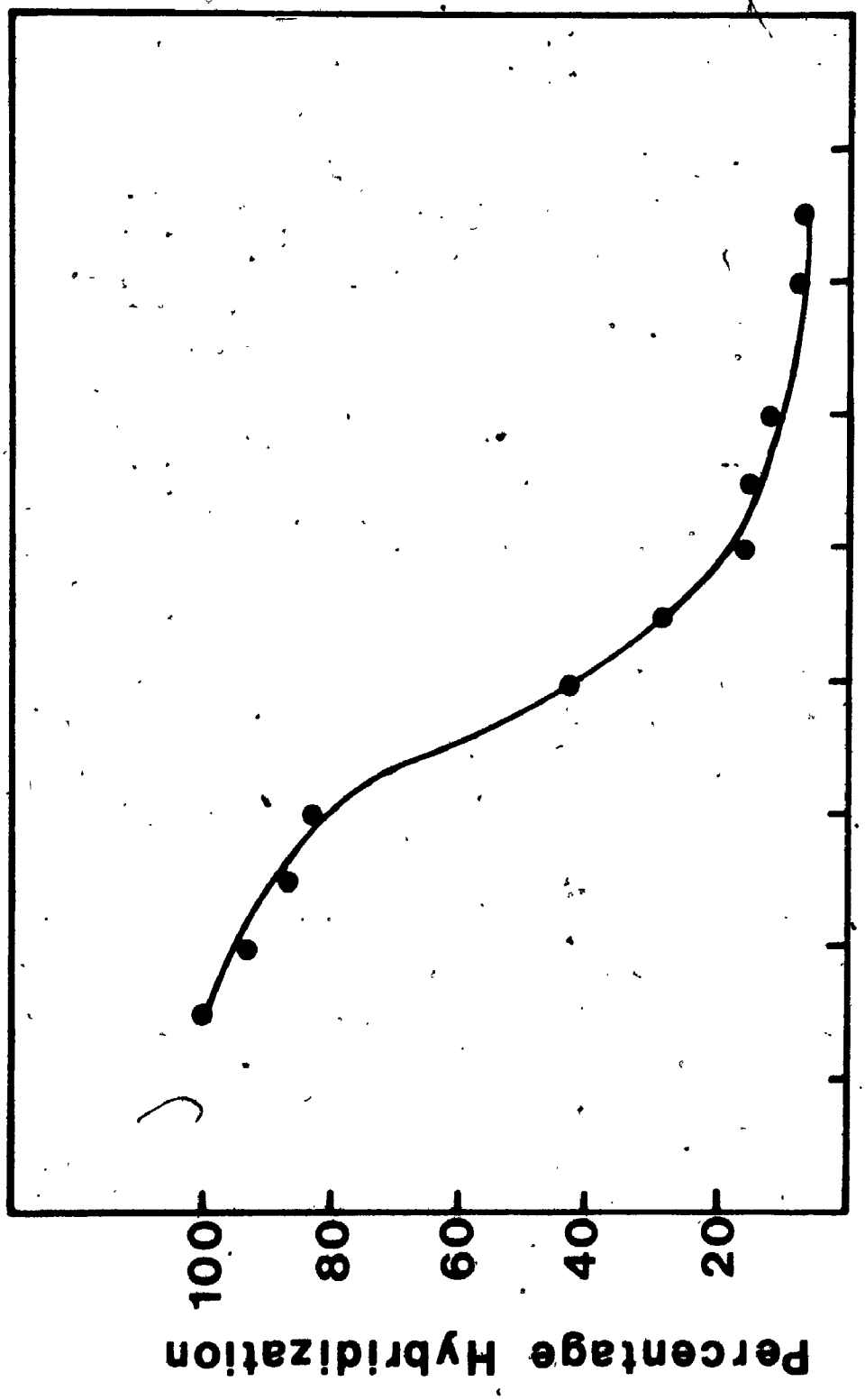
2.3.8 Relationships between Potexviruses and Other Selected Plant Viruses

The members of the potexvirus group have been classified as such because they share morphological properties and exhibit varying degrees of serological cross-reactivity (Harrison et. al., 1971; Koenig and Lesemann, 1978). Additionally, physical studies have indicated that members within this group share the same architecture. In each case, viral particles exhibit approximately 9 protein subunits per turn (Tollin et. al., 1979, 1980, 1981; Richardson et. al., 1981).

The morphological relatedness of this virus group, however, does not appear to be a reliable indicator of the functional behavior of the corresponding RNAs. On the basis of in vitro translation it appears that viruses within this group can be further divided into 2 groups, those whose genomic RNA directs the synthesis of their respective coat protein and those

Figure 14 Formation of unlabelled cDNA - ^{125}I-CYMV RNA hybrids.

^{125}I-CYMV RNA (4000 cpm; approximately 9 ng) was added to 0.05 ml reactions containing serial dilutions of unlabelled cDNA. The formation of hybrids and the determination of their resistance to RNAse A and T1 was as described in Materials and Methods, Section 2.2.16.


Percentage Hybridization
100
80
60
40
20
250
62.5
15.6
3.9
0.97
0.24
0.06
0.015
cDNA (ng)

whose do not. As a means of analyzing the possible relationships between the RNA genomes of this plant virus group, the method of hybridization of RNA with complementary DNA (cDNA) was employed.

Since most potexvirus RNAs are not polyadenylated at their 3'-termini they cannot serve as templates for oligo-dT primed cDNA synthesis. Therefore random nucleotides generated by DNAse I digestion of calf thymus DNA were used as primers for the reverse transcription reaction (see Materials and Methods, Section 2.2.13). To ensure that the cDNA generated by this method was representative of the RNA template, unlabelled cDNA (prepared to CYMV RNA) was hybridized with ^{125}I-labelled CYMV RNA. Hybrid formation was then monitored by resistance to RNAse A and T1. Figure 14 illustrates that essentially 100% protection of the ^{125}I-RNA was achieved indicating that the cDNA represented the entire template. Furthermore, the absence of discrete plateaus in the hybridization curve generated by dilution of the cDNA suggested that the cDNA was not grossly enriched for one or more regions of the genome. Therefore, using this method cDNA was prepared using PMV, CYMV, BCV, FTMV and VMV RNAs as templates. In each case, the ^{32}P-labelled cDNAs exhibited an average size of 400-500 nucleotides as judged by electrophoresis on an alkaline agarose gel (not shown).

The extent of nucleotide sequence homology between an RNA and a cDNA probe was determined by measuring the amount of ^{32}P-cDNA that became resistant to S1 nuclease by virtue of forming a double-stranded structure with the test RNA. Hybridizations using both heterologous and homologous RNAs were driven to a Rot of 13.5 mole.sec/l which is approximately 6-fold beyond the point at which the homologous reaction was complete as determined by hybridization kinetics (data not shown). Table 2 summarizes the data obtained. As would be expected, each cDNA tested showed complete homology with its respective RNA. However, when each cDNA was hybridized to a heterologous RNA, no significant homology was found. The slight homology found when PMV cDNA was used as probe against heterologous RNA's under high salt conditions (0.6 M Na^+) (Table 2, top row) was eliminated using the same probe under low salt conditions (0.2 M Na^+). This would indicate that these slight homologies were due to mismatched regions which were stable at the higher but not the lower salt concentrations. We suspect that the small homologies indicated by hybridization of the BCV probe to several heterologous RNAs are also due to mismatched regions. In addition the results indicate that no sequence homology exists between these members of the potexvirus group and

TABLE 2 Summary results of excess RNA -cDNA hybridization

ESTIMATED PERCENTAGE SEQUENCE HOMOLOGY

RNA Used For cDNA Preparation	RNA Used for Hybridization Reaction									
	PMV	CYMV	BCV	VMV	FTMV	PVX	CCMV	BMV	TMV	NONE
PMV	100[a] (4200)[c]	0	4.8	2.4	1.6	0	0	1.2	0	(48)[d]
CYMV	0	100 (2200)	0	0	0	0	0	0	0	(81)
BCV	0	2.6	100 (7700)	4.7	0	5	0	0	0	(51)
VMV	0	0	0	100 (16,800)	0	0	0	0	0	(422)
FTMV	0	0	0	0	100 (12,600)	0	0	0	0	(332)
PMV	100[b] (8900)	0	0	0	0	0	0	0	0	(167)

a) cDNA-RNA hybrids formed under high salt conditions (0.6M Na^+) were assayed with S1 nuclease and the estimated % sequence homologies calculated as described in Materials and Methods (Section 2.2.15).

b) cDNA-RNA hybrids formed using a PMV cDNA probe under conditions of low salt (0.2 M Na^+). The estimated % sequence homologies were calculated as in a).

c) S1 nuclease-resistant cpm obtained in the homologous reaction.

d) S1 nuclease-resistant cpm in the reaction without RNA. These have been subtracted from the other results before calculation of the percentages

diverse unrelated viruses such as cowpea chlorotic mottle virus (CCMV), brome mosaic virus (BMV) and tobacco mosaic virus.

2.4 Discussion

This work presents evidence that the genomic RNAs of PMV and CYMV direct the synthesis in two cell-free systems of polypeptides which are apparently identical to their respective coat proteins isolated from purified virus particles. In each case, the apparent molecular weight and antigenic determinants of the 22,000 dalton and 20,000 dalton *in vitro* polypeptides specified by PMV and CYMV RNAs respectively, resemble closely those of their respective coat proteins. The peptide map of the 20,000 dalton product specified by CYMV RNA also supports this conclusion. In addition, preliminary experiments indicate that the 22,000 dalton product synthesized *in vitro* from PMV RNA is capable of assembling with authentic coat protein and thus is functionally very similar to it (data not shown).

The *in vitro* translation of the genomic RNA of each potexvirus member tested resulted in the synthesis of numerous polypeptides with molecular weights greater than that of their respective coat proteins. The nature and function of non-structural proteins specified by plant viral genomes is unclear. Presumably these proteins may function in viral replication, regulation

of host metabolism and/or cell-to-cell movement of the virus within the plant. A 30,000 dalton non-structural protein specified by the genome of tomato strains of tobacco mosaic virus has recently been identified as a factor mediating cell-to-cell movement of virus in the leaf (Leonard and Zaitlin, 1982).

The analysis of the higher molecular weight products directed by PMV and CYMV demonstrated that these products clearly differ from their respective coat proteins by several criteria. First, they are not precipitable by antibodies prepared to their respective virus nor are they part of the virus particle (our unpublished work). Second, peptide mapping experiments show that tryptic peptides of PMV-specified products pA and pB are similar to each other, but differ from that of pC. Partial proteolysis of pA and pB has confirmed that the sequence of pB and probably of many of the minor products of translation is likely nested within that of pA. Similarly, partial proteolysis of the 182,000 dalton and the 76,000 dalton polypeptides directed by CYMV show they are related in part. We have been unable to demonstrate the conversion of the smaller into the larger protein during in vitro translation of either PMV or CYMV by use of yeast amber or ochre

suppressor tRNA's (data not shown) or by altering the concentration of magnesium ion. Therefore, "readthrough" of leaky termination codons which has been demonstrated for TMV RNA (Pelham, 1978) does not seem to explain the relationship between these proteins. It is yet to be established whether such products exist in vivo. It is noteworthy, however, that the 165,000 dalton in vitro translation product directed by TMV RNA is found in vivo (Scalla et al., 1978, Siegel et al., 1978). Thus it is conceivable that the 182,000 dalton protein encoded by CYMV and the pA product of PMV possess in vivo counterparts.

The synthesis of coat protein from the genomes of PMV and CYMV contrasts with the translational behavior of other members of the potexvirus group. Analysis of the in vitro translation products directed by the RNA's of VMV, BCV and FTMV failed to reveal polypeptides with electrophoretic mobilities similar to their respective coat proteins. These viruses therefore mimic PVX, the type member of the potexvirus group. Ricciardi et al. (1978) and Wodnar-Filipowitz et al. (1980) have proposed that the translational strategy of PVX resembles that of TMV where the coat protein cistron is not normally accessible for translation in the intact viral RNA. Presumably, coat protein synthesis in vivo would involve the production of a sub-genomic RNA in a manner analogous to that described for TMV RNA (Hunter

et. al., 1976). Without evidence to the contrary, the synthesis of the coat proteins of viola mottle, barrel cactus and foxtail mosaic viruses may also rely on the production of a sub-genomic RNA in vivo. Alternatively, in vitro conditions may be suboptimal for coat protein synthesis by these RNAs. Conceivably, this could result from a very strong folding of the RNA.

The analysis of RNA extracted from CYMV-infected plants, however, suggests that a low molecular weight RNA species is not generated in vivo during CYMV infection. CYMV coat protein synthesis therefore most likely results from the direct translation of the genomic RNA. The in vitro translation of coat protein from a high molecular weight RNA fraction derived from CYMV infected plants supports this view. By analogy, it would seem logical that PMV coat protein synthesis follows this mechanism.

The finding that the morphological relatedness of this group appears not to be a reliable indicator of the functional behavior of the corresponding RNAs led us to examine the relationship of these viral genomes at the level of nucleotide sequence. Using RNA-cDNA hybridization we found that no homology exists between the genomes of all members of the potexvirus group tested, nor with other unrelated viruses. Definitive potexvirus members do show serological relatedness although the relationship appears to be distant (Koenig

and Lesemann, 1979). The serological relatedness, however, is based on the capsid protein which for potexvirus members constitutes 8 - 10% of the total coding capacity of their respective genomes. Furthermore, since serological identity would involve only that portion of the coat protein exposed to the surface of the viral particle, it may not be surprising that within the limits of detection no homology could be noted. The lack of sequence homology between members of the potexvirus group as detected by cDNA-RNA hybridization is not unique. Kummert et al. (1978) demonstrated that three serologically related members of the tymovirus group, namely turnip yellow mosaic virus, eggplant mosaic virus and Andean potato latent virus share no homology at the level of nucleotide sequence. Similarly, no sequence homology could be found between most sub-groups of tobacco mosaic virus (tobamovirus group), between three comoviruses, or between two potexviruses (Van de Walle and Siegel, 1982, Palukaitis and Symons, 1980). Perhaps, this noted lack of sequence homology reflects some evolutionary mechanism whereby mutation of a parental strain resulted in viruses with some advantage in a particular host. This advantage could be the acquisition of sequences which match the preferred codon usage of the host and/or enhance replication of the virus. Additionally, adaptation to

different replication strategies is exemplified by CYMV RNA whose replication may take place in the nucleus (de Zoeten and Schlegel, 1967).

CHAPTER 3 - ORGANIZATION OF GENETIC INFORMATION AS DETERMINED BY CELL-FREE TRANSLATION OF PARTIALLY ENCAPSIDATED VIRAL PARTICLES

3.1 Introduction

The *in vitro* reconstitution of PMV (Erickson *et al.*, 1976, Abouhaidar and Bancroft, 1978, Erickson *et al.*, 1978, Erickson and Bancroft, 1978a, Erickson and Bancroft, 1978b) and CYMV (Bancroft *et al.*, 1979, Abouhaidar, 1981) from their purified constituents is a polar process. PMV assembly *in vitro* begins with a rapid initiation phase during which PMV coat protein recognizes and encapsidates an adenylate rich region within 200 residues proximal to the 5' end of the RNA (Abouhaidar and Bancroft, 1978, Erickson and Bancroft, 1978b) Similarly, initiation of encapsidation of CYMV RNA by its coat protein leads to protection of the 5' terminal 7-methylguanosine residue from nuclease attack (Abouhaidar, 1981). Following the rapid initiation phase is a slower elongation phase in which coat protein polymerizes along the RNA in the 5' to 3' direction ultimately generating fully encapsidated particles. Such particles are indistinguishable from native virus on the basis of their morphology and sedimentation coefficients. They are also infectious (Erickson and Bancroft, 1978a, Bancroft *et al.*, 1979).

As a consequence of the kinetics of assembly *in vitro*, reconstitution with less than stoichiometric ratios of coat protein to RNA (20:1 (wt/wt)) generates partially encapsidated RNA templates. In all cases, it is the 5' terminal portion of the RNA which is complexed with coat protein leaving the 3' extremity uncovered. RNA tails protruding from incomplete particles have been visualized in platinum-shadowed preparations of such particles (Abouhaidar and Bancroft, 1978).

This chapter describes the translation of such partially assembled viral particles and demonstrates that template activity is retained even when the 5' terminus of the RNA is masked.

3.2 Materials and Methods

3.2.1 Chemicals

All chemicals and enzymes were described in Chapter 2.

3.2.2 Viral Reconstitution

PMV was reconstituted from purified RNA and coat protein (isolated as described in Section 2.2.2) in 0.01 M Tris-HCl, pH 8.0 as previously described by Erickson and Bancroft (1978a). Similarly CYMV was reconstituted from its constituent components in 0.01 M Tris-HCl, pH 7.5 as described (Bancroft *et al.*, 1979).

Briefly, 50 µg of the appropriate RNA from a 1 mg/ml stock solution was added to a one ml reaction containing 1 mg/ml of protein in 0.01 M Tris buffer at the appropriate pH. Assembly was carried out at 25° C for 30 minutes and stopped by chilling on ice. Partially encapsidated particles were formed by using less than stoichiometric (20:1) amounts of coat protein in the reconstitution reaction. Particles so formed were added without purification to one of the cell-free systems for protein synthesis to assay template activity or were examined by electron microscopy. Particle length distributions at each ratio of coat protein to RNA were constructed as described (Erickson and Bancroft, 1978)

3.2.3 Translation and Analysis of In Vitro Products

Conditions for translation and analysis of products by SDS-gel electrophoresis were those described in Chapter 2, Sections 2.2.4, 2.2.5 and 2.2.6. To quantitate the incorporation into specific polypeptides, the bands of interest were sliced from dried gels using the exposed x-ray film as a guide, rehydrated in H_2O, then dissolved in 1 ml 30% H_2O_2 at 50° over a period of 24-30 hours. The digested gel was dispersed in 20 ml of Aquasol ® (NEN) and counted after at least one hour's adaptation to darkness.

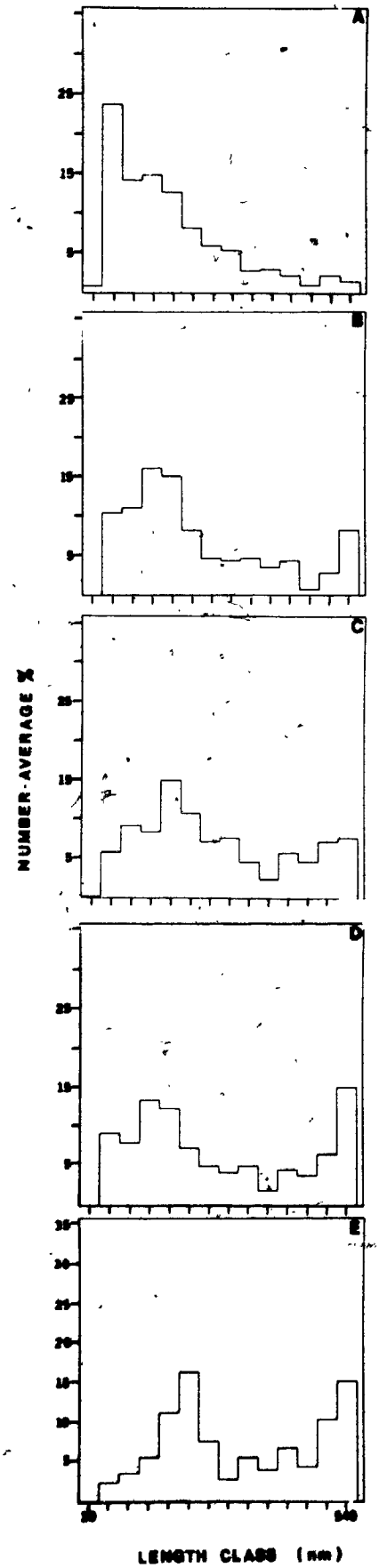
3.3 Results

3.3.1 Encapsidation of PMV RNA

A set of partially encapsidated particles using coat protein to RNA ratios of 2:1, 5:1, 10:1, 15:1 and 20:1 was prepared as described in Materials and Methods. The extent of encapsidation in each case was determined by electron microscopic examination of each preparation and the measurement of the lengths of over 250 particles in each field. Figure 15 illustrates the frequencies with which different particle lengths occur in each of the partially encapsidated preparations. At a low ratio of coat protein to RNA, 2:1 (Figure 15a), the most frequent ribonucleoprotein particle length was 60 nm. The number average length of the particles is, however, approximately 180 nm, corresponding to encapsidation of one third of the RNA. Less than 1.5% of the particles were fully encapsidated (i.e., are 540 nm long). The amount of RNA not encapsidated under these conditions was not assessed. At higher ratios of coat protein to RNA (5:1 and 10:1) there is a shift in the distribution of particle sizes to a number average length of roughly 180 - 220 nm and there is a greater percentage of complete viral particles (Figure 15b and c). Finally, at still higher ratios (15:1, Figure 15d and 20:1, Figure 15e) a bimodal distribution of particle lengths exists between partially encapsidated particles

Figure 15 Histograms of particle length distributions of partially encapsidated PMV DNA assembled at various coat protein to RNA ratios.

PMV RNA was reconstituted in 0.01 M Tris, pH 8.0, as described in Materials and Methods with the following ratios of coat protein to RNA: (a) 2:1, (b) 5:1, (c) 10:1, (d) 15:1 and (e) 20:1. After 30 min at 25° samples were taken for electron microscopy as described by Erickson and Bancroft (1978b). The lengths of over 250 particles in each reconstitution were measured and the data were processed as described by these authors. The data are plotted as number-average (%) as a function of measured particle length. Fully reassembled and/or native PMV particles are 540 nm in length. The length class scale is in 40 nm divisions.


A
B
C
D
E
NUMBER-AVERAGE %
LENGTH CLASS (mm)

typically 220 nm in length and fully completed particles. As would be expected, the latter class is more abundant at the higher ratios than at the lower ones. The material in the completed incubations where the ratio of coat protein to RNA is low (2:1 and 5:1) probably consists of naked RNA, initiated particles, and particles typically one third complete. Presumably at higher ratios of coat protein to RNA (10:1 and above) the fraction of uncoated RNA present at the end of the reconstitution reaction decreases and the resultant particles are mixtures of initiated, partially encapsidated (typically 30-40%) and fully reconstituted particles. It should be noted that a particle 180 nm in length represents the masking by coat protein of approximately 2300 nucleotide residues whose coding capacity would correspond to a protein of approximately 90,000 molecular weight.

3.3.2 The Translation of Partially Encapsidated PMV RNA

The partially encapsidated ribonucleoprotein particles described above were used as templates in either *in vitro* translation system. We found that as the ratio of coat protein to RNA in the reconstitution incubation increased, the incorporation of methionine into acid precipitable material in the subsequent translation assay decreased. Table 3 presents these data

TABLE 3 Template Activity of PMV RNA Encapsidated to Different Extents[a,b]

Template[a]	pH for Encapsidation[b]	Coat Protein: RNA[b]	Most Frequent particle sizes (nm)	Additions to Incubation	No. Avr. Length of Distribution	Relative Activity[c]
PMV RNA	8.0	-	-	-	-	100
PMV RNA	8.0	-	-	-	-	100
PMV RNA	8.0	2:1	60	-	181.3	30
PMV RNA	8.0	4:1	N.D.[d]	-	N.D.[d]	12
PMV RNA	8.0	5:1	140-180	-	255.9	10
PMV RNA	8.0	10:1	180-220	-	281.2	8
PMV RNA	8.0	15:1	180-54	-	295.0	7
PMV RNA	6.0	2:1		-	-	4
PMV RNA	-	-	-	PMV coat protein, 0.2 mg	-	100
PMV	-	-	-	-	-	2

[a] 5.0 µg of PMV RNA (native, partially encapsidated, or in intact viral particles) was used as a template in the wheat germ system (See Materials and Methods).

[b] PMV RNA was reconstituted with purified PMV coat protein at the indicated ratio of coat protein to RNA in 0.01 M Tris-HCl, pH 8.0 (see Abouhaidar and Bancroft, 1978 for details) or in 0.01 M MES pH 6.0 according to Erickson and Bancroft (1978a).

[c] Relative activity is defined as the total incorporation of ^{35}S-methionine per 0.05 ml incubation into hot trichloracetic acid-precipitable material in the sample of interest, relative to that obtained with untreated PMV RNA as template (line 1). In the experiment illustrated, 100% corresponds to 5.7×10^3 cpm. The background in the absence of added RNA of 3.3×10^3 cpm has been subtracted from all samples.

[d] N.D., not determined.

for the wheat germ system; the reticulocyte lysate programmed by aliquots of the same material behaved comparably. In view of the controls discussed below, the observed decrease in template activity reflects the disappearance of translatable sequences on the template as it is increasingly encapsidated, and not non-specific inhibition by free coat protein or completed virus.

The following control experiments were undertaken to validate the encapsidation-translation experiments. First, native viral particles were tested and found to have no template activity in the wheat germ system. PMV alone failed to stimulate incorporation of methionine beyond the background (Table 3). Moreover, electrophoretic examination of the "products" in an extract programmed with viral particles revealed no identifiable bands (Figure 17g). We conclude that PMV RNA within the complete virus is not available for translation. Moreover, it is unlikely that the virus would dissociate in the ionic environment of the cell-free system. Secondly, purified coat protein, added directly to the cell-free system programmed with PMV RNA in a 40 fold excess by weight over PMV RNA had no inhibitory or stimulatory effect on incorporation (Table 3). In addition, the spectrum of labelled products displayed by electrophoresis was the same as in an untreated control (data not shown). This argues against assembly occurring during the translation assay. Indeed,

Figure 16 Electron micrographs of nucleoprotein particles formed by PMV RNA and PMV coat protein assembled in 10 mM Tris-HCl at pH 6.0 (A) and pH 8.0 (B). The bar represents 100 nm. This photograph was the kind gift of Dr. J. Erickson and Dr. J.B. Bancroft.

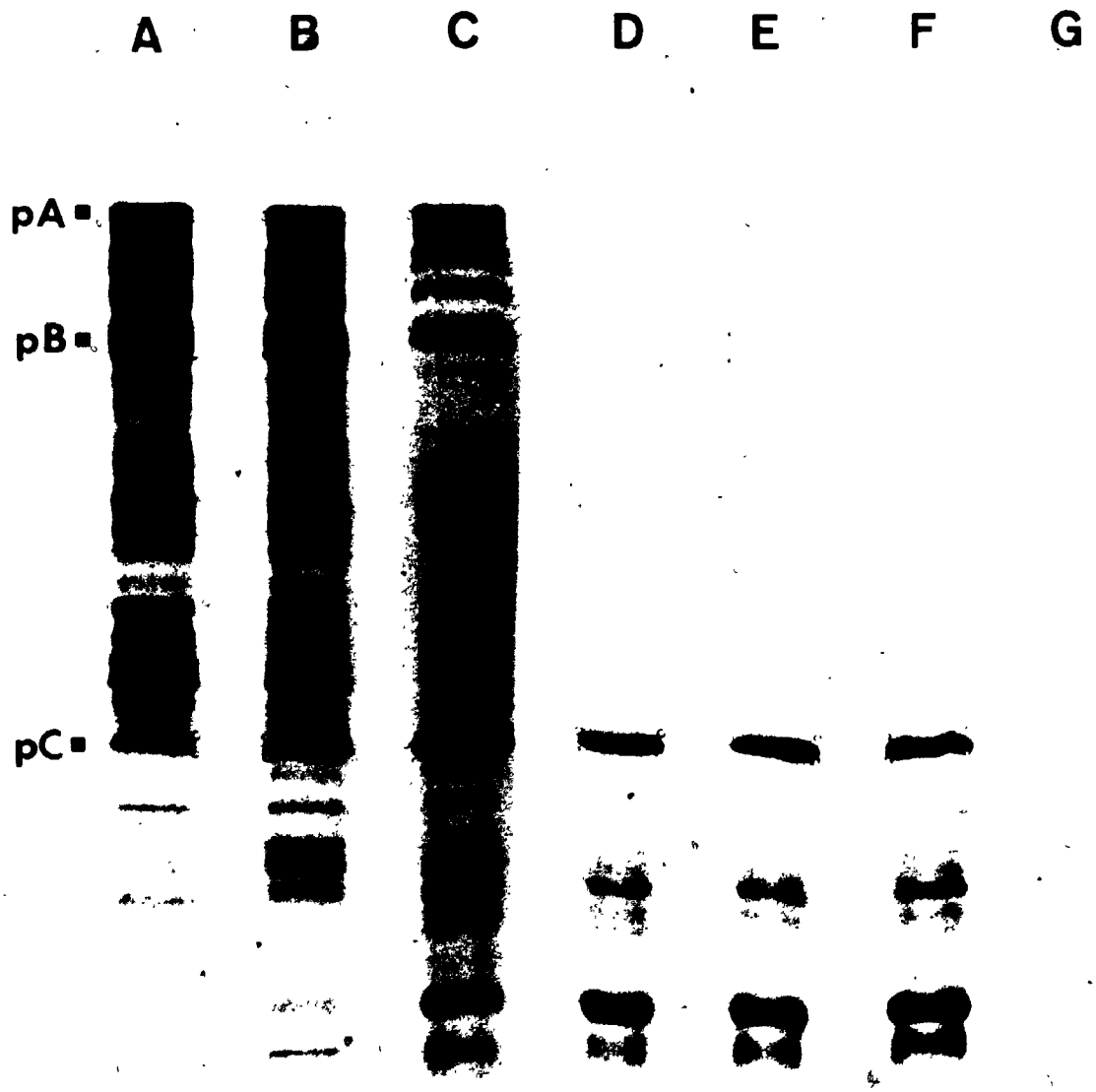

A

the ionic strengths encountered in the cell-free system would be strongly inhibitory to viral reconstitution (Erickson and Bancroft, 1978a). This result also indicates that at the concentrations tested, free coat protein does not act as an inducer or repressor of the synthesis of any of the major in vitro products.

Two further control experiments were undertaken which relate to the specificity of assembly. It is known that PMV coat protein will bind to PMV RNA at pH 6.0 (Erickson, Abouhaidar and Bancroft, 1978). Under these conditions, the polarity of assembly is lost; moreover, at this pH PMV coat protein encapsidates any single stranded RNA or DNA (Erickson and Bancroft, 1979). In marked contrast to ribonucleoprotein particles formed at pH 8.0, those formed at pH 6.0 are morphologically segmented, sensitive to ribonuclease and have altered sedimentation properties (Figure 16). The addition of such aberrant particles assembled at a coat protein to RNA ratio of 2:1 at pH 6.0, to the wheat germ cell-free system reduced the incorporation of methionine to values below background (Table 3). No discernable products were apparent after electrophoretic analysis (data not shown). As an additional test of the requirement for specific assembly PMV coat protein was incubated with TMV RNA in 0.01 M Tris-HCl, pH 8.0. Binding of PMV coat protein to TMV RNA does not occur under these conditions (Erickson et al., 1978). The

Figure 17 Electrophoretic separation of products synthesized in the wheat germ cell-free system with partially encapsidated PMV RNA as template.

The assembly of partially encapsidated PMV subparticles and their subsequent translation in the wheat germ system are described in Materials and Methods. Aliquots from each incubation were applied to a 12.5% SDS-polyacrylamide gel and the products resolved by electrophoresis. The templates, in each case containing 5 µg PMV RNA in a reaction of 0.05 ml are: (a) purified PMV RNA; (b - f), PMV RNA partially encapsidated with purified coat protein at the following ratios of coat protein to RNA: (b) 2:1, (c) 5:1, (d) 10:1, (e) 15:1 and (f) 20:1, (g) native PMV particles.

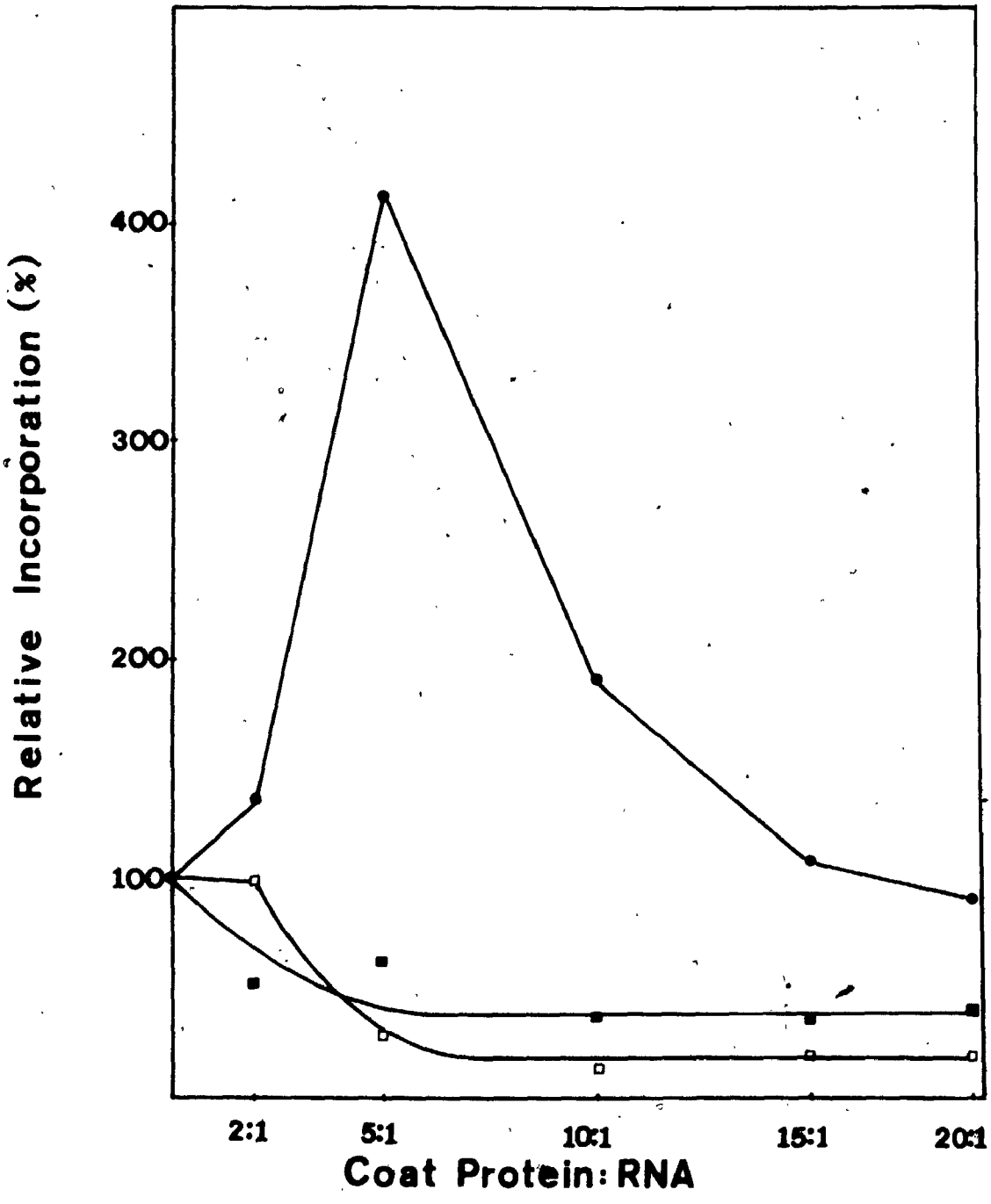

A
B
C
D
E
F
G
pA
pB
pC

presence of PMV coat protein did not alter the spectrum of products synthesized in vitro from this template in a subsequent translation experiment relative to untreated control RNA. Taken together, these controls indicate that coat protein is inhibitory to the translation of PMV RNA alone, and only when bound to it specifically.

The products synthesized from partially encapsidated templates were examined by electrophoresis. It is clear that as the fraction of coat protein in the particle increases, the PMV subparticles lose their ability to direct the synthesis of the high molecular weight products pA and pB, as well as all the minor products whose molecular weights are greater than 22,000. Most of the partially encapsidated PMV subparticles retained the ability to program the synthesis of pC, the PMV coat protein. (Figure 17, lanes b - d, corresponding to coat protein to RNA ratios of 2:1, 5:1 and 10:1). Loss of the ability to synthesize pC occurs only at higher ratios of coat protein to RNA, 15:1 and 20:1 (Figure 17, lanes e and f, respectively). For a given preparation of partially encapsidated RNAs, the results of the translation assay were essentially the same in both cell-free systems. More quantitative results were obtained by cutting out segments of the dried gel corresponding to the bands pA, pB and pC, and counting them (see Materials and Methods, Section 3.2.3). These results shown in Figure 18, are presented

Figure 18 Relative yields of pA, pB and pC from different partially encapsidated templates.

The bands corresponding to pA, pB and pC in the gel illustrated in Figure 17 were cut out, dissolved, and counted as described in Materials and Methods. The results are expressed as the percentage of counts in the band of interest, relative to the counts in the same band in lane a (purified PMV RNA as template) as a function of the coat protein to RNA ratio during reconstitution. Comparable results were obtained when the entire experiment was repeated. Symbols: Relative incorporation(%) per band in pA ■ ; in pB □ ; and in pC ● .


Relative Incorporation (%)
400
300
200
100
2:1
5:1
10:1
15:1
20:1
Coat Protein: RNA

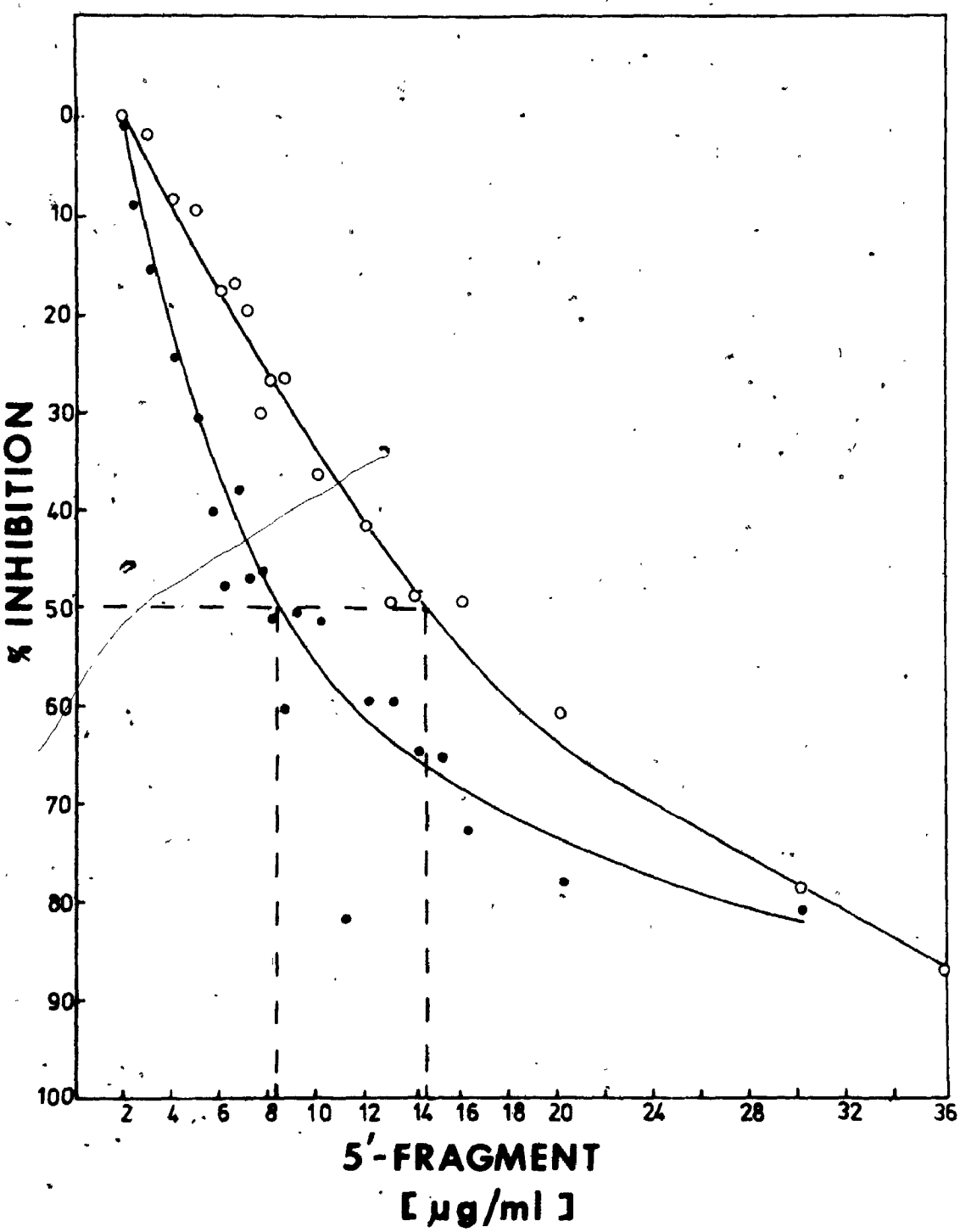
as the percentage of the radioactivity found in the same band in the unencapsidated control. The relative synthesis of the high molecular weight products pA and pB decreased sharply as the ratio of coat protein to RNA in the prior encapsidation reaction increased. More interestingly, the relative synthesis of pC was enhanced 4-fold in this experiment and by as much as 8-fold in others at intermediate ratios of coat protein to RNA (i.e., 5:1 and 10:1). At higher ratios of coat protein to PMV RNA during the encapsidation reaction (i.e., at ratios greater than 10:1), the subsequent synthesis of coat protein (pC) declined as the fraction of completed PMV particles increased. Since the assembly of PMV is polar and unidirectional (Abouhaidar and Bancroft, 1978), we conclude that the coding sequences for pA and pB, whose synthesis are first to be inhibited are localized towards the 5' end of PMV RNA. Conversely, the coding sequences for pC must be localized more towards the 3' end of PMV, or at least in a part of the genome which is not masked in the partial encapsidation reactions. Possible reasons for the enhancement of the synthesis of pC with PMV subparticles relative to native viral RNA are discussed below.

3.3.3 Relative Efficiencies of Translation of PMV RNA and of Partially Encapsidated PMV

The preceeding experiments demonstrated that partially encapsidated PMV RNA has some sequences for translation in vitro and that these sequences include the coat protein cistron. To determine whether the available sequences in partially encapsidated particles are as efficiently recognized by ribosomes as native PMV RNA, the effect of a competitor on the translational yield of each template was assayed. While m^7Gp could be used as a competitive inhibitor of ribosome binding, we chose instead to use a fragment approximately 600 nucleotide residues in length. This oligonucleotide was obtained by digestion of PMV subparticles reconstituted at a coat protein to RNA ratio of 2:1 with T1 ribonuclease (Abouhaidar and Bancroft, 1978). This fragment contains the 5' terminal cap structure (Abouhaidar and Bancroft, 1978). By itself, this fragment failed to stimulate incorporation in the wheat germ cell-free system; rather it reproducibly depressed endogenous incorporation to about half its usual level. Its advantage was that it was effective as an inhibitor at much lower concentrations than m^7Gp as its empirical Ki in my hands was .50 nM compared to a Ki for m^7Gp of 1 mM. Since each molecule of template is translated once,

Figure 19 Competitive inhibition of translation of purified or of partially encapsidated PMV RNA.

An equal amount (5 μg) of either PMV RNA or partially encapsidated PMV RNA made at a coat protein to RNA ratio of 10:1 was translated in the wheat germ cell-free system. Increasing amounts of a 600 residue long oligonucleotide containing the 5' end of PMV RNA prepared as described (Abouhaidar and Bancroft, 1978) were added to the translational assays. The incorporation of radioactivity into acid-precipitable form was measured after 90 min incubation at 25°. The results are plotted as the percentage inhibition relative to the incorporation in the absence of inhibitor as a function of the final concentration of the inhibitory fragment. The open circles denote PMV RNA as template while closed circles denote PMV subparticles assembled at a coat protein to RNA ratio of 10:1.


% INHIBITION
0
10
20
30
40
50
60
70
80
90
100
2
4
6
8
10
12
14
16
18
20
24
28
32
36
5'-FRAGMENT
[µg/ml]

on average, the translational yields in the presence of the inhibitory fragment are a crude measure of its inhibition of initiation and ribosome binding.

Figure 19 illustrates the incorporation of labelled methionine into acid precipitable material dependent on either intact native PMV RNA or on PMV partially encapsidated at a coat protein to RNA ratio of 10:1 in the presence of increasing amounts of inhibitory fragment. For native PMV RNA, 14.3 µg/ml of inhibitory fragment was required to effect 50% inhibition of incorporation. About half this concentration, 8.6 µg/ml elicited 50% inhibition of translation of partially encapsidated PMV RNA. Our results are consistent with the notion that partially encapsidated PMV RNA contains sequences which can bind to ribosomes and translational components with only slightly weaker affinities than native PMV.

3.3.4 The Integrity of PMV RNA during Translation

The preceeding experiments demonstrate that partially encapsidated PMV RNA retains some sequences available for translation *in vitro* and that these sequences include the coat protein cistron. The translation of the coat protein cistron might occur either by initiation at an internal translational initiation site at the 5' side of the coat protein

Figure 20 Analysis of the integrity of PMV RNA as a function of time of incubation in the wheat germ extract.

50 µg of PMV RNA was added to a 500 µl wheat germ reaction as described in Materials and Methods, Section 2.2.4 with the exception that 25 µM methionine replaced ^{35}S-methionine. Aliquots of 100 µl were taken at 15, 20 and 60 minute intervals then phenol extracted and ethanol precipitated. After recovery by centrifugation, samples were subjected to electrophoresis on a 1% agarose gel containing 10 mM methylmercury hydroxide, transferred to Ultrablot and then hybridized with ^{32}P -PMV cDNA as described in Materials and Methods, Section 2.2.14. The resulting autoradiogram illustrates PMV incubated in the wheat germ system for the following times: Lane a, 15 minutes; Lane b, 30 minutes; Lane c, 60 minutes.


PMV
RNA
28S
18S

cistron or alternatively via translation of RNA fragments generated by cleavage of the RNA in the in vitro translation systems.

To test the integrity of PMV RNA in the wheat germ in vitro translation system PMV RNA was added to a scaled up reaction mixture and aliquots of the reaction extracted with phenol at 15 minute intervals. The extracted RNA was then subjected to electrophoresis in 1% (w/v) agarose gels containing 10 mM methyl mercury hydroxide (Bailey and Davidson, 1976) and subsequently transferred to Ultrablot ® paper (Alwine et al., 1979, see Materials and Methods, Chapter 2, Section 2.2.14). Bound RNA was then examined for PMV RNA specific sequences by hybridization with ^{32}P-cDNA transcribed from the PMV RNA template (i.e., by "Northern" blotting). Figure 20 illustrates that PMV RNA is rapidly degraded in the wheat germ system. Within 15 minutes (lane a) under standard translation conditions, PMV RNA is converted to fragments of approximately two-thirds of the full length RNA. Full length PMV RNA is however, still detectable. Incubation of the RNA in the wheat germ system for greater lengths of time (lanes b and c) results in the complete conversion of the RNA to subgenomic fragments. This finding suggests that translation in vitro may occur in part from RNA fragments which are capable of spurious translational initiation. The translation of RNA fragments might also

explain why the minor products of PMV RNA directed translation increase with increasing time of incubation in the wheat germ extract (data not shown).

3.3.5 The In Vitro Translation of Partially Encapsidated CYMV RNA

As an extension of our experiments with PMV RNA, we tested the ability of partially encapsidated CYMV RNA to act as a template for protein synthesis. CYMV coat protein also initiates assembly specifically at the 5'-terminus and subsequently polymerizes along the RNA in the 5' to 3' direction (Abouhaidar, 1981). We prepared partially encapsidated particles at coat protein to RNA ratios of 2:1, 5:1 and 10:1. Upon their translation in the wheat germ system, these particles exhibited a progressive loss in template activity in proportion to the extent of encapsidation (of PMV) (data not shown). Figure 21 illustrates the electrophoretic separation of products directed by partially encapsidated templates in the wheat germ system.

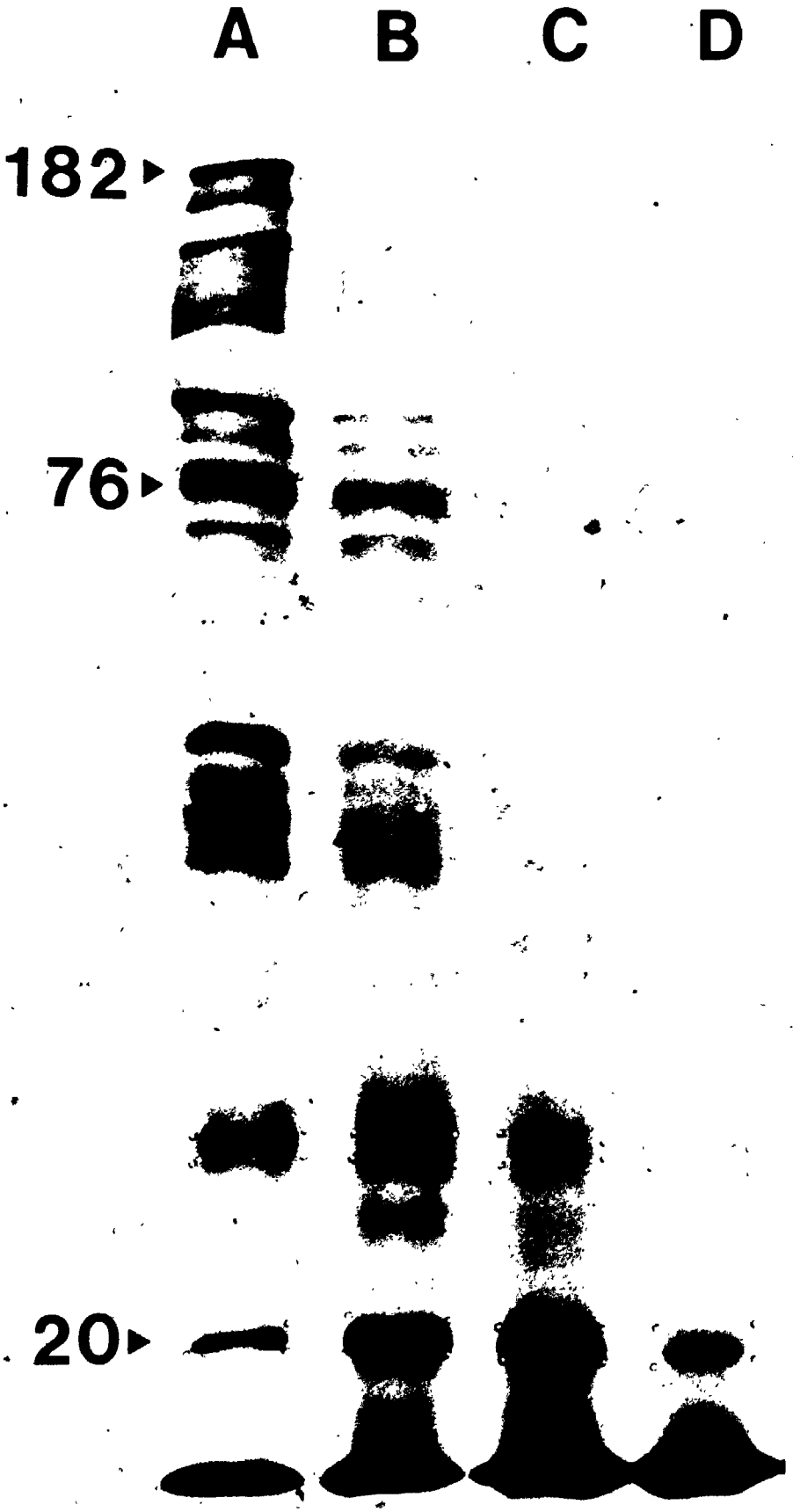
Lanes b and c demonstrate clearly that synthesis of CYMV coat protein is enhanced relative to the non-encapsidated control (lane a). Concommitantly, there is a decrease in the 182,000 dalton and 76,000 dalton products. At a coat protein to RNA ratio of 10:1, the synthesis of coat protein declines. These results imply that the coding sequences for the CYMV coat

Figure 21 The electrophoretic separation of translational products directed by partially encapsidated CYMV RNA.

The assembly of partially encapsidated CYMV subparticles and their subsequent translation in the wheat germ systems are described in Materials and Methods. Aliquots of each incubation were applied to a 10% SDS-polyacrylamide gel and the products resolved by electrophoresis.

The templates used in each case are:

a) purified CYMV RNA; (b - d), CYMV RNA partially encapsidated with purified coat protein at the following times of coat protein to RNA: (b) 2:1, (c) 5:1, (d) 10:1. The positions of the three major products are noted.


A
B
C
D
182
76
20

protein are localized towards the 3'-terminus of the CYMV genome resembling very closely the localization of the coat protein in PMV RNA.

3.4 Discussion

We have demonstrated that limited encapsidation of PMV and CYMV RNAs (i.e., at coat protein of 2:1 and 5:1) inhibits the synthesis of the respective high molecular weight products. In view of the polarized assembly of PMV and CYMV, the coding sequences for these polypeptides must be situated immediately distal to the site of initiation of encapsidation at the 5'-terminus of their respective RNAs. Under the same conditions, the synthesis of the coat protein, is markedly enhanced. This could be due to the availability of otherwise limiting components in the extracts which then become available for the synthesis of their coat proteins. Alternatively, the partial encapsidation of the viral RNAs could alter their secondary structure to favour the translation of the coat protein cistrons. This latter possibility seems unlikely, since heating PMV RNA in the presence of EDTA or magnesium ions followed by quick cooling or pretreatment of the RNA with 5 mM methylmercury hydroxide (Payvar and Schimke, 1979) and subsequent translation in vitro does not mimic the results obtained with partially encapsidated templates (data not shown). Of course we cannot rule out rapid

refolding of the template once added to the in vitro translation system. More substantial encapsidation which occurs at coat protein to RNA ratios of greater than 10:1 generates templates inactive in the synthesis of high molecular weight products. In addition, with increasing encapsidation there is a gradual inhibition in the synthesis of coat protein on either template. This is consistent with the location of the coat protein cistrons distal to those of the high molecular weight products. Hunter et. al. (1976) have shown that in TMV the coat protein cistron is also located towards the 3'-terminus of the viral RNA.

One implication of the successful synthesis of coat protein from partially encapsidated RNA is that initiation of coat protein synthesis may occur at a site distinct from the primary initiation site at the capped 5' end which presumably is the site used for the synthesis of the higher molecular weight products. The competition experiment illustrated in Figure 19 is consistent with the presence of a second translational initiation site in the 3' portion of PMV RNA. The same experiment suggests that the efficiency of this site is somewhat lower than that of the primary site. The enhancement in coat protein synthesis during translation of partially encapsidated templates suggests that

competition between these two translational initiation sites could be a factor in regulating the synthesis of the structural and non-structural proteins.

We have found that PMV RNA is cleaved within 15 minutes incubation in wheat germ extracts to approximately two-thirds its size but is stable thereafter. This suggests that translation may occur in part from fragmented RNA since incorporation of labelled amino acids into TCA-precipitable material is linear over a 90 minute period (data not shown). Therefore, as the data stand, two interpretations with regard to the synthesis of coat protein have approximately equal validity. First, coat protein is translated from fragments of the virion RNA that are produced during incubation in the cell-free system. Second, the coat protein is translated by internal initiation of the virion RNA. Possibly both of these mechanisms are functional during translation in vitro; however, it is curious that fragmentation would lead to the synthesis of coat protein with some RNAs but not others when the same extracts were used. Additionally, it is noteworthy that total RNA extracted from CYMV-infected plants (Figure 11) gave no indication of fragmentation or subgenomic RNA derived from CYMV RNA in vivo.

CHAPTER 4 - THE CONSTRUCTION OF cDNA CLONES TO PMV AND CYMV RNAs

4.1 Introduction

The translation products directed by the genomic (Chapter 2) and partially encapsidated (Chapter 3) PMV and CYMV templates suggested that the structural organization of coding sequences of these potexviruses was similar to that of TMV (Hunter et. al., 1976). First, the coding sequences for two high molecular weight non-structural proteins, whose sequences overlap, reside towards the 5' end of the genome. Secondly, the coding sequences which specify their respective coat proteins are localized towards the 3' end of the genome. Although the structural organization of coding sequences is similar among these viruses, the expression of coat protein in vitro from the genomic RNAs of PMV and CYMV suggests that, unlike TMV, their coat protein cistrons are accessible to translation. The question of whether coat protein synthesis results from translation of their respective genomic RNAs or from LMC RNAs generated in the in vitro translation systems remains unsolved.

In this chapter, we report our attempts to construct double-stranded DNA copies of PMV and CYMV RNA and their cloning in E. coli using pBR322 as a vector. We envisaged that such clones would be invaluable for several reasons. First, cDNA clones could be used to confirm the

structural organization of the coding sequences of the PMV and CYMV genomes. Secondly, cDNA clones containing the coat protein sequence of PMV and/or CYMV would provide a specific probe to analyze the nature of the RNA from which coat protein is expressed. Thirdly, cDNA prepared to specific regions of the genome would provide a more sensitive probe to determine whether small regions of homology exists between the genomes of members of the potexvirus group.

4.2 Materials and Methods

4.2.1 Chemicals

Radiochemicals were purchased from New England Nuclear (Boston, Mass.), tryptone and powdered yeast extract were from Difco (Detroit, Mich.). Oligo-dT_{12-18} and Oligo-dG_{12-18} were purchased from Collaborative Research (Waltham, Mass.). Tetracycline was obtained from Sigma and ampicillin was provided by Dr. R. Behme. Reagents for electrophoresis were obtained from Bio-Rad. All other chemicals were reagent grade and were obtained from either Sigma (St. Louis, MO.) or Fisher (Fair Lawn, N.J.). Enzymes were obtained from the following sources: AMV reverse transcriptase (a kind gift of Dr. J. Beard, Life Sciences Inc.), Ava I, Cla I, Pvu II, Sma I, Kpn I, Hinf I, Xba I, Eco RI and terminal transferase were obtained from Bethesda Research Laboratories Inc.

(Bethesda, Maryland). Pst I, Sph I, T4 DNA ligase were from Boehringer-Mannheim (Canada), polynucleotide kinase, Bgl II, and Bam H1 were from NEN (Boston, Mass.) and DNA polymerase I, Klenow fragment was from New England Biolabs (Beverly, Mass.). S1 nuclease was from Miles Laboratories.

4.2.2 Strains

The strains used in this work were all derivatives of Escherichia coli K12. Strain HB101 (hsm^-, hsr^-, $recA^-$, gal^-, pro^-, str^R) and the bacterial plasmid pBR322 were kindly provided by Dr. H. Boyer, University of California Medical Center, San Francisco. Strain RR1 (a $recA^+$ derivative of HB101) and bacterial plasmid pBR325 were kindly provided by Dr. D. Edwards and Dr. T. Linn, respectively (University of Western Ontario).

4.2.3 Media

Cultures were grown in L-broth or in M9 medium (Miller, 1972) supplemented as required with thiamine (10 mg/liter), glucose (2 g/liter), casamino acids (3 g/liter), ampicillin (25 mg/liter) or tetracycline (25 mg/liter). Antibiotic solutions were made fresh prior to each use. Cultures were stored at -20°C in supplemented M9 containing 50% (v/v) glycerol.

4.2.4 Construction of cDNA clones

4.2.4.1 First-Strand cDNA Synthesis

The synthesis of near to full length single-stranded DNA complementary to viral RNA sequences was based on several general procedures (Ullrich et al., 1977, Buell et al., 1978, Monahan et al., 1976). Reactions of 1.0 ml volume were prepared in siliconized 1.5 ml Eppendorf micro test tubes. A standard reaction mixture contained 100 mM Tris-HCl, pH 8.3, 10 mM $MgCl_2$, 30 mM β-mercaptoethanol, 1 mM each of dATP, dGTP, dTTP and dCTP, 0.5 mCi α-^{32}P-dCTP (410 Ci/mmole, Amersham), 10 µg/ml of either Oligo-dT_{12-18} or Oligo-dG_{12-18} and viral RNA at 50 µg/ml. In some cases, the RNA was incubated at room temperature with 2.5 mM methyl mercury hydroxide (Payvar and Schimke, 1979) for 3 minutes prior to its addition to the assembled components listed above containing, however, 150 mM β-mercaptoethanol instead of 30 mM. The reaction was initiated with the addition of 2.9 units of avian myeloblastosis virus reverse transcriptase per µg of RNA template in the reaction. This concentration of enzyme reflects the units of reverse transcriptase per input RNA which produced the maximal yield of cDNA (Refer to Figure 23). After incubation at 42°C for 60 minutes, the reaction was terminated by the addition of 20 mM EDTA, pH 8.0. Subsequently, the reaction was deproteinized by the

addition of one-half volume of phenol saturated with TEN-9 (20 mM Tris-HCl, pH 9.0, 1 mM EDTA, 100 mM NaCl), followed by one-half volume of chloroform: isoamyl alcohol (24:1) (Goodman and MacDonald, 1979). After vortexing, the aqueous phases were recovered by centrifugation in an Eppendorf centrifuge at 12,000 rpm for 30 seconds. The lower organic phase was again extracted with one-half volume of TEN-9 and the aqueous phase again treated with an equal volume of chloroform: isoamyl alcohol (24:1), vortexed and the phases separated as before. The two aqueous phases were combined and loaded directly on a Bio-gel ® A 1.5 m column (0.9 x 15 cm). Seven drop fractions (approximately 300 µl) were collected at room temperature. Fractions containing the cDNA-RNA hybrid were localized by Cerenkov radiation, pooled, adjusted to 0.2 M NaCl and precipitated at -20°C for 12 hours after addition of 2.5 volumes of 95% (v/v) ethanol. Precipitates were recovered by centrifugation in a Sorvall ® HB-4 rotor at 10,000 rpm for 60 min at 4°C and washed twice with 70% (v/v) ethanol prior to resuspension. Yields were calculated according to the following formula:

$$\% \text{ yield} = \frac{(\text{cpm incorporated}_{t60} - \text{cpm incorporated}_{t0})}{\text{S.A. (cpm/µmole)}} \times \text{mass of 1 µmole of incorporated deoxynucleoside monophosphate} \times 4 \Big/ \text{mass input RNA} \times 100$$

4.2.4.2 Second-Strand cDNA Synthesis

The synthesis of a second DNA strand complementary to and primed from the first DNA strand was accomplished using either AMV reverse transcriptase or DNA polymerase I (Klenow fragment). When DNA Polymerase I (Klenow fragment) was used, the purified cDNA-RNA hybrid was resuspended in 0.5 mM EDTA, pH 7.5, then heated at 100°C for 2 minutes. Immediately thereafter, the sample was quick-cooled in an ice-water bath, then added directly to an assembled reaction containing the following components: 100 mM Hepes-NaOH (pH 6.9), 70 mM KCl, 5 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM each of dATP, dGTP, dCTP and dTTP, 1 unit of DNA Polymerase (Klenow fragment) per 10 ng cDNA and cDNA at 2 µg/ml. The reaction was then incubated at 15°C for up to 18 hours. (Bothwell et. al., 1981). This incubation time yielded the maximum length of second strand material. When AMV reverse transcriptase was used for second strand synthesis, the first cDNA strand was purified from the cDNA-RNA hybrid by hydrolysis of the RNA component in 0.1 N NaOH at 70°C for 20 minutes (Goodman & MacDonald, 1979). After neutralization with HCl and ethanol precipitation the cDNA was added at a concentration of 40 µg/ml to the following reaction: 50 mM Tris-HCl, pH 8.3, 8 mM $MgCl_2$, 20 mM dithiothreitol, 0.4 mM each of dATP,

dGTP and dTTP, 0.1 mM dCTP and AMV reverse transcriptase at 934 units/ml (Humphries et. al., 1978). The reaction was incubated at 42°C for 2 hours.

Both the DNA polymerase and the AMV reverse transcriptase reactions were terminated by addition of EDTA, pH 8.0 to 20 mM. Purification of the double-stranded cDNA by phenol extraction and column chromatography was accomplished in the same manner described for first strand synthesis. The yield of second strand was calculated as previously described.

4.2.4.3 S1 Nuclease Digestion

A consequence of priming second strand synthesis from the first strand is the generation of a hairpin loop at one end of the double-stranded cDNA which consists of unpaired single-stranded DNA (Ullrich et. al., 1977). This hairpin loop structure was removed from the double-stranded cDNA by digestion with Aspergillus oryzae single-strand specific nuclease S1. Digestions were carried out for 30 minutes at 25°C in a reaction mixture of 0.1 ml or less containing 30 mM sodium acetate, ph 4.5, 0.3 M NaCl, 4.5 mM $ZnCl_2$, 5 µg/ml double-stranded cDNA and S1 nuclease at 1200 units/ml (Ullrich et. al., 1977, Goodman and MacDonald, 1975). This concentration of S1 nuclease was the maximal amount

which would not degrade linear pBR322 (data not shown). The reaction was terminated by addition of 1 M Tris-HCl, pH 9.0 to 100 mM and 0.25 M EDTA, pH 8.0 to 25 mM.

The reaction was then phenol extracted as described previously, then extracted twice with 8 volumes of ether. The double-stranded cDNA contained in the aqueous phase was then ethanol precipitated and recovered as previously described. In some cases after phenol extraction, the aqueous DNA-containing phase was loaded directly onto a Bio-gel A150 m® (1.0 cm x 35 cm) column equilibrated in 0.15 M NaCl, 1 mM EDTA. Appropriate fractions were pooled and ethanol precipitated at -20°C.

4.2.4.4 Tailing of duplex cDNA and vector pBR322

The 3'-terminal ends of duplex cDNA were extended with homopolymeric tracts of deoxycytidylate residues using calf thymus terminal deoxynucleotidyl transferase (Roychoudhury et al., 1976). The reaction was carried out in enzyme excess using the following reaction conditions: 0.14 M cacodylic acid, 0.03 M Tris, pH 6.9, 100 µM dCTP, 25 µCi ^{3}H-dCTP, 100 µM DTT, 10 pmoles cDNA 3' ends per ml, 1 mM $CoCl_2$ and approximately 4 units of terminal deoxynucleotidyl transferase per pmole 3' DNA ends. The reaction was incubated at 37°C. The reaction was stopped after 5 to 10 min by placing the sample on ice from which an aliquot was removed and precipitated with TCA. If the reaction had not progressed sufficiently

it was returned to 37°C. Generally the reaction was terminated after 15 to 30 residues were added. The vector pBR322 was linearized by Pst I digestion. Typically 10 µg of pBR322 was added to 50 µl of restriction buffer composed of 10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl and 6 mM β-mercaptoethanol. The DNA was then digested with 12 units of Pst I for 6 hours. The reaction mixtures were extracted with phenol: chloroform: isoamyl alcohol (25:24:1) followed by an ether extraction of the aqueous phase. The DNA was then ethanol precipitated as previously described. The 3'-terminal ends were similarly extended with deoxyguanidylate residues under identical reaction conditions with the exception that dGTP substituted for dCTP. Again, the reaction was terminated after the addition of 15 - 30 residues/3' end.

When tailing of the vector or insert was acceptable the reaction was stopped by the addition of 0.1 volumes of 0.25 M EDTA, pH 8.0. The reaction was then extracted with phenol: chloroform: isoamyl alcohol (25:24:1) as previously described. The samples were then precipitated with ethanol.

4.2.4.5 Annealing of Tailed Insert and Vector pBR322

After recovery by centrifugation, the tailed vector and insert sequences were mixed in equimolar amounts or in amounts up to a 5-fold molar excess of vector in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA and 100 mM

NaCl at a total DNA concentration of 1 µg/ml. Annealing was then achieved by successive incubations at 65°C for 5 minutes, 42°C for 2 hours, 30°C for one hour and 15°C for 1 hour. Samples were made, 0.2 M in NaCl and 10 µg/ml yeast RNA, then precipitated at -20°C with 2.5 volumes of 95% (v/v) ethanol.

4.2.5 Cloning double-stranded cDNA by restriction and ligation

Digests of both double-stranded cDNA and the plasmids pBR322 and pBR325 were performed at 37°C for 6 - 12 hours in a standard restriction buffer containing 10 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$ and 6 mM β-mercaptoethanol. NaCl was added separately in accordance with the enzyme's requirements specified by the supplier. In general, a two to threefold excess of enzyme was used in each experiment. After digestion samples were extracted with phenol: chloroform: isoamyl alcohol (25:24:1) and ether, then recovered by ethanol precipitation as previously described.

Following digestion and recovery, linearized plasmid DNAs were treated with alkaline phosphatase (Chaconas and van de Sande, 1980) to prevent their recircularization. Reactions of 0.1 ml contained 50 mM Tris-HCl, pH 9.5, 1 mM $MgCl_2$, 1 mM $ZnCl_2$ 10 mM spermidine, 10 µg linearized plasmid DNA and 1 unit of calf intestine alkaline phosphatase.

Digestions were performed at 37°C for 30 minutes, after which time an additional 1 unit of calf intestine alkaline phosphatase was added. After 60 minutes total incubation, the reaction was made 20 mM in EDTA, pH 8.0, then extracted and ethanol precipitated as previously described. Samples were washed three times with 70% ethanol prior to resuspension in 10 mM Tris-HCl; 1 mM EDTA, pH 7.5 to a concentration of 50 ng/µl.

The linearized plasmid vector and restriction enzyme digested double-stranded cDNA were then ligated in a 20 µl reaction with the following composition: 50 mM Tris-HCl, pH 7.5, 0.04% (w/v) gelatin, 10 mM DTT, 0.26 mM ATP, 50 ng of digested double-stranded cDNA, 150-190 ng of linearized plasmid DNA and 1 - 2 units of T4 DNA ligase. The assembled reaction was incubated at 15°C for a minimum of 6 hours, then stored at -20°C until used for transformation.

4.2.6 Transformation of E. coli strains RR1 and HB101

During the course of this work numerous transformation procedures were tested in order to attain the maximal transformation efficiency possible (i.e. number of transformants/µg DNA added). In this regard, we found the procedure of Kushner, (1978) to be superior. Briefly, a 10 ml culture of E. coli strain HB101 or RR1 was grown in L-broth to an OD_{600} = 0.4 (approximatly 3 x

10^8 cells/ml). Two ml of culture (6 x 10^8 cells) was centrifuged at 8000 rpm for 10 minutes in an SS34 rotor. The supernatant was removed immediately and the cell pellet gently resuspended in 1 ml of 10 mM MOPS, pH 7.0 containing 10 mM RbCl. The suspended cells were immediately centrifuged as above. The supernatant was removed quickly and the pellet resuspended in 1 ml of 100 mM MOPS, pH 6.5, 50 mM $CaCl_2$, and 10 mM RbCl (Buffer B). The cells were held on ice for 30 minutes, centrifuged as above, and resuspended in 1.2 ml of buffer B. 3 µl of dimethylsulfoxide and up to 0.2 µg of DNA in 20 µl were then added. The mixture of cells and DNA was incubated on ice for 30 minutes, shocked at 43°C for 30 seconds and finally diluted with L-broth to a final volume of 5 ml. The cells were subsequently incubated at 37°C for 60 minutes to allow expression of antibiotic resistance. From 50 µl to 100 µl of the transformed cell suspension was plated with 2.5 mls of 0.75% (w/v) overlay agar on 100 mm L-broth agar plates containing 20 g/ml tetracycline.

Typical transformation efficiencies using the plasmid pBR322 were 1.6 x 10^6 and 2.4 x 10^7 transformants/µg DNA in E. coli strains HB101 and RR1 respectively.

4.2.7 Isolation of Plasmid DNA

Cultures harbouring the plasmid of interest were grown to mid-log phase (OD_{600} of 0.5) in L-broth or supplemented M9 medium prior to the addition of chloramphenicol to 160 µg/ml. Cells were harvested 14 to 16 hours later. Circular plasmid DNA was isolated by dye-buoyant density centrifugation of the cleared lysate (Clewell, 1972). Plasmid DNA was ultimately dissolved in 10 mM Tris-HCl, 1 mM EDTA, pH 7.5 at concentrations of 200 to 400 µg/ml and stored at 4°C.

4.2.8 Electrophoresis of Nucleic Acids

Polyacrylamide gel electrophoresis was carried out as described by Maniatis et. al. (1975), using the TBE (90 mM Tris base, 90 mM borate, 25 mM EDTA, pH 8.3) buffer system. Electrophoresis was carried out at either 40V (constant voltage) for 15 hours or 100V for 6 hours in vertical gels. Neutral or alkaline agarose gel electrophoresis was executed in buffer systems composed of 40 mM Tris base, 20 mM acetic acid and 2 mM EDTA or 30 mM NaOH, 2 mM EDTA respectively (McDonnel et. al, 1977). Horizontal gels were run at 40V (constant voltage) for 15 hours. After electrophoresis, gels were stained with 0.5 µg/ml ethidium bromide prior to visualization of DNA under U.V. light at 302 n.m. The alkaline agarose gel system was neutralized by soaking the gel in 0.5 M Tris-HCl, pH 7.5 prior to staining.

DNA was extracted from polyacrylamide gel slices by the diffusion method of Maxam and Gilbert (1979) and from neutral agarose gels with the chaotropic agent $NaClO_4$ followed by retention of the DNA on glass-fiber filters (Chen and Thomas, 1980).

4.2.9 In Situ Colony Hybridization

4.2.9.1 Immobilization of DNA on Nitrocellulose

Cell colonies containing plasmids with cDNA inserts were detected using modifications (Chang et al., 1978, Buell et al., 1979 and Wickens et al., 1979) of the in situ colony hybridization procedure (Grunstein and Hogness, 1975). Individual tetracycline-resistant, ampicillin-sensitive colonies were inoculated into 2 ml of L-broth and 10 µg/ml tetracycline and grown at 37°C overnight. 2 µl of each culture was spotted in ordered arrays on nitrocellulose filters placed on L-broth agar plates containing 10 µg/ml tetracycline. The plates were incubated at 37°C until colonies were 3 to 4 mm in diameter (approximately 24 hours). Subsequently, the plasmid DNA of the filter-bound colonies was amplified by transferring the filter onto L-broth agar plates containing 10 µg/ml tetracycline and 250 µg/ml chloramphenicol, followed by further incubation at 37°C

for 18 hours. Colonies were then lysed *in situ* by placing the nitrocellulose filter for 10 minutes on a pad of 4 layers of Whatman® 3 MM paper saturated in 0.5 N NaOH. For neutralization and immobilization of the DNA on the filter, the nitrocellulose filter was transferred sequentially to a series of similar pads saturated in 1 M Tris-HCl, pH 7.5, (twice, 5 minutes), then 0.5 M Tris-HCl, pH 7.5, 1.5 M NaCl (twice, 10 minutes). The filter was washed with 95% (v/v) ethanol, air dried for 15 minutes, then baked at 80°C for 3 hours *in vacuo*. Filters were then stored at room temperature until used.

4.2.9.2 Preparation of Labeled Nucleic Acids

i) End-labeling

^{32}P-end-labeled viral RNA fragments were prepared as described by Gergen *et. al.*, 1979. First, up to 5 µg of viral RNA was partially hydrolyzed by incubation at 90°C for 30 minutes in 90 µl of 5 mM Tris-HCl, pH 9.5, 10 µM EDTA and 0.1 mM spermidine. This partial hydrolysate was transferred to an eppendorf microtest tube containing 50 µCiγ-^{32}P-ATP dried *in vacuo*. The ATP concentration was adjusted to yield a final concentration of 1 µM. The reaction was adjusted to 50 mM Tris-HCl, pH 9.5, 5 mM DTT and 10 mM $MgCl_2$. 0.5 µl (5 units) of polynucleotide kinase was then added and the reaction incubated at 37°C for 30 minutes. The reaction

was terminated by the addition of 20 mM EDTA, pH 8.0 and extraction with phenol: chloroform: isoamyl alcohol as previously described. The end-labeled material was purified by gel filtration on a 0.9 cm x 30 cm Bio-gel ® A 1.5 m column as previously described (Section 4.2.4.1). After purification the end-labeled material was stored frozen until used. Routinely, specific activities of 0.7 - 1.0 x 10^6 cpm/µg RNA were obtained.

ii) Nick Translation

DNA fragments extracted from gels were nick-translated using the protocol provided by Bethesda Research Laboratories in their Nick Translation Kit. At the end of the reaction labeled DNA was purified by either chromatography on a 0.9 x 25 cm column of Bio-gel A 1.5 m ® equilibrated in 10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0 or by three successive precipitations with 10 mM spermine (Hoopes and McClure, 1981).

4.2.9.3 Hybridization of Immobilized DNA with Labeled RNA

Hybridizations were executed in 150 x 15 mm silane-coated glass petri dishes. Prior to hybridization, filters were incubated at 65°C in 15 ml of buffer H1 composed of 5 x SSC, 1 x Denhardt's solution (Denhardt, 1966) (0.02% (w/v) Ficoll 400, 0.02% (w/v) polyvinyl pyrrolidone, and 0.02% (w/v) bovine serum albumin), 100

µg/ml E. coli tRNA, 100 µg/ml poly A and 0.02% (w/v) SDS (Wickens et al., 1979). After 12 hours this solution was replaced with 10 mls of buffer H1 containing ^{32}P-end-labeled-fragmented RNA (5.0×10^5 - 1.0×10^6 cpm), and incubated at 65°C for 24 hours. After hybridization, the filters were washed six times (1 hour each) in 20 mls of buffer H1 then twice (30 minutes each) in 20 mls of 2 x SSC at room temperature. Filters were subsequently incubated in 10 mls of 2 x SSC containing 10 µg/ml RNAse A for 30 minutes at room temperature followed by one wash in 20 mls of 2 x SSC for 30 minutes at room temperature. Finally, filters were blotted on Whatman ® 3 MM paper, air dried, then used to expose Kodak ® X-Omat AR film with the aid of an intensifying screen for 12 to 48 hours at -70°C.

4.2.9.4 Hybridization of Immobilized DNA with Labeled DNA

Prior to incubation with labeled probe, filters containing the immobilized DNA were incubated for 12 hours at 68°C in 15 ml of buffer H2 (150 mM Tris-HCl, pH 8.0, 0.75 M NaCl, 5 mM EDTA, 1 x Denhardts, and 0.1% (w/v) SDS) (Hanahan and Meselson, 1980) containing 100 µg/ml poly A and 1 mg/ml sheared and denatured calf thymus DNA. After this period, this mixture was replaced with 10 ml of hybridization buffer containing DNA fragments labeled with ^{32}P by nick translation. The

filters were again incubated at 68°C for 12 - 24 hours. After hybridization the filters were washed five times (1 hour each) in 100 ml of 60 mM Tris-HCl, pH 8.0, 0.3 M NaCl, 2 mM EDTA and 0.5% (w/v) SDS at 68°C. This was followed by 4 washes (15 minutes each) with 3 mM Tris base at room temperature (Hanahan and Meselson, 1980). The filters were dried and autoradiographed as before.

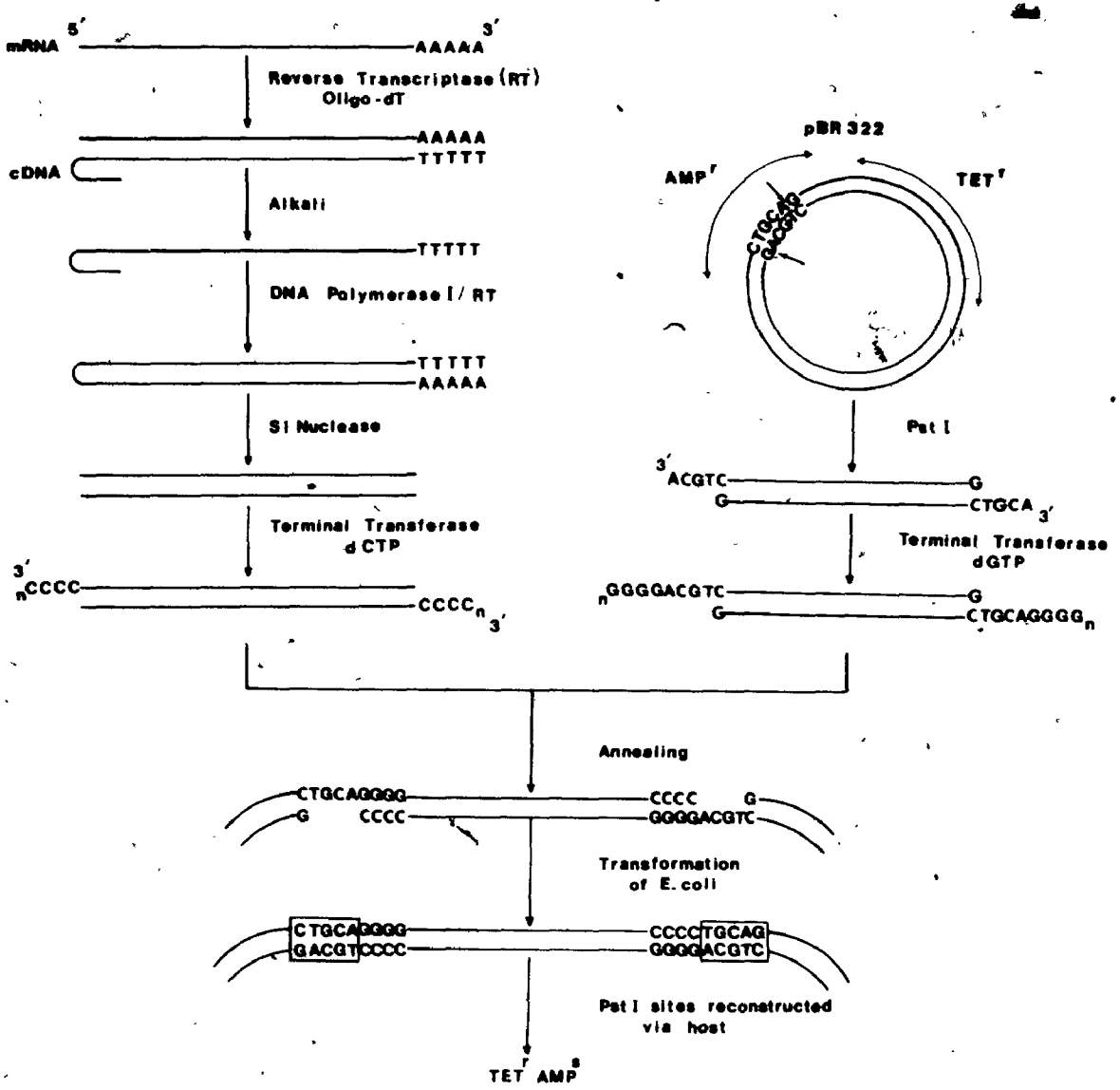
4.3 Results

4.3.1 Characterization of Enzymatic Reactions

i) First-Strand Synthesis

Figure 22 illustrates the numerous enzymatic steps required to convert a single-stranded RNA template into a double-stranded DNA with ends suitable for cloning into a vector, in this case the plasmid pBR322. A survey of the literature indicates that no consensus exists for optimal reaction conditions for the first enzymatic step in the series, the transcription of a single-stranded DNA complementary to its RNA template by avian myeloblastosis virus reverse transcriptase. Among the parameters which may affect this reaction, the quality of the reverse transcriptase, the enzyme to RNA template ratio, and secondary structure in the template itself are probably most important. We therefore examined these parameters as follows. Serial dilutions of the AMV reverse

Figure 22 General scheme of the synthesis of double-stranded DNA complementary to poly A^+ RNA and its subsequent ligation into pBR322.

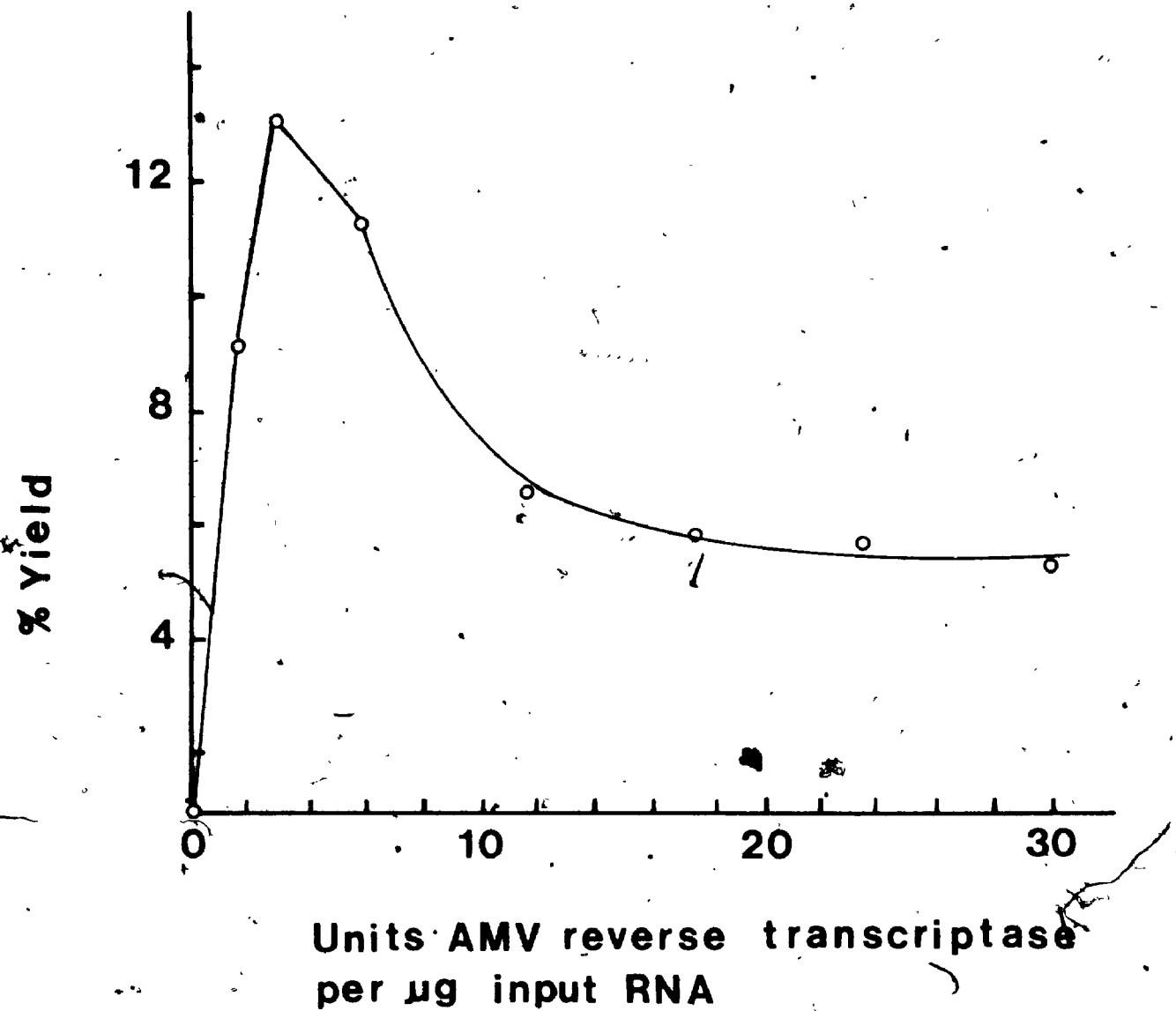

mRNA 5′
AAAAA 3′
Reverse Transcriptase (RT)
Oligo-dT
AAAAA
TTTTT
cDNA
Alkali
TTTTT
DNA Polymerase I / RT
TTTTT
AAAAA
S1 Nuclease
Terminal Transferase
d CTP
3′ nCCCC
CCCCn 3′
pBR 322
AMPr
TETr
CTGCAG
GACGTC
Pst I
3′ ACGTC
G
G
CTGCA 3′
Terminal Transferase
dGTP
nGGGGACGTC
G
G
CTGCAGGGGn
Annealing
CTGCAGGGG
CCCC G
G CCCC
GGGGACGTC
Transformation
of E. coli
CTGCAGGGG
GACGTCCCC
CCCCTGCAG
GGGGACGTC
Pst I sites reconstructed
via host
TETr AMPs

transcriptase were tested against a fixed amount of CYMV RNA to determine the ratio of enzyme to RNA which provided the maximal yield of transcript. Figure 23 illustrates that the maximal yield was obtained at 2.9 units of AMV reverse transcriptase/µg RNA. The yield rapidly declined however as the concentration of enzyme in the reaction increased beyond the optimum. This decline may be due to the presence of a contaminating ribonuclease activity in the AMV reverse transcriptase preparation. Nonetheless, the inclusion of ribonuclease inhibitors such as sodium pyrophosphate, vanadyl ribonucleoside complex or human placental ribonuclease inhibitor in the reverse transcription reaction did not increase the transcript yield even at the optimal enzyme/RNA ratio (data not shown). In fact, human placental RNAse inhibitor (2 - 10 µg ml) had no effect on yield whereas sodium pyrophosphate (1 - 5 mM) and vanadyl-ribonucleoside complex (2-5 mM) were found to be inhibitory. Recently, it has been suggested that vanadyl ribonucleoside complex may be helpful if used below 1 mM (Maniatis et. al., 1982).

Another factor which may affect transcript yield and size is the secondary structure of the template RNA. To examine this, we treated CYMV RNA with methyl-mercury hydroxide (Payvar and Schimke, 1979) prior to its addition into the reverse transcriptase reaction. Table 4 illustrates that treatment of CYMV RNA with 2.5 mM

Figure 23 The effect of AMV reverse transcriptase concentration on the yield of first-strand cDNA.

25 µl reverse transcription reactions were assembled on ice and each contained components at concentrations listed in Materials and Methods, Section 4.2.4.1 with the exceptions that the CYMV RNA template was added to a concentration of 80 µg/ml, Oligo-dT to 100 µg/ml and ^{3}H-dCTP (0.2 Ci/mmole) substituted for ^{32}P-dNTP. The reaction was initiated by the addition of 5 µl of AMV reverse transcriptase (11,700 U/ml) or 5 µl of an appropriate dilution of the enzyme in enzyme storage buffer (0.2 M KPO_4, pH 7.2, 50% glycerol, 0.2% Triton X-100 and 2 mM DTT). Samples were incubated for 60 minutes at 42°C, then TCA precipitated, filtered and counted. Yields were calculated as described in Materials and Methods, Section 4.2.4.1.


% Yield
12
8
4
0
10
20
30
Units AMV reverse transcriptase
per µg input RNA

methylmercury hydroxide (line 3) enhanced the transcript yield over that obtained with no treatment. Treatment with increasing concentrations of methylmercury hydroxide (lines 4 and 5) resulted in lower transcript yields, those at 10 mM being below the control (line 2). The addition of β-mercaptoethanol to 150 mM rather than to 30 mM in the reverse transcription reaction was necessary to complex free methylmercury. This increased concentration of reducing agent as shown in Table 4 (line 2) has no adverse effect on the reverse transcription reaction. To determine whether methylmercury hydroxide treatment had any effect on transcript size, the transcripts were resolved by electrophoresis on alkaline 1% agarose gels. Molecular weight markers were provided by a HindIII digest of λ plac 5 DNA and a HindIII and HincII digest of pBR322. Figure 24, lane b demonstrates that pre-treatment of the RNA with 2.5 mM methylmercury hydroxide substantially increases the proportion of transcripts whose size is 6600 nucleotides which roughly corresponds to the full length of the RNA. Examination of the transcripts made from untreated RNA (Figure 24, lane a) reveals that the transcripts are heterogenous in length. The presence of several discrete transcripts of less than full length suggests that secondary structure in the RNA may arrest reverse transcription prematurely. We cannot, however, rule out the suggestion (Payvar and Schimke, 1979) that methylmercury hydroxide may also act as a

Table 4. The effect of methylmercury hydroxide on first-strand synthesis:

Condition[a]	Total cpm ($\times 10^5$) Incorporated[b]	% yield[c]
No treatment, 30 mM β-mercaptoethanol	1.63	13.4
No treatment, 150 mM β-mercaptoethanol	1.67	13.7
2.5 mM methylmercury hydroxide[d]	2.10	17.2
5.0 mM methylmercury hydroxide	1.71	14.0
10.0 mM methylmercury hydroxide	1.40	11.5

a. Reaction mixtures were assembled on ice omitting CYMV RNA which was added last. 5 μg of RNA was treated with an appropriate concentration of methylmercury hydroxide or an equivalent volume of water and then transferred to the reaction mixture within one minute (Payvar and Schimke, 1979). Reactions were incubated at 42°C for 60 minutes then stopped by the addition of EDTA, pH 8.0, to 20 mM and chilling on ice.

Table 4. cont'd

b. Total incorporation was determined by precipitation of 10 µl aliquots of reactions with 5% (w/v) TCA. Values represent the average of duplicate samples.

c. A yield of 100% would correspond to 1700 pmoles of dCMP incorporated per pmole RNA whose total length is assumed to be 6800 nucleotides. Specific activity of dCTP was 3.0×10^8 cpm/µmole.

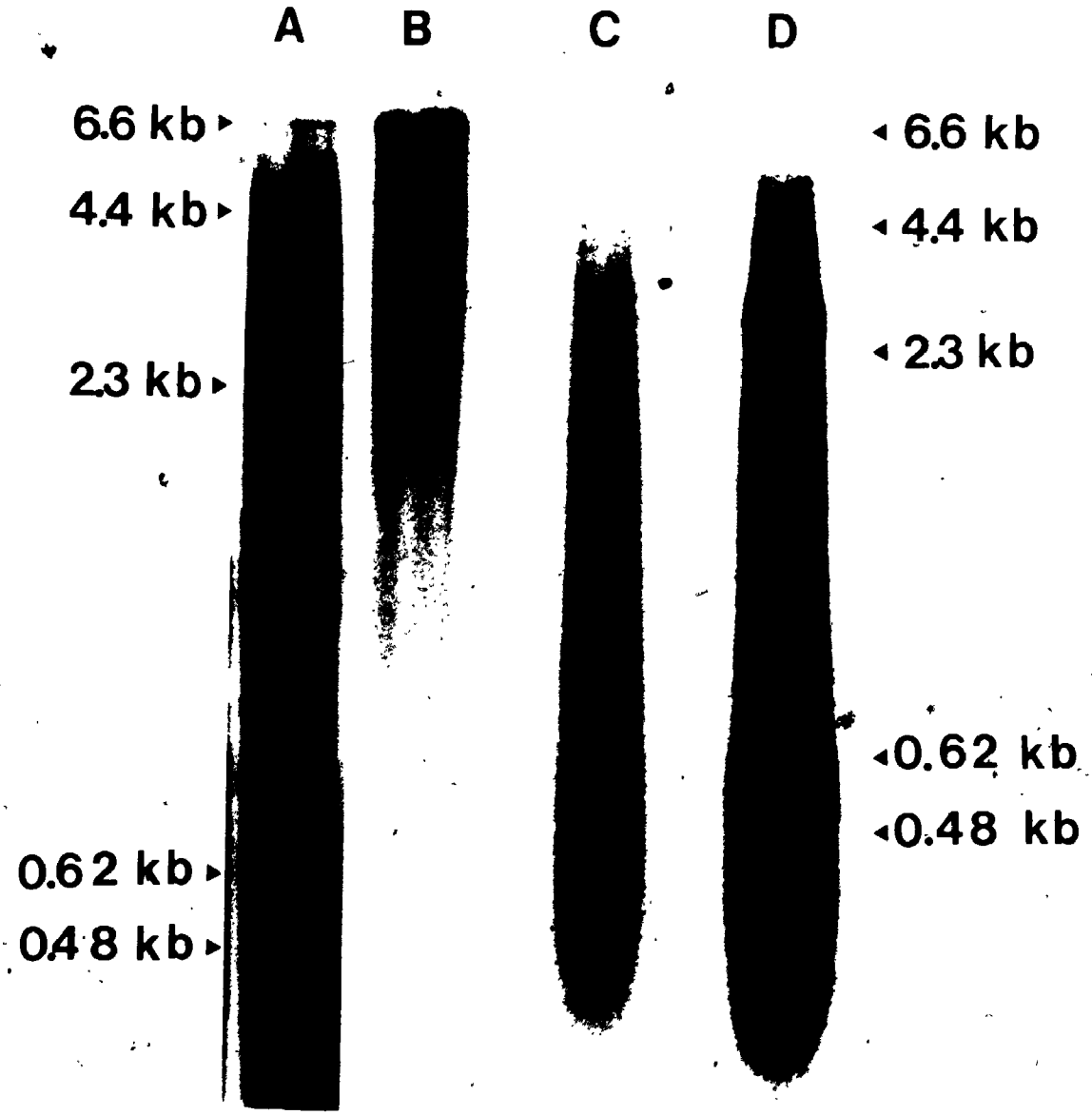
d. All reactions with methylmercury hydroxide treated RNA contained 150 mM β-mercaptoethanol.

nuclease inhibitor. Similarly, Figure 24 displays the size distribution of transcripts made in the presence of 2.5 mM methylmercury hydroxide by internal priming of the non-polyadenylated RNA of PMV. Lane c illustrates the size distribution of transcripts made from PMV RNA using Oligo-dT_{12-18} as a primer for the enzyme. The resultant transcripts are heterogeneous in size, with no discrete transcripts formed. Also, the majority of the transcripts appear to be below 1000 nucleotides in length. The priming of reverse transcription of PMV RNA with Oligo-dG_{12-18} also resulted in a very heterogenous transcript population (Lane d). Although a large proportion of transcripts are below 1000 nucleotides, transcripts from 2000 to 4000 nucleotides are evident.

The treatment of CYMV RNA with methylmercury hydroxide prior to first-strand synthesis was found to be necessary in order to produce full length transcripts. As a consequence of this observation, the heterogeneous size distribution of transcripts made from PMV RNA are likely due to multiple internal sites to which the primer hybridizes rather than premature arrest of transcription. The transcript yield, although adequate, was relatively low in comparison to other studies but may be a consequence of the sequence of these particular templates. Under identical conditions, the transcript yield from PMV RNA (Oligo-dT or dG primed) never exceeded 6% whereas that from the CYMV RNA was maximally 17%.

Figure 24 Electrophoretic separation of cDNA made from CYMV or PMV RNA.

Single stranded ^{32}P-labeled cDNAs were prepared in 50 µl standard reaction mixtures containing 150 mM β-mercaptoethanol, then purified (Materials and Methods, Section 4.2.4.1) prior to electrophoretic separation. Approximately 10 000 Cerenkov cpm were applied to each lane of a 1% alkaline agarose gel. Lanes a and b illustrate the size distribution of cDNA products made from either CYMV RNA (lane a) or CYMV RNA treated with 2.5 mM methylmercury hydroxide prior to addition to the reaction (lane b). Lanes c and d illustrate the size distribution of cDNA products made from PMV RNA in the presence of Oligo-dT_{12-18} (lane c) or Oligo-dG_{12-18} (lane d) as primers. In both reactions, PMV RNA was treated with 2.5 mM methylmercury hydroxide prior to addition. Molecular weight markers provided by a HindIII digest of λplac 5 DNA and a HindIII and HincII digest of pBR322 are noted in the margins.


A
B
C
D
6.6 kb
4.4 kb
2.3 kb
0.62 kb
0.48 kb
6.6 kb
4.4 kb
2.3 kb
0.62 kb
0.48 kb

ii) Second-Strand Synthesis

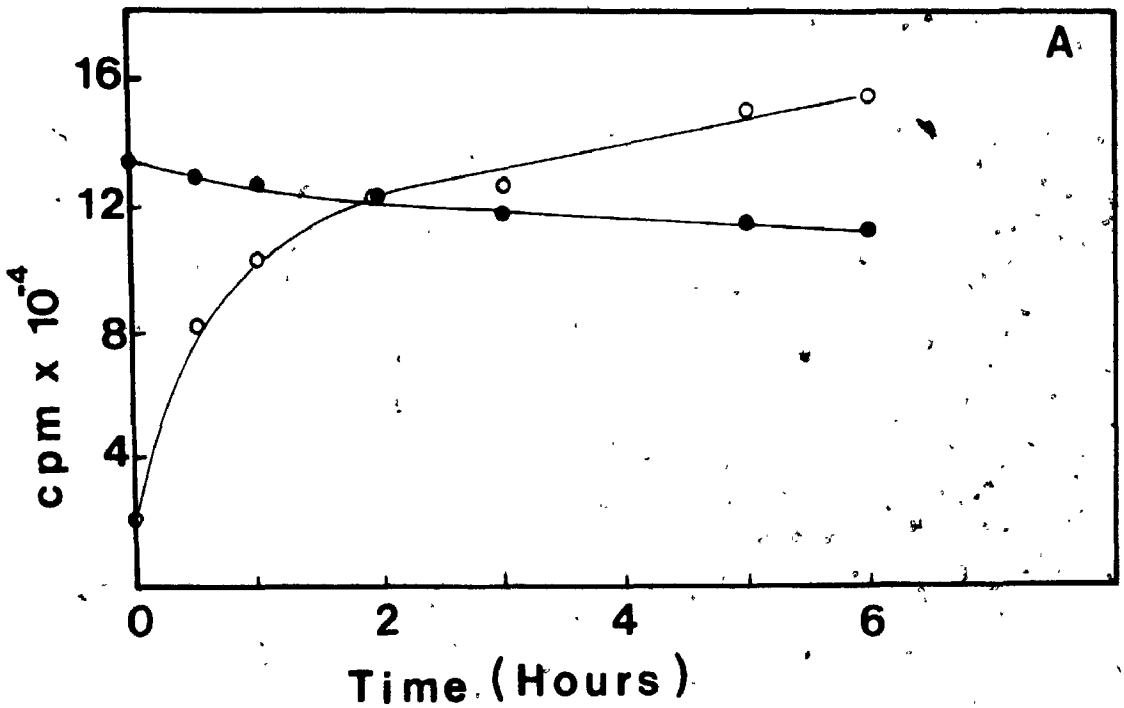
A property of first-strand cDNA synthesis is the generation of a double-stranded 3'-terminal hairpin structure (Leis and Hurwitz, 1972) (refer to Figure 22). This hairpin structure can be utilized as a primer for the synthesis of second-strand cDNA using AMV reverse transcriptase, DNA Polymerase I (POL-1) or the large fragment of DNA Polymerase I (POL-1A) lacking the 5' → 3' exonuclease activity. (Efstratiadis et. al., 1976, Ullrich et. al., 1977)

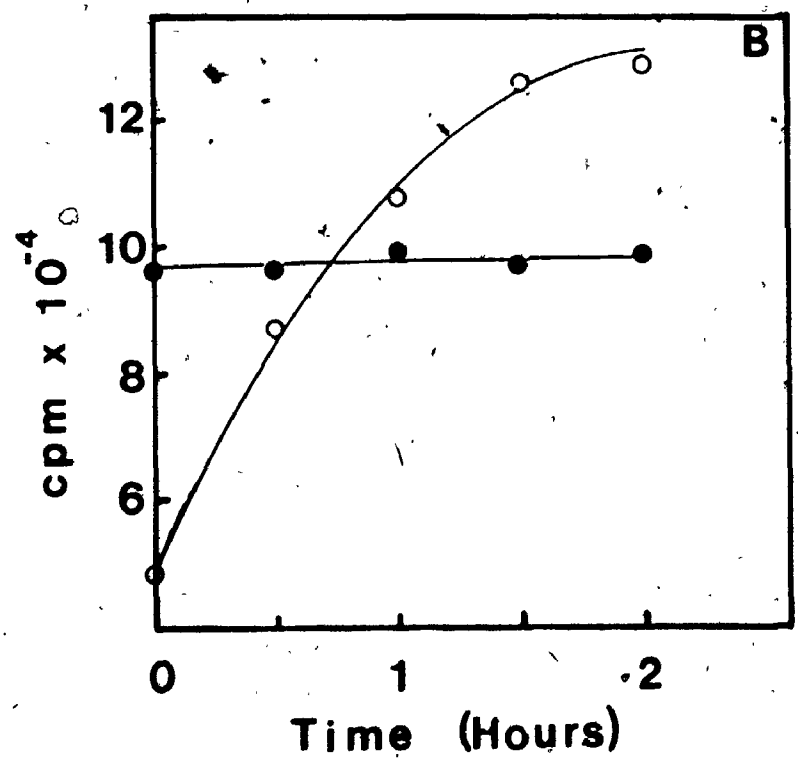
The ^{32}P-labeled cDNA was purified by gel filtration then released from the RNA (see Materials and Methods, Section 4.2.4.2), prior to use as a template for second-strand synthesis. Figure 25 illustrates the time course of ^{3}H-dCMP incorporation into CYMV second-strand material using either POL-1A (Figure 25a) or AMV reverse transcriptase (Figure 25b).

The POL-1A reaction appears to be approaching the completion of synthesis by six hours while that catalyzed by AMV reverse transcriptase is complete after two hours. The fate of the first-strand cDNA during second-strand cDNA synthesis was monitored in each case. As shown in Figure 25a there is a slight loss of TCA precipitable material from the first-strand cDNA when POL-1A was used in second-strand cDNA synthesis. This indicated that nucleolytic activity may be present in the

Figure 25 The time course of second-strand cDNA synthesis using either AMV reverse transcriptase or POl-1A.

0.5 µg of purified ^{32}P-labeled CYMV cDNA was added to either the POl-1A or AMV reverse transcriptase reaction mixtures (Materials and Methods, Section 4.2.4.2) containing ^{3}H-dCTP (0.2 Ci/mmole). The POl-1A reaction (Panel a) was incubated at 15°C whereas the AMV reverse transcriptase reaction (Panel b) was incubated at 42°C. Aliquots of the reactions were taken at the indicated times, then TCA precipitated, filtered and counted. Open circles correspond to second-strand cDNA synthesis (^{3}H cpm) whereas closed circles represent the first-strand cDNA (^{32}P cpm).


A
16
12
8
4
cpm x 10^{-4}
0
2
4
6
Time (Hours)


B
12
10
8
6
cpm x 10^{-4}
0
1
2
Time (Hours)

POL-1A preparation. Within the period of second-strand cDNA synthesis using AMV reverse transcriptase (Figure 25b) the first-strand cDNA appears stable.

The test of Goodman and MacDonald (1979) was applied to determine whether the product generated after second-strand cDNA synthesis was in fact double-stranded DNA with a single-stranded hairpin at one end. As shown in Table 5, row 1, the cDNA generated with AMV reverse transcriptase in second-strand synthesis was almost completely double-stranded as indicated by almost complete resistance to S1 nuclease digestion of both the first (98% resistance) and second strands (97% resistance). Second-strand synthesis catalyzed by POL-1A, however, resulted in molecules where approximately 30% of the first-strand cDNA is susceptible to the action of S1 nuclease. In contrast, the second-strand cDNA was totally resistant to digestion. This indicated that a complete second-strand transcript was not made with POL-1A. Denaturation of the double-stranded cDNA prepared by either method followed by S1 nuclease treatment (Table 5, row 2) resulted in 40-50% resistance to digestion. This result indicated in accordance with the results of Goodman and MacDonald (1979) that the double-stranded cDNA once denatured had the ability to renature due to the presence of the single-stranded hairpin. This conclusion was substantiated by a third test in which the double-stranded cDNA was first treated with S1 nuclease

to remove the postulated single-stranded hairpin. Subsequently, the first and second strands were separated by denaturation then subjected to a second S1 nuclease treatment. A second S1 nuclease treatment should digest both strands completely as their rate of reannealing should be slow. When double-stranded cDNA generated using AMV reverse transcriptase in second-stranded synthesis was subjected to this treatment (Table 5, row 3)' approximately 15% nuclease resistance in both the first and second strand was maintained. Similarly, the first and second strands of double-stranded cDNA whose second-strands were synthesized using POL-1A were approximately 13% resistant to S1 nuclease digestion. This reaction was repeated using 1000 units of S1 nuclease in both digestions with similar results (not shown). The S1 nuclease resistance of first-strand cDNA prior to second-strand synthesis (Table 5, column 5) was 13%. This may suggest that individual strands have internal secondary structure which rapidly reanneal after denaturation. These results indicate that using either AMV reverse transcriptase or POL-1A in second-strand synthesis, 70% or greater of the second strands synthesized are complementary to the first-strand template. The behavior of first and second strands is consistent with a hairpin loop at one end which is susceptible to cleavage with S1 nuclease.

Table 5. S1 nuclease resistance of first and second strand products.

Condition	Percentage S1 nuclease Resistance				
	AMV[a]		POl-1A[b]		Single[g]
	First Strand	Second Strand	First Strand	Second Strand	Stranded cDNA
S1 nuclease[c] treated	98 (950)[f]	97 (1500)	74 (1090)	100 (1380)	13 (1800)
Denatured[d] then S1 nuclease treated	51	44	49	47	
S1, denatured, [e] Second S1 nuclease treatment	15	16	13	14	

a. Second-strand synthesis was accomplished using AMV reverse transcriptase.

b. POl-1A was used in second strand synthesis.

Table 5. cont'd

c. Approximately 2000 Cerenkov cpm of presumed double-stranded cDNA from either the AMV reverse transcriptase of P01-1A reaction was added to one ml of S1 buffer (Materials and Methods, Section 4.2.4.3.containing 10 µg/ml denatured calf thymus DNA. This mixture was then split into two 495 µl reactions. To one was added 300 units of S1 nuclease and the other received an equivalent volume of S1 dilution buffer (Materials and Methods). Both were incubated at 37°C for 60 minutes, then TCA precipitated, filtered and the radioactivity in each determined.

d. Reactions were prepared as in c. with the exception that duplicate reactions were heated to 100°C for 3 minutes then quick cooled in an ice-water bath prior to S1 nuclease treatment.

e. Reactions were prepared and executed as in c. After S1 nuclease treatment samples were denatured as in d, then treated with an additional 300 units of S1 nuclease prior to quantification.

Table 5. cont'd

f. Figures in parentheses represent the approximate cpm corresponding to 100% resistant.

g. The cDNA component of the column purified cDNA-RNA hybrid was purified free of RNA by treatment with 0.1 M NaOH at 70°C for 20 minutes. After neutralization and ethanol precipitation, the cDNA was resuspended in water and an aliquot treated with S1 nuclease as in c.

iii) Tailing of double-stranded cDNA

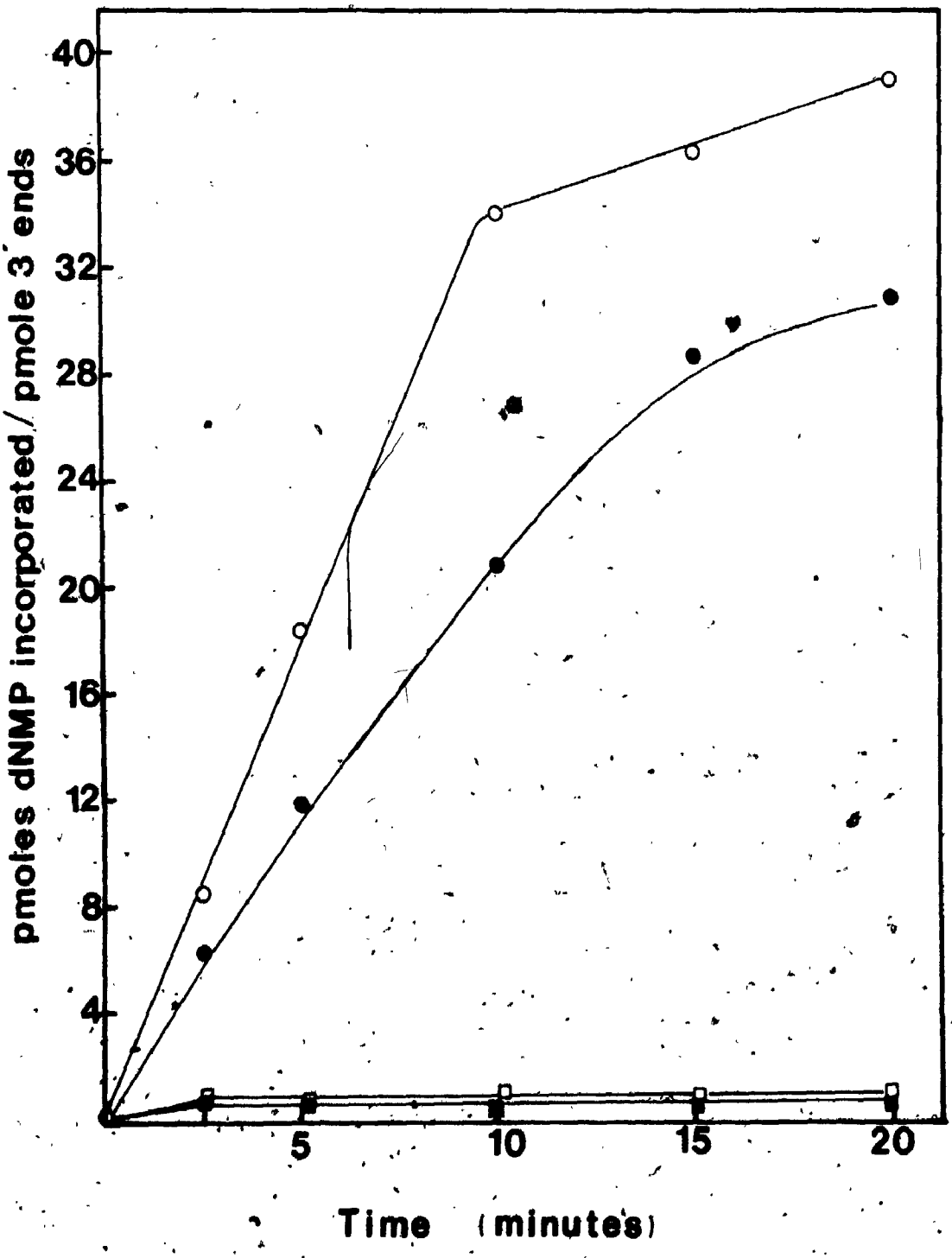
The ends of this double-stranded cDNA must be modified in order to clone it into a vector such as the plasmid pBR322. To do this, we have used G-C tailing (Otsuka, 1981) (refer to Figure 22). The enzyme terminal deoxynucleotidyl transferase was used to add deoxyguanidylate residues onto the protruding 3' ends of Pst I digested pBR322 and similarly deoxycytidylate residues onto the 3' ends of the double-stranded cDNA.

Figure 26 illustrates a typical example of the time course of addition of dGMP and dCMP respectively to the ends of pBR322. Reaction conditions were established for each cDNA preparation and each enzyme lot used during the course of this study. The addition of dCMP residues to E. coli tRNA was also tested since it was often used as a carrier in precipitation of the cDNA. As illustrated in Figure 26, tRNA was not a substrate for the enzyme. Similarly in the absence of DNA, terminal deoxynucleotidyl transferase did not convert dGTP into TCA precipitable material.

Once tailed, the vector and insert were annealed together and then used in transformation of Escherichia coli (refer to Materials and Methods, Section 4.2.4.5 and 4.2.6)

Figure 26. Time course of homopolymer addition to linearized pBR322 directed by terminal deoxynucleotidyl transferase.

140 µl reaction mixtures were assembled as described in Materials and Methods, Section 4.2.4.4. The reactions contained either 1.4 pmoles 3' ends of Pst I digested pBR322 or 10 µg of E. coli tRNA. 6 units of terminal deoxynucleotidyl transferase were used in each reaction. Homopolymer addition was monitored by TCA precipitation of 20 µl aliquots of the reactions at the times indicated. ○ pmole dGMP incorporated/3' end of Pst I digested pBR322, ● pmoles dCMP incorporated/3' end of Pst I digested pBR322, □ pmoles dGMP incorporated in the absence of DNA, ■ pmoles dGMP incorporated with E. coli tRNA as substrate.


pmoles dNMP incorporated / pmole 3' ends
40
36
32
28
24
20
16
12
8
4
5
10
15
20
Time (minutes)

4.3.2 Analysis of PMV clones

Tetracycline-resistant, ampicillin-sensitive bacterial colonies were screened for the presence of sequences complementary to PMV RNA by *in situ* colony hybridization (Materials and Methods, Section 4.2.9). Figure 27 illustrates the hybridization of ^{32}P-end labeled, fragmented PMV RNA to bacterial transformants containing inserts which were generated using Oligo-dT_{12-18} as primer in first-strand synthesis. Transformants which exhibited a more intense hybridization signal were assumed to contain plasmids with larger inserted DNA sequences. Such colonies were selected and plasmids prepared from 30 ml cultures of each (Materials and Methods, Section 4.2.7). The inserted DNA sequences were released from each plasmid by digestion with restriction endonuclease *Pst* I. Table 6 lists the sizes of the cloned DNA determined by electrophoresis. Only two plasmids, pPMV 6 and pPMV 8 were found to contain inserts greater than 1 Kb. Insert sizes, however, correlated well with the size of the S1 nuclease-treated ds cDNA used as substrate in the terminal transferase reaction (Figure 28). The same strategy was applied to clones generated from double-stranded PMV cDNA whose first-strand was primed with Oligo-dG_{12-18}. In this case, however, inserts from all transformants tested were found to be less than 500 base pairs in size (data not shown). This did not correlate

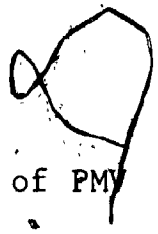
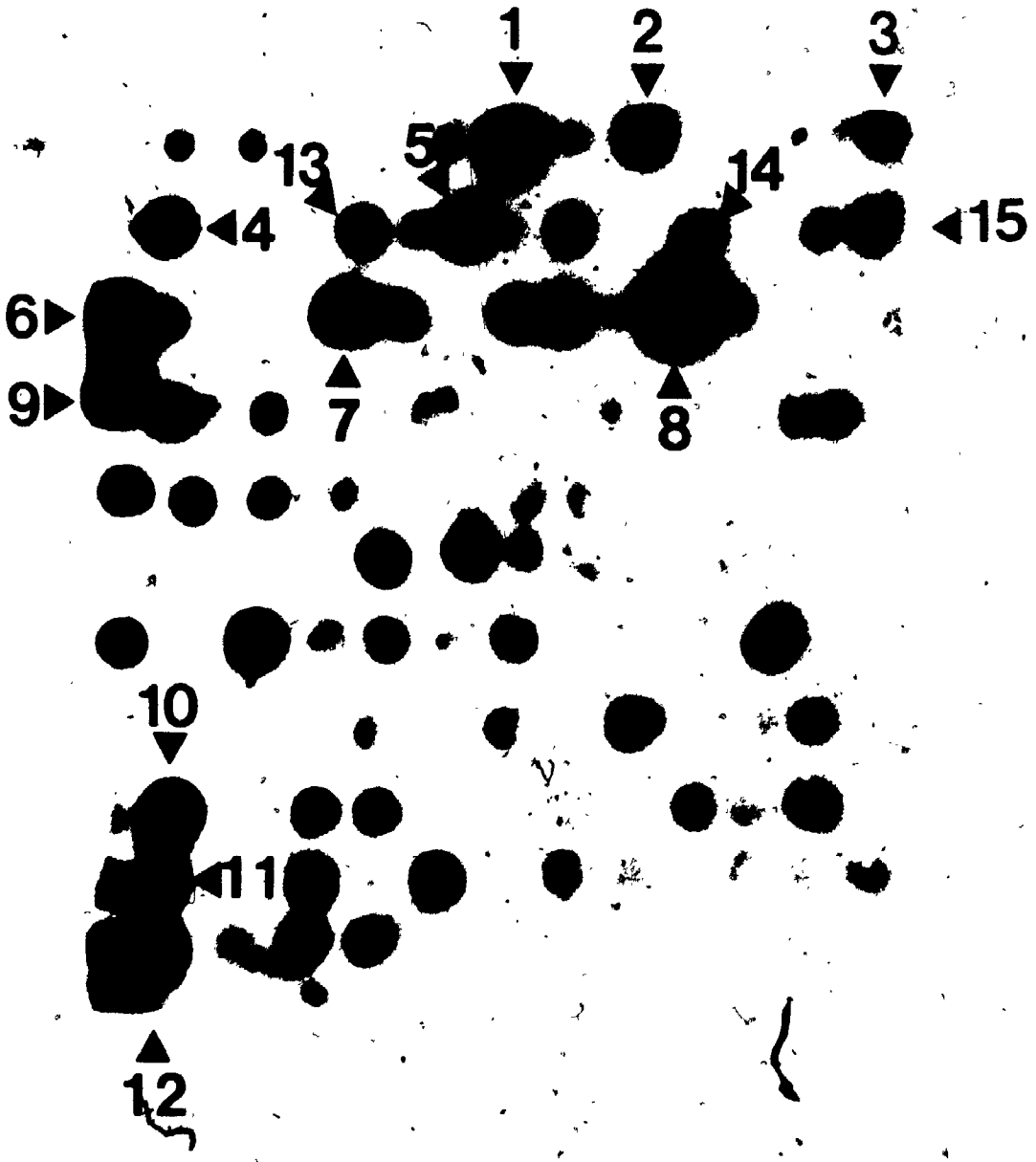


Figure 27 In situ colony hybridization of PMV clones using ^{32}P-end labelled fragmented PMV RNA as probe.

^{32}P-end labeled fragmented PMV RNA (5×10^5 cpm) was hybridized to the DNA of bacterial transformants immobilized on nitrocellulose filters (Materials and Methods, Section 4.2.9). Positive colonies (i.e. those showing visible hybridization) selected for further characterization and the number assigned to each (pPMV 1 through 15) is noted. Negative colonies represent plasmids which probably contain extremely small inserts.


1
2
3
5
13
14
4
15
6
9
7
8
10
11
12

well with the size of S1 nuclease treated double-stranded cDNA (Figure 29, lane g). Instead, S1 nuclease treated double-stranded cDNA displayed four discrete bands on a 1% neutral agarose gel of 2800, 1600, 980 and 680 base pairs. The 2.8 Kb double-stranded cDNA was partially purified on a column of Bio-gel ® A15Q m, then treated with E. coli DNA ligase to seal any nicks in the DNA. This DNA was then tailed with dCMP residues by terminal transferase, and annealed to dGMP tailed pBR322. All cells transformed with this cDNA were again found to have inserts of less than 500 base pairs in size (data not shown). This result implied that this particular terminal transferase preparation might have a DNA nicking activity which would result in internal tailing of the double-stranded cDNA.

To circumvent this problem, we avoided terminal transferase altogether. Rather we generated a cDNA suitable for cloning by digesting it with restriction enzymes. Aliquots of the Oligo dG-primed PMV double-stranded cDNA were digested with various restriction enzymes as shown in Figure 29 and Table 7. What we wanted ideally was a fragment with ends suitable for cloning whose size approached 2.8 Kb, the maximum size of the PMV ds cDNA. Of all digestions analyzed, that with Eco RI and HindIII (Figure 29, lane b) appeared most suitable, producing a cleavage product of 2.5 Kb which did not result from cleavage with either Eco RI (lane a)

Figure 28 The electrophoretic separation of double-stranded PMV cDNA after S1 nuclease treatment.

Approximately 5000 Cerenkov cpm of S1 nuclease treated double-stranded PMV cDNA whose first-strand cDNA was made using Oligo-dT_{12-18} as primer was separated on a 1% (w/v) alkaline agarose gel as described in Materials and Methods, Section 4.2.8. On completion of electrophoresis, the gel was neutralized and 3mm fractions sliced with a razor blade. Radioactivity in individual fractions was monitored by Cerenkov radiation.

Markers provided by a HindIII x Eco RI digest of λ plac 5 were visualized by staining of a separate lane with ethidium bromide. The positions to which fragments of known size migrated is noted.

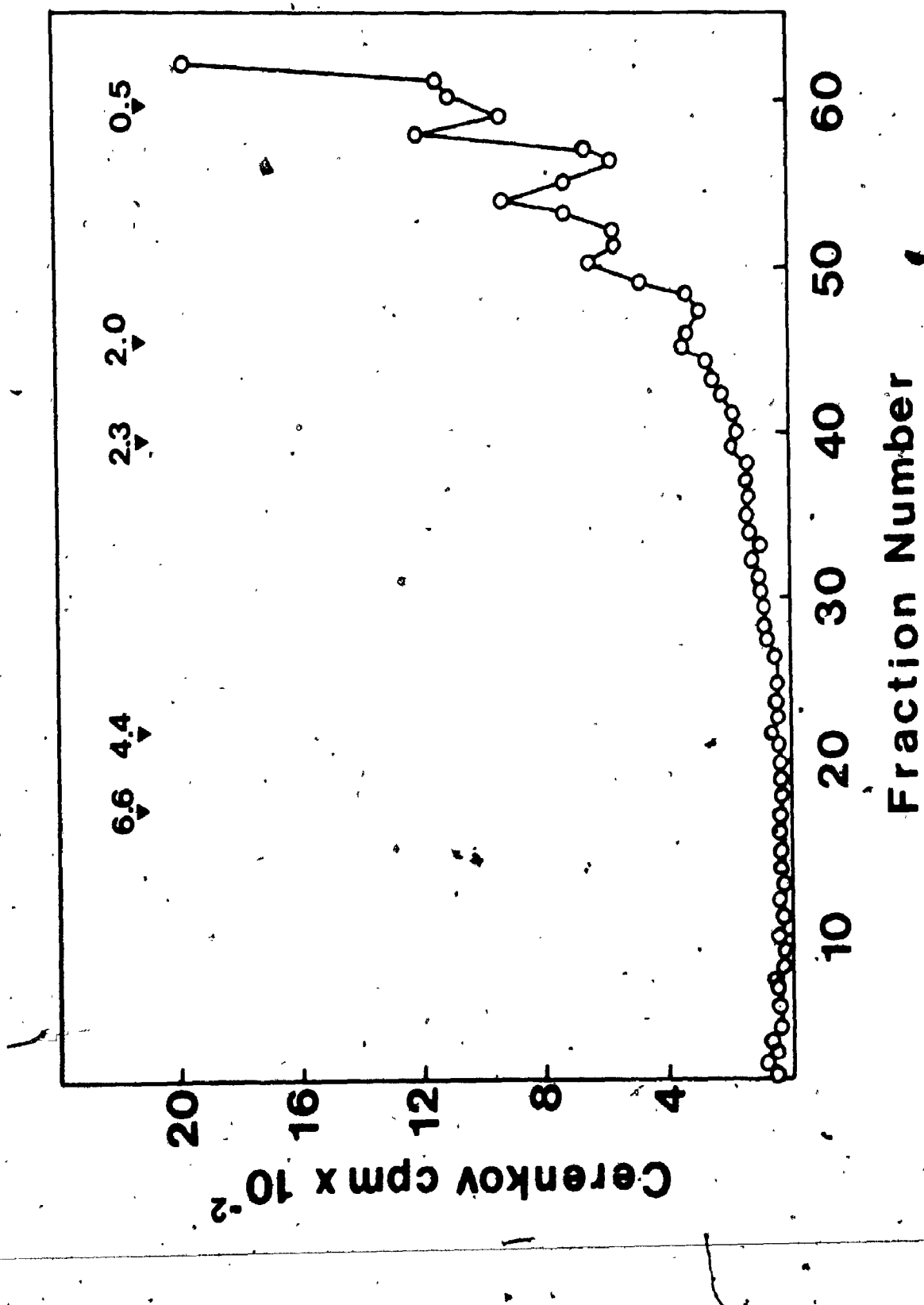

Cerenkov cpm x 10^{-2}
20
16
12
8
4
10
20
30
40
50
60
Fraction Number
0.5
2.0
2.3
4.4
6.6

Table 6. Sizes of cloned PMV cDNA inserts

Plasmid	Size of cloned cDNA[a] (in Kb)
pPMV 1	0.2
pPMV 2	0.35
pPMV 3	0.2
pPMV 4	0.6
pPMV 5	0.8
pPMV 6	1.2
pPMV 7	0.5
pPMV 8	1.6
pPMV 9	0.45
pPMV 10	0.35
pPMV 11	0.2
pPMV 12	0.8
pPMV 13	0.2
pPMV 14	0.6
pPMV 15	0.3
pPMV 16	0.2
pPMV 17	0.4
pPMV 18	0.4
pPMV 19	0.2
pPMV 20	0.5

Table 6. cont'd

a. Sizes were determined by electrophoresis of Pst I digested DNA samples on 1.5% agarose gels. Molecular weight standards were produced by digestion of λ plac 5 with Eco RI and HindIII and digestion of pBR322 with HinfI.

or HindIII (lane c) alone. Accordingly, 50 ng of PMV ds cDNA digested with Eco RI and HindIII was ligated to 190 ng of pBR322 cleaved with the same enzymes (Materials and Methods, Section 4.2.5). Transformants were obtained at an efficiency of $1.3 \times 10^3/\mu g$. Seventy percent of those tested were tetracycline-sensitive and ampicillin-resistant, consistent with their containing DNA inserted into the vector. Plasmids were extracted from sixteen such colonies chosen at random. Each plasmid was digested with Eco RI and HindIII and the cleavage products separated by electrophoresis on a 1% (w/v) agarose gel (data not shown). Of these, only pEHPMV1 contained a 2.5 Kb insert as judged against an Eco RI and HindIII digest of λ plac 5 DNA as size markers. All other clones were less than 0.7 Kb in size.

Since all clones derived from PMV RNA were generated by internal priming, it was reasonable to assume that the cloned DNA corresponded to the 5' half of PMV RNA. To test whether the cloned sequence in pEHPMV1 was also present in clones generated using Oligo-dT_{12-18} as primer in first-strand synthesis, we again used in situ colony hybridization. The 2.5 Kb Eco RI-HindIII insert from pEHPMV1 was purified by electrophoresis, and labeled by nick translation (Materials and Methods, Section 4.2.9.2.ii.) This labeled DNA was then used as a probe to screen the pPMV series of clones for homologous

Figure 29 Electrophoretic separation of oligo-dG primed PMV double-stranded cDNA digested with restriction enzymes.

Approximately 10,000 Cerenkov cpm of S1 nuclease treated PMV double-stranded cDNA was added to each 20 µl reaction containing 1 µg of λ plac 5 and standard restriction buffer (Materials and Methods, Section 4.2.5). Samples were allowed to digest for 6 hours at 37°C. DNA fragments were then separated by electrophoresis on a 1% neutral agarose gel. After electrophoresis the gel was stained with ethidium bromide and the positions of restriction fragments of λ plac 5 noted. The gel was then dried and exposed to x-ray film for 12 hours.

Restriction digests were as follows: lane a, Eco RI; lane b, Eco RI + HindIII; lane c, HindIII; lane d, Bam HI; lane e, Sal I; lane f, Bam HI + HindIII; lane g, Oligo-dG primed double-stranded PMV cDNA after S1 nuclease treatment.

The size of the major fragments (in Kb) of lane g are noted in the margin. The arrow between lanes b and c indicates the 2.5 Kb Eco RI + HindIII fragment described in the text.

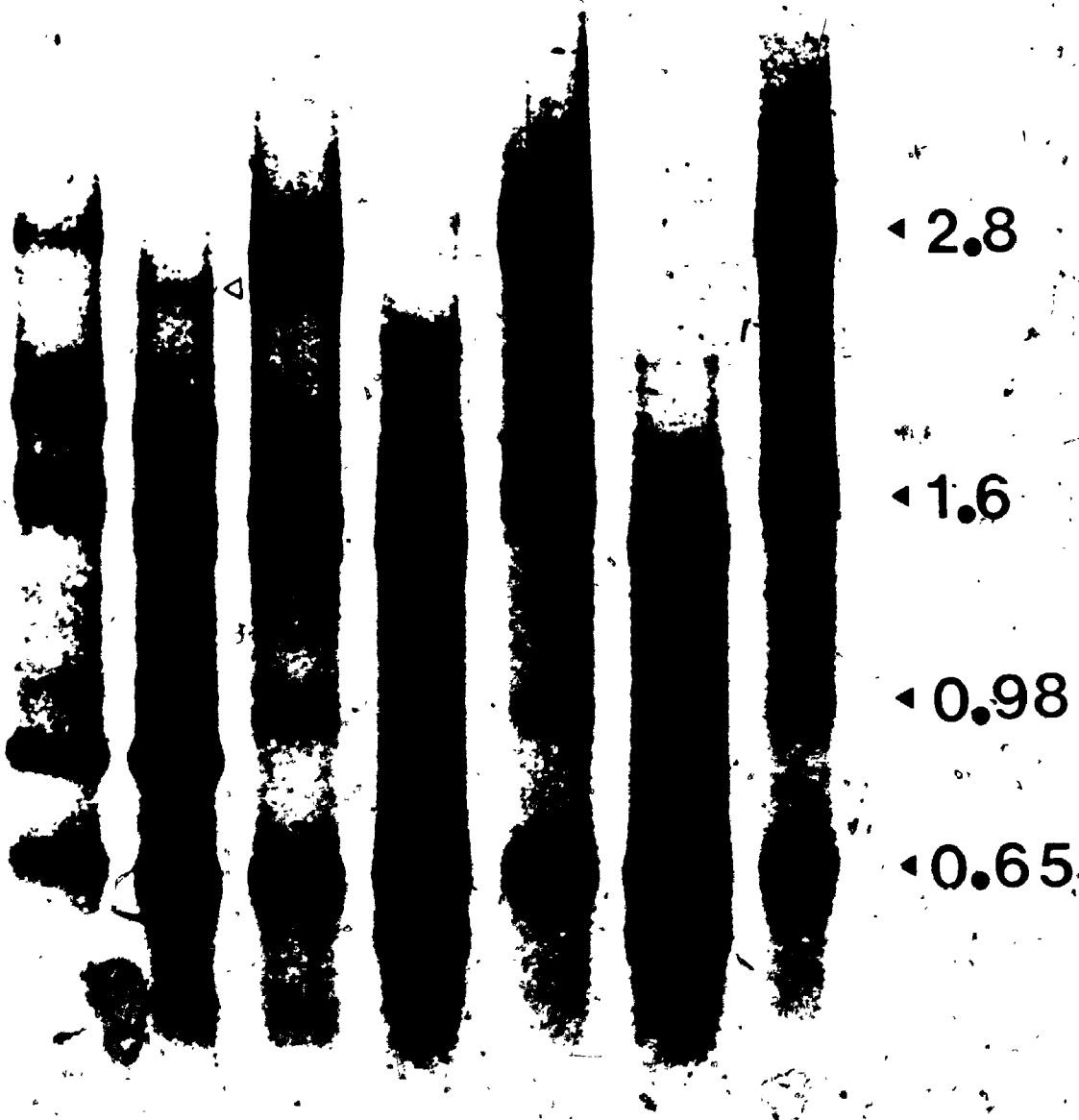

A
B
C
D
E
F
G
2.8
1.6
0.98
0.65

Table 7. Digestion of oligo dG-primed PMV cDNA with restriction enzymes[a].

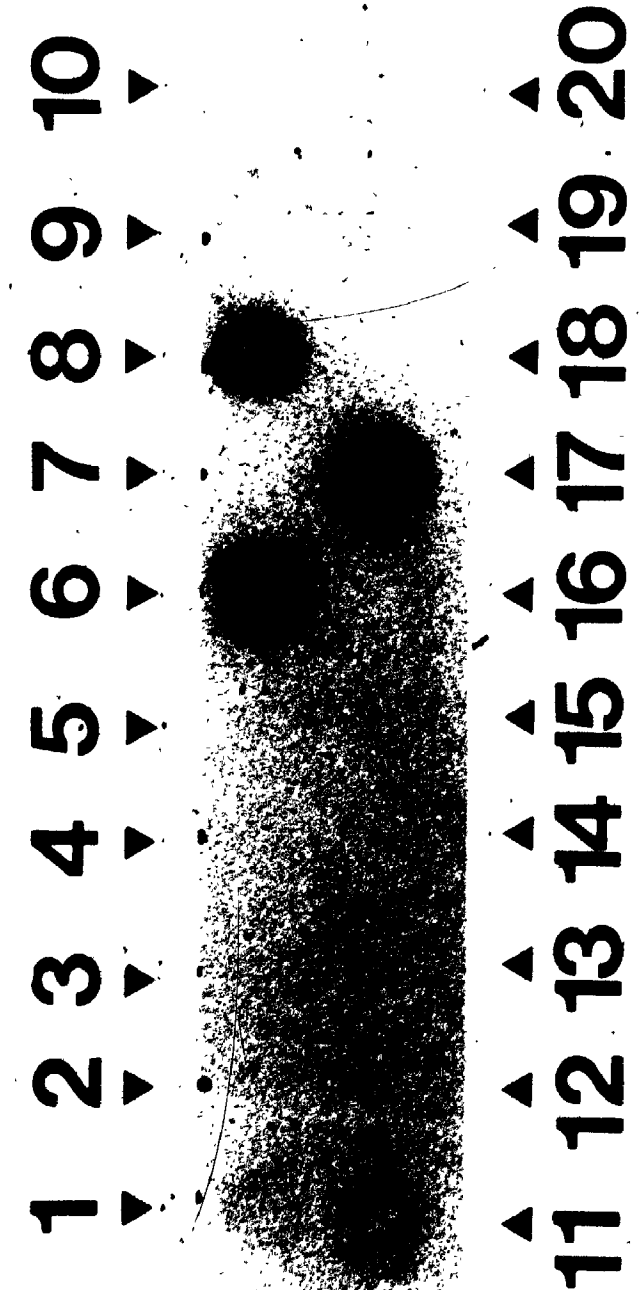
Cleavage by:	Major Discrete Products[b] (in bp)
	2800 + 1600 + 980 + 650
Eco RI	2800 + 2100 + 1880 + 1600 + 830 + 650
Eco RI + HindII	2500 + 2100 + 1800 + 1500 + 1400 + 1000 + 830 + 650
HindIII	2700 + 1500 + 1400 + 1000 + 650
Bam HI	2300 + 1700 + 1400 + 1150 + 650 + 560
Sal I	2800 + 1600 + 980 + 650
Bam HI + HindIII	2300 + 1700 + 1400 + 1150 + 650 + 560

a. Restriction digests were performed on 10,000 Cerenkov cpm of the double-stranded cDNA with λ plac 5 in the reaction as an internal standard to insure that complete digestion had occurred.

b. Sizes were determined by electrophoresis in a 1% agarose gel (Figure 29) with the digestion products of λ plac 5 providing standards.

Figure 30 In situ colony hybridization of selected pPMV clones using the insert of pEH PMV 1 as probe.

The 2.5 Kb insert of pEH PMV 1 was extracted from a 1% neutral agarose gel and labeled with ^{32}P-dCTP by nick translation. 1 x 10^5 Cerenkov cpm of labeled DNA was hybridized to the DNA of clones pPMV 1 through 20 immobilized on nitrocellulose filters as described in Materials and Methods, Section 4.2.9.


1 2 3 4 5 6 7 8 9 10
11 12 13 14 15 16 17 18 19 20

sequences. Figure 30 illustrates that the pEHPMV insert hybridized to pPMV6, pPMV8 and pPMV17. This probe also hybridized weakly to pPMV1, pPMV11 and pPMV19.

4.3.3 Further Characterization of PMV Clones

Four plasmids pEHPMV1, pPMV5, pPMV6 and pPMV8 were chosen for further study, pPMV6 and pPMV8, since they contained the largest inserts (Table 6) and shared sequences with pEHPMV1. pPMV5 since it contained an 800 bp insert and displayed no homology with pEHPMV1 and therefore was expected to contain unique sequences. Restriction maps of these plasmids were constructed to facilitate their comparison and to determine their orientation within the vector. Table 8 indicates which restriction enzymes were able to cleave within the inserted sequence of each of these plasmids. The insert in pEHPMV1 was cleaved by Bam HI, Sph I, Pst I, Sma I and twice with Ava I. pPMV8 contained an Ava I, an Sph I and a HindIII site within the insert. pPMV6 contained two Ava I sites, an Sph I and a Sma I site within its insert. In conjunction with the colony hybridization the sharing of restriction sites among pEHPMV1, pPMV6 and pPMV8 is consistent with the idea that the insert sequences in these three plasmids are nested. Only pPMV5 contained a Bg1II site, clearly distinguishing its insert from those in the other three plasmids

Table 8. Screening for restriction enzymes which cleave within the inserts of recombinant plasmids[a].

Restriction Enzymes[b,c]	Plasmid			
	pEHPMV 1	pPMV 5	pPMV 6	pPMV 8
HindIII	–	–	–	+
Bam HI	+	+	–	–
Ava I	++	–	++	+
Cla I	–	–	–	–
Sph I	+	–	+	+
Pvu II	–	–	–	–
Pst I	+	–	–	–
Eco RI	–	–	–	–
Bgl II	–	+	–	–
Sma I	+	–	+	–
Kpn I	–	–	–	–
Xba I	–	–	–	–

Table 8. cont'd

a. Most of the restriction enzymes tested are known to cleave the vector pBR322 once (Figure 31). If no restriction site were present within the insert, the plasmid would be linearized resulting in a single DNA fragment. Conversely, if an restriction site were present in an insert two DNA fragments would result. The restriction enzymes Bgl II, Sma I, Kpn I and Xba I do not cleave pBR322. Thus, if the inserts contained a restriction site specific for these enzymes, the plasmid was linearized.

b. Restriction recognition sequences for these enzymes are given in Appendix I.

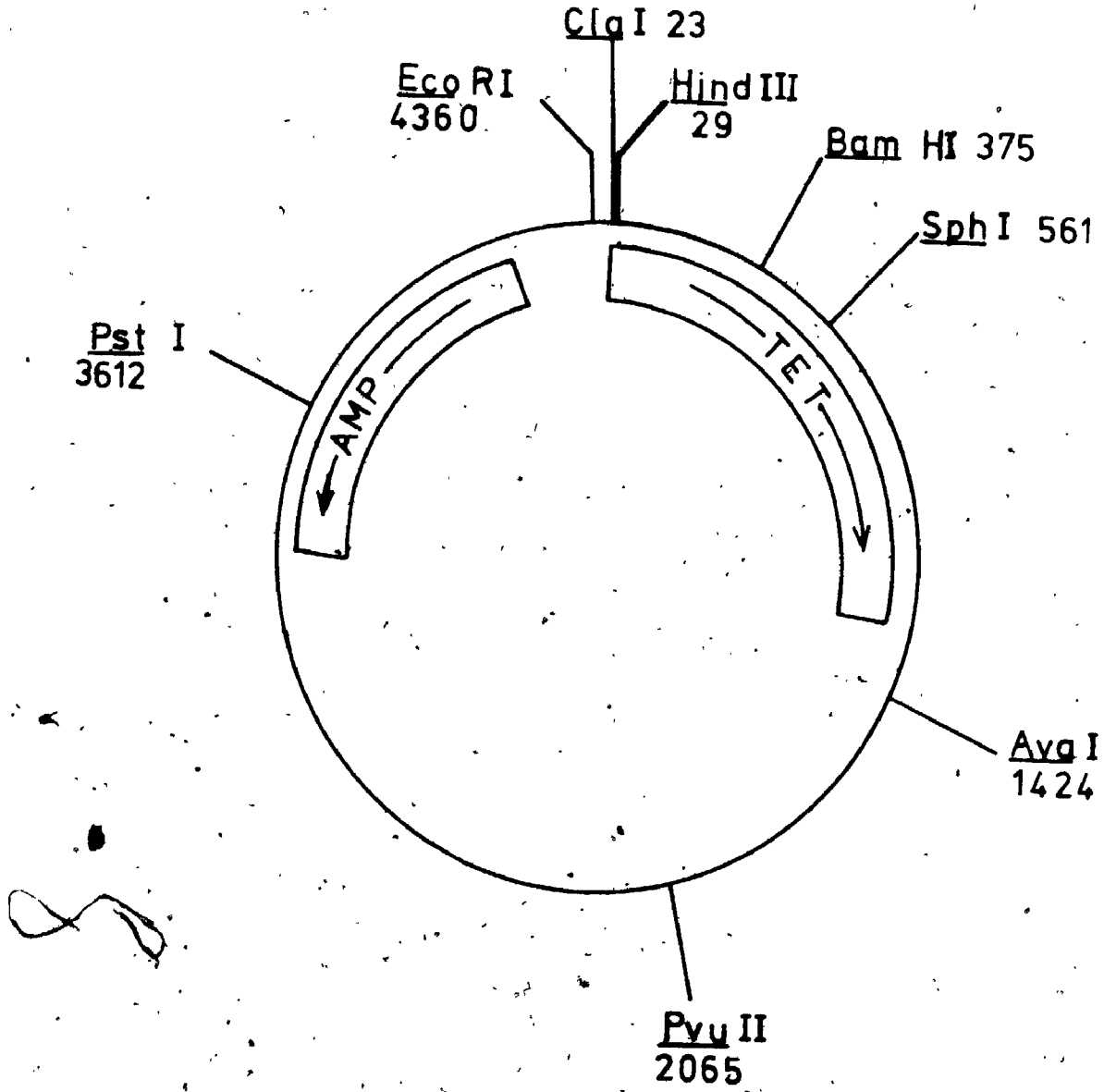
c. All restriction digests were performed with an excess of enzyme and analyzed on 1% agarose gels. Restriction enzymes which cleaved within the insert are represented by +, those which did not by -.

The orientation of these restriction sites was then determined for each plasmid by using multiple digestions. Since the inserted DNA of pEHPMV1 has unique ends the restriction sites within the insert can be mapped relative to the Eco RI or HindIII termini. Figure 32 illustrates the electrophoretic separation of DNA fragments produced by multiple digestion of pEHPMV1. The corresponding sizes of the DNA fragments are given in Table 9. The cleavage of pEHPMV1 with Sma I (lane b) linearized the plasmid to produce a single band of approximately 6.8 Kb (lane b). Further digestion of the Sma I cleaved plasmid with Eco RI (lane c) released an 1150 base pair fragment.

This fragment size therefore represents the distance from the Eco RI end of the insert to the Sma I cleavage site. Similarly, digestion of Sma I cleaved pEHPMV1 with HindIII (lane d) released a 1375 bp fragment. This fragment size therefore represents the distance from the HindIII end of the insert to the Sma I cleavage site. Both the 1150 and 1375 bp fragments were released upon digestion of the vector with Sma I, Eco RI and HindIII (lane h).

Other restriction sites were also mapped relative to the Eco RI and HindIII sites. Digestion with Ava I (lane i) produced three fragments of 4000, 2150 and 620 bp. Ava I cleaves pBR322 once at coordinate 1424 (Figure 31). The distance from the Ava I site to the

Figure 31 Cleavage coordinates in pBR322 of restriction enzymes used in this study.
Compiled from Sutcliffe (1979) and Roberts (1982).


Cla I 23
Eco RI
4360
Hind III
29
Bam HI 375
Sph I 561
Pst I
3612
AMP
TET
Ava I
1424
Pvu II
2065

HindIII site in the vector is 1395 bp. Subtracting this value from 2150 yields the result that one Ava I in the insert in pEHPMV1 is 755 bp from the HindIII site at the vector-insert junction. A more precise measurement from an Ava I-HindIII digestion of pEHPMV1 (Figure 32, lane k) suggests a distance of 600 bp. This latter site was confirmed by resolving Ava I-HindIII fragments on polyacrylamide gels. The 620 base pair fragment resulting both in the Ava I and Ava I plus HindIII digest must therefore represent a fragment with Ava I ends. This places the second Ava I cleavage site approximately 1200 bp from the HindIII site at the insert-vector junction. Digestion of pEHPMV with Eco RI and Ava I (Figure 32, lane j) confirms the disposition of Ava I sites within the insert (see the map in Figure 33). Data in Table 9 shows that Sph I, Bam HI and Pst I sites were found 210, 344 and 570 bp respectively from the Eco RI site of the insert-vector junction. Confirmatory digests were also performed to ensure that the positions of restriction sites were correct, for example using Sma I in combination with Pst I or Ava I or Bam HI (Figure 32, lanes e, f and g respectively). Interestingly, the Sma I-Ava I digest (lane f) produced the same fragment sizes as that produced by digesting pEHPMV1 with Ava 1 alone (lane i). The sequence recognition site of Sma I is 5' - CCCGGG -3' and that of Ava I is 5'- C Py C G Pu G - 3'. Therefore, it is likely that the unique Sma I site

Figure 32 Resolution of restriction digestion products of pEH PMV 1.

Approximately 2 µg of plasmid DNA was digested with 3 units of the appropriate restriction enzyme for 6 hours at 37°C. The digests were then subjected to electrophoresis on a 1% agarose gel together with the standards noted below. Digests are as follows: a. Undigested pEH PMV 1, b. Sma I, c. Sma I + Eco RI, d. Sma I + HindIII, e. Sma I + Pst I, f. Sma I + Ava I, g. Sma I + Bam HI, h. Sma + Eco RI + HindIII, i. Ava I, j. Ava I + Eco RI, k. Ava I + HindIII, l. Sph I, m. Sph I + Eco RI, n. HindIII + Eco RI cleaved λplac 5 DNA.

The sizes of fragments produced by Eco RI and HindIII digestion of λplac 5 are noted in the margin.

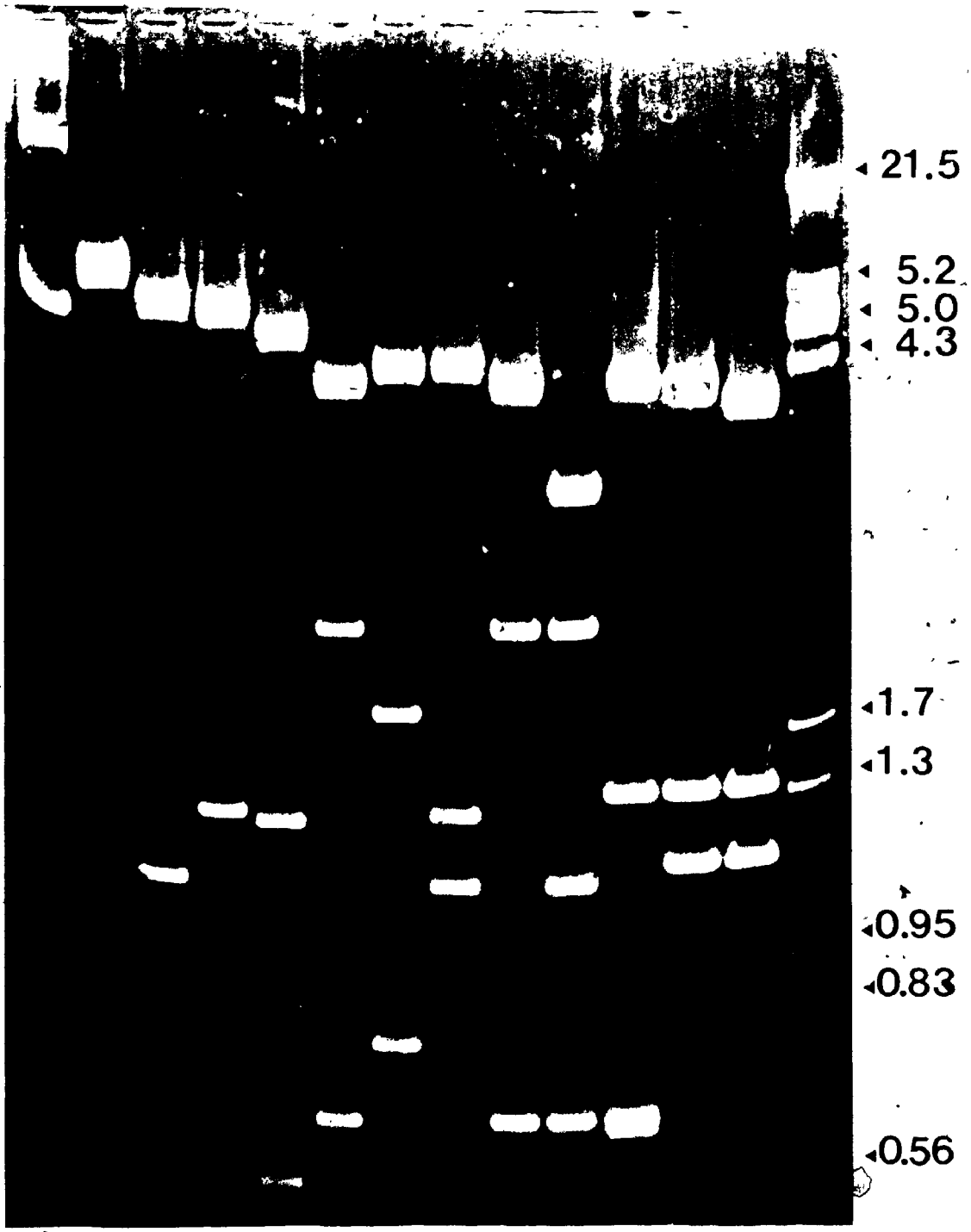

A B C D E F G H I J K L M N
21.5
5.2
5.0
4.3
1.7
1.3
0.95
0.83
0.56

Table 9. Fragment Sizes produced by restriction enzyme digests of plasmids containing PMV inserts

Plasmid	Cleavage by [a]	Products, (bp) [c,d]
pEHPMV 1	Sma I + Eco RI	5200, 1150
	Sma I + HindIII	5100, 1375
	Sma I + Pst I	4800, 1300, 520
	Sma I + Ava I	4100, 2150, 620
	Sma I + Bam HI	4200, 1700, 740
	Sma I + Eco RI + HindIII	4300, 1350, 1100
	Ava I	4000, 2150, 620
	Ava I + Eco RI	3050, 2150, 1150, 620
	Ava I + HindIII	4000, 1400, 620, 600
	Sph I	3800, 1380, 1200
	Sph I + Eco RI	3600, 1380, 1200
	Eco RI + Sph I [b]	280
	Eco RI + Bam HI [b]	344
	Eco RI + Pst I [b]	570
	Pst I + Bam HI [b]	230
	Ava I + Pst I [b]	620, 520

Table 9. cont'd

pPMV 8	Pst I	4300, 1300
	HindIII	4500, 930
	HindIII + Pst I	3500, 1150, 780
	HindIII + Ava I	3000, 1400, 930, 600
	Pst + Ava I	2100, 750, 550
pPMV 6	Pst I	4300, 1100
	Sma I + Pst I	4300, 700, 400
	Ava I	2500, 2100, 620
	Ava I + Pst I	2100(d), 620, 400
	Sma I + Sph I	3900, 1500, 500
pPMV 5	Pst I	4300, 840
	Bam HI	3200, 1700
	Pvu II + Bam HI	1800, 1700, 1680
	Pvu II + Bgl II	3500, 1650
	Bam HI + Pst I [b]	575, 265
	Bgl II + Pst I [b]	725, 115
	Bam HI + Bgl II [b]	150
	Bam HI + Bgl II + Pst I [b]	575, 150, 115

Table 9. cont'd

a. 2 μg of plasmid was used in each restriction digest. Since the buffer used for Sma I digestions is incompatible with other restriction enzymes, Sma I digested plasmid DNA was phenol extracted and ethanol precipitated prior to digestion with a second enzyme.

b. Digestions which produced fragments less than 600 bp were resolved on 6% polyacrylamide gels using HinfI-cleaved pBR322 as a marker. Fragment sizes shown are only these which were resolved by the gel.

c. Digestion products resolved on 1% agarose gels were determined using an Eco RI + HindIII digest of λ plac 5 as molecular size markers.

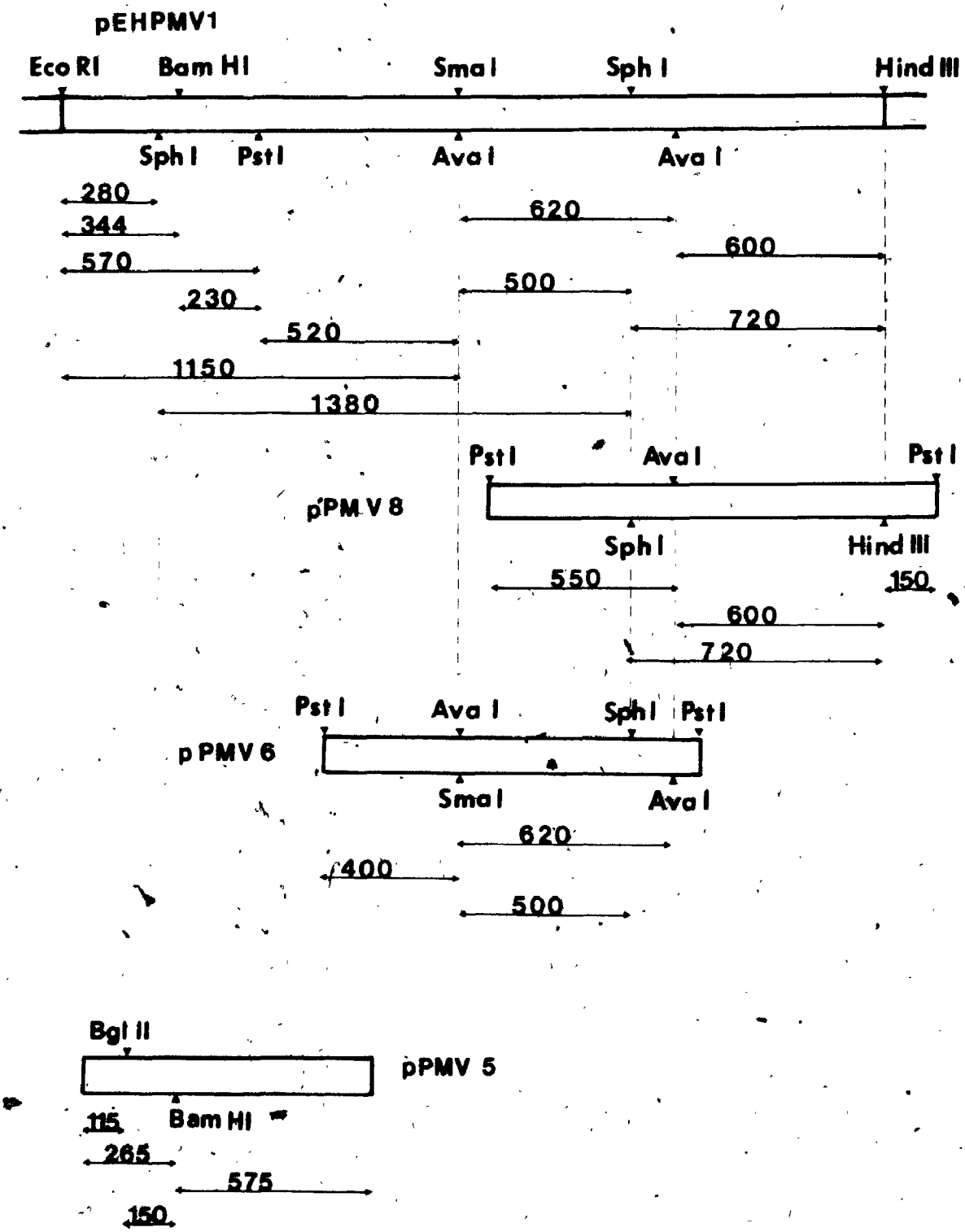
d. DNA fragments underlined with a solid line indicate vector sequences. Those which contain both vector and insert sequences are underlined with a broken line and those which represent insert sequences are not underlined. (d) represents a doublet.

is congruent with the Ava I site closest to the Eco RI site. The final compilation of restriction sites in the insert of pEHPMV1 and their distances are given in Figure 33.

The restriction mapping of inserts cloned into the Pst I site of pBR322 was treated similarly. For example, digestion of pPMV8 with Pst-1 released the insert of 1300 bp from the vector. Cleavage with HindIII, however, released a 930 base pair fragment. The distance from the Pst I site (coordinate 3612) to that of the HindIII site of the vector (coordinate 29) is 779 base pairs. The 930 base pair distance therefore represents the vector HindIII-Pst I distance plus the distance from the Pst I site to the Hind III site of the insert. The HindIII site in the insert is therefore 151 bp from one Pst I site. Further digestions with HindIII and Ava I confirmed that the Ava I site is 600 base pairs from the HindIII site as was found in pEHPMV1. This, coupled with the colony hybridization results suggests strongly that this HindIII-Ava I fragment is the same as that found in pEHPMV1. The sizes of fragments produced by digestion of pPMV5 and pPMV6 are given in Table 9 and the deduced orientation displayed in Figure 33. The restriction map of pPMV5 established that this 840 base pair insert was distinct from that of pEHPMV1, whereas the sequences of pPMV6 and pPMV8 fall within those of pEHPMV1. In total then we obtained clones representing approximately 50% of

Figure 33 Partial restriction maps of pEH PMV 1, pPMV 5, pPMV 6, and pPMV 8.

Restriction fragment sizes and their orientation were constructed from the data shown in Table 9.


pEHPMV1
Eco RI
Bam HI
Sma I
Sph I
Hind III
Sph I
Pst I
Ava I
Ava I
280
344
570
230
520
1150
1380
620
600
500
720
Pst I
Ava I
Pst I
pPMV 8
Sph I
Hind III
550
150
600
720
Pst I
Ava I
Sph I
Pst I
pPMV 6
Sma I
Ava I
620
400
500
Bgl II
pPMV 5
115
Bam HI
265
575
150

the PMV genome (3300 base pairs). The manner in which these plasmids were generated, that is, using internal priming of first strand cDNA synthesis suggests that these inserts represent the 5' end of the RNA. Attempts to attach a poly A tail to PMV RNA in order to facilitate a more conventional strategy have failed. Thus cloning of the rest of PMV RNA is a task for the future.

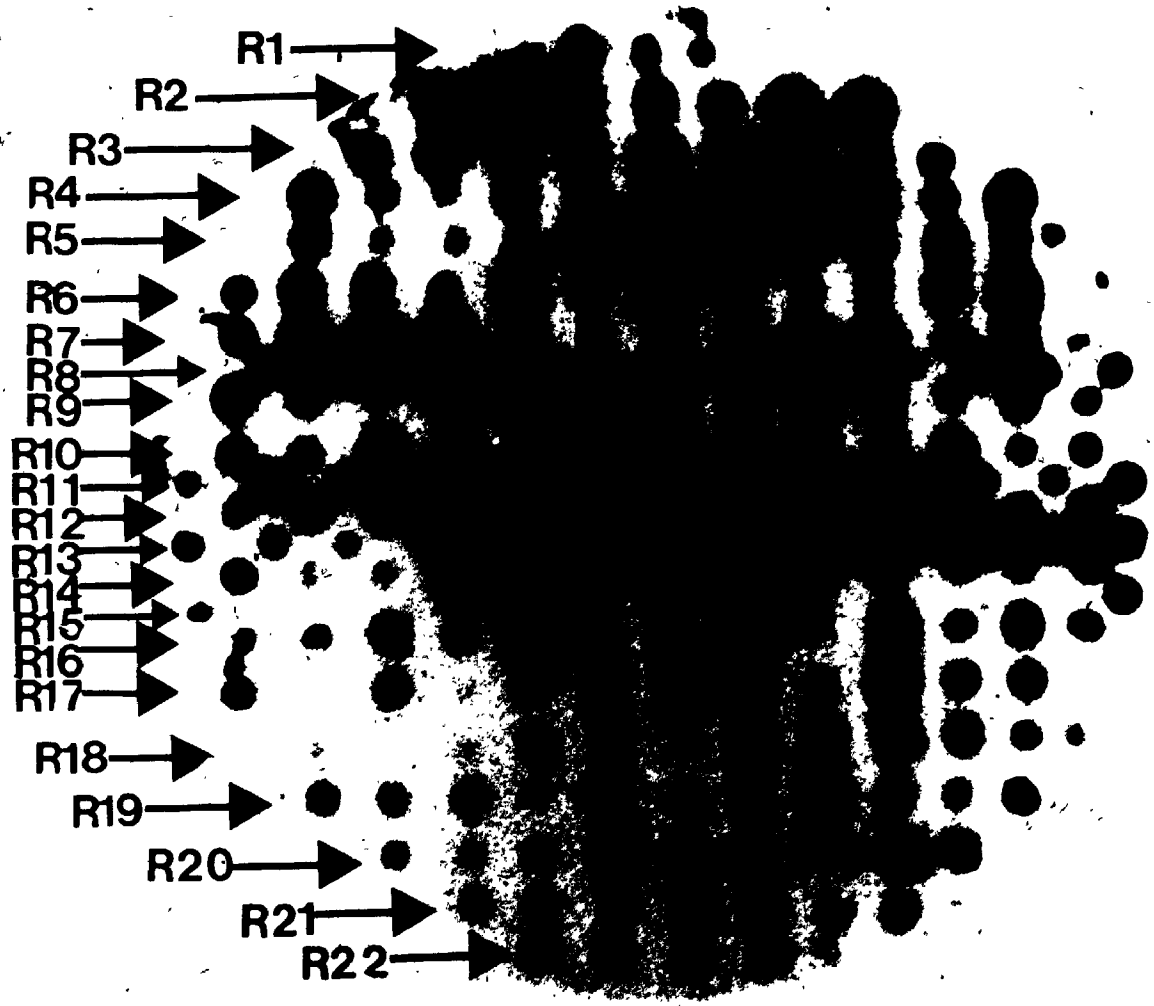
4.3.4 Analysis of CYMV clones

Transformation of Escherichia coli with pBR322 into which double-stranded cDNA derived from CYMV RNA had been annealed at the Pst I site resulted in tetracycline resistant, ampicillin sensitive colonies. The analysis of these clones followed the same initial strategy as was applied to the analysis of PMV clones.

First, colonies were screened for the presence of sequences complementary to CYMV RNA by in situ colony hybridization using ^{32}P-labeled fragmented CYMV RNA as probe. Figure 34 illustrates that most colonies tested contained sequences which hybridized with CYMV RNA. The intensity of the hybridization signal was again assumed to be an indicator of the size of the inserted DNA sequence. Plasmids were extracted from 30 ml cultures of those colonies which displayed the most intense hybridization signals. The inserted DNAs were released by digestion of the plasmid with Pst I then sized by gel electrophoresis. Comparison of the insert sizes (Table

Figure 34 In situ colony hybridization of bacterial transformants with ^{32}P-end labeled fragmented CYMV RNA.

Tetracycline ^{R}, ampicillin ^{S} bacteria generated by transformation with plasmids containing CYMV double-stranded cDNA inserts were screened by in situ colony hybridization with 1.0 x 10^{6} cpm ^{32}P-end labeled fragmented CYMV RNA as described in Figure 27. pCYMV colonies were numbered as follows:


R1
R2
R3
R4
R5
R6
R7
R8
R9
R10
R11
R12
R13
R14
R15
R16
R17
R18
R19
R20
R21
R22

R I						60	61	62	63					
R 2				64	65	66	67	68	69	70				
R 3			71	72	73	74	75	80	81	82	83			
R 4		84	85	86	87	88	89	90	91	92	93	94		
R 5		95	96	97	98	99	100	101	102	103	104	105	106	
R 6	107	108	109	110	111	112	113	114	115	116	117	118	119	
R 7	120	121	122	123	124	125	126	127	128	129	130	131	132	
R 8	266	267	268	269	270	271	272	273	274	275	276	277	278	
R 9	133	134	135	136	137	138	139	140	141	142	143	144	145	
R 10	146	147	148	149	150	151	152	153	154	155	156	157	158	
R 11	279	280	281	282	283	284	285	286	287	288	289	290	291	292
R 12	159	160	161	162	163	164	165	166	167	168	169	170	171	
R 13	293	294	295	296	297	298	299	300	301	302	303	304	305	306
R 14	172	173	174	175	176	177	178	179	180	181	182	183	184	
R 15	307													308
R 16	185	186	187	188	189	190	191	192	193	194	195	196	197	
R 17	198	199	200	201	202	203	204	205	206	207	208	209	210	
R 18		211	212	213	214	215	216	217	218	219	220	221	222	
R 19		223	224	225	226	227	228	229	230	231	232	233		
R 20			244	245	246	247	248	249	250	251	252	253		
R 21				254	255	256	257	258	259	260				
R 22					261	262	263	264	265					

10) with the hybridization intensity of the corresponding colony (Figure 34) demonstrates that there was close agreement between the intensity of the hybridization signal and the insert size. Again, the sizes of the inserts did not agree with the size of the S1 nuclease treated double-stranded cDNA used as substrate in the terminal transferase reaction (Figure 35). This again suggested that the terminal transferase used may have been contaminated with a DNA nicking activity which resulted in internal tailing of the double-stranded cDNA.

To circumvent the use of terminal transferase samples of the S1 nuclease treated double-stranded cDNA shown in Figure 35 were digested with one of the restriction endonucleases Pst I, Eco RI, Bam HI or HindIII. Without further sizing, 50 ng of each digest was ligated with 150 ng of similarly digested and dephosphorylated vector. The restriction map of pBR322 (Figure 31) shows that insertion of Bam HI or HindIII fragments into their respective cleavage sites in pBR322 should inactivate the tetracycline gene. Similarly, insertion of Pst I fragments into the Pst I site in pBR322 should inactivate the ampicillin gene. The cleavage site for Eco RI in pBR322, however falls between the two drug resistant markers. Accordingly, Eco RI fragments were cloned into the plasmid pBR325 (Bolivar, 1978), a derivative of pBR322 which contains a unique Eco RI site in a gene conferring chloramphenicol resistance.

Figure 35 Electrophoretic separation of double-stranded CYMV cDNA after S1 nuclease treatment.

Approximately 10,000 Cerenkov cpm of double-stranded CYMV cDNA after S1 nuclease treatment (Materials and Methods, Section 4.2.4.3) was separated by size by electrophoresis on a 1% (w/v) agarose gel under alkaline conditions. The bands were visualized by autoradiography of the dried gel.

Molecular size markers were provided by a HindIII digest of λplac 5 and their sizes (in Kb) noted in the margin.


6.6
4.4
2.3

Table 10. Sizes of cloned CYMV cDNA inserts.

Plasmid		Size of cloned cDNA (in Kb)[a]
pCYMV	16	0.90
	17	0.55
	21	1.6
	25	0.20
	47	0.47
	48	0.59
	49	0.4
	60	0.65
	64	0.47
	65	2.0
	67	0.20
	69	0.74, 0.57
	70	0.75
	74	0.5
	80	0.62
	81	N.C. (f)
	84	0.24
	91	1.1
	92	0.44, 0.34, 0.26
	93	0.7
	94	1.0
	95	0.6
	99	0.8
	100	0.7
	101	1.0
	105	N.C.
	108	0.34
	111	0.355
	118	1.1

Table 10. cont'd

	122	0.44
	123	0.31
pCYMV	139	0.42
	140	0.2
	141	0.2
	162	1.05
	169	0.45
	171	0.85
	172	0.3
	175	0.92
	189	0.21
	262	0.40
	263	0.75
	269	0.56
(b) pCP	16	0.62
	17	0.6
	43	1.1
(c) pCE	13	0.80
	56	0.90
	62	0.78
	81	N.C.
	91	N.C.
(d) pCH	87	0.54
	153	0.54
(e) pCB	125	0.88

Table 10. cont'd

(a) Sizes were determined by electrophoresis of Pst I digested DNA samples on 1.5% (w/v) agarose gels.

(b) pCP clones are those in which Pst I digested ds CYMV cDNA was ligated with Pst I cleaved pBR322. Inserts were released by digestion with Pst I.

(c) pCE clones are those in which Eco RI cleaved ds CYMV cDNA was ligated with Eco RI cleaved pBR325. Inserts were released from the vector by digestion with Eco RI.
Similarly pCH (d) and pCB (e) clones refer to ds CYMV cDNA cleaved with HindIII and Bam HI respectively and ligated with pBR322 similarly cleaved.

(f) N.C. refers to clones whose inserts were not released from the vector by restriction.

The bacterial transformants which grew after transformation and selection for the appropriate antibiotic resistances were further screened by *in situ* colony hybridization using ^{32}P-end labeled fragmented CYMV RNA to select for those colonies which contained the largest inserts (not shown). Those colonies were grown, their plasmids isolated and their inserts released by digestion with the appropriate restriction enzymes. The sizes of the cloned DNA inserts are also shown in Table 10.

To our disappointment, the size of any individual cloned insert represented no more than 30% of the 6800 nucleotide CYMV genome. However, the sum of the insert sizes (Table 10), if all fragments were different, would span this genome more than three times.

As a means of further characterizing the cloned CYMV sequences selected plasmids were grown in large scale cultures, the plasmids extracted, purified on CsCl gradients then subjected to digestion with numerous restriction enzymes. Table 11 lists those restriction enzymes which cleaved within the inserted sequence of each of the plasmids tested. Comparison of the restriction enzyme cleavage sites within each insert indicated that some cloned inserts did contain sequences representing different regions of the CYMV genome. For example, the 2000 bp insert of pCYMV65 had recognition sequences for the enzymes *Ava* I and *Kpn* I, whereas the

1600 bp insert of pCYMV21 contained recognition sequences for Sph I and Eco RI. Since no Pst I sites were found in these inserts, the two Ava I cleavage sites of the 1100 bp insert of pCP43 which was cloned as a Pst I fragment must be unique. Similarly, the Eco RI site in the 880 bp insert with Bam HI ends derived from pCB125 must be unique. Also a small 210 bp insert derived from pCYMV 189 contained sites for both Xba I and Pvu II clearly differentiating this insert from that of other clones. These 5 recombinant plasmids span a total of 5790 bp which represents 85% of the CYMV genome.

The orientation of restriction sites within these inserts was then determined using multiple restriction enzyme digestions (Table 12). Since the ends of each insert have identical restriction enzyme recognition sites, the orientation of sites within the insert was mapped relative to their corresponding restriction site and/or the Pvu II site in the vector. For example, digestion of pCYMV 65 with Ava I and Pst I resulted in three fragments of 2200 (a doublet), 1075 and 960 bp. The distance from the Pst I site where the fragment was inserted moving clockwise to the Ava I site of the vector is 2174 bp. Moving counterclockwise from the other Pst I site through the Pvu II site to the Ava I site of the vector is 2188 bp. The 1075 bp and 960 bp fragments therefore represent the distances from the Ava I site in the 2000 bp insert to the Pst I sites at either

Table 11. Restriction enzymes which cleave within the inserts of recombinant CYMV plasmids [a].

	Restriction Enzymes										
Plasmid	Bam HI	Ava I	Cla I	Sph I	Pvu II	Sma I	Kpn I	Xba I	Pst I	Eco RI	HindIII
pCYMV21	-	-	-	+	-	-	-	-	-	+	-
25	-	-	-	-	-	-	-	-	-	-	-
49	-	-	-	-	-	-	-	-	-	-	-
60	-	-	-	-	-	-	-	-	-	+	+
65	-	+	-	-	-	-	+	-	-	-	-
81	-	-	-	-	-	-	-	-	-	-	-
91	-	+	-	+	+	-	+	+	-	-	-
101	-	-	-	-	+	-	-	+	-	-	-
118	-	-	-	+	+	-	-	+	-	+	+
139	-	-	-	-	+	-	-	+	-	-	-

Table 11. cont'd

	141	-	-	-	-	-	-	-	-	-	-	-
	162	-	-	-	-	-	-	+	-	-	-	-
	171	+	-	-	-	-	-	-	-	-	+	-
	172	-	-	-	-	-	+	-	-	-	-	-
	176	-	-	-	-	+	+	-	+	-	+	+
	189	-	-	-	-	-	+	-	+	-	-	-
pCE	56	-	-	-	-	+	-	-	-	-	-	+
pCB	125	-	-	-	-	-	-	-	-	-	+	-
pCP	43	-	++	-	-	-	-	-	-	-	-	-

a. + indicates a single cleavage, ++, two sites of cleavage.

end. The orientation of the Ava I site relative to the vector cannot be discerned through this digestion alone. The orientation of the Ava I restriction site was therefore determined relative to the orientation of the Kpn I site. Digestion of pCYMV 65 with Kpn I and Pst I divided the insert into two fragments, 1250 and 700 bp in length. Digestion with Pvu II and Kpn I also yielded two fragments of 3600 and 2900 bp. Since Kpn I does not cleave within the vector the 2900 bp fragment therefore represents the distance from the Pvu II site of the vector to the Kpn I site within the insert. Subtracting the distance from the Pvu II site to the Pst I site (1547 bp) in the vector, results in a calculated distance of 1353 bp. The Kpn I site is accordingly 1250 bp from the left end of the insert and 700 bp from the right end. The Kpn I- Ava I distance (340 bp) within the insert is therefore consistent with the placement of the Ava I site 960 bp from the left end of the insert. The orientations of restriction sites in other inserts were determined using similar logic and are shown in Figure 36. The orientations of the restriction sites in each insert as well as their relative separation confirmed that each was located in different regions of the CYMV genome.

To address the problem of how these clones are oriented with respect to one other and with respect to the genome we again resorted to in situ colony hybridization. Essentially we "walked" from a fixed point

Table 12. Fragment Sizes produced by restriction enzyme digests of plasmids containing CYMV inserts.

Plasmid	Cleavage by	Products (bp)[a,b]
pCYMV 21	Pst I	4300, 1600
	Sph I	3250, 2600
	Sph I + Pst I	3100, 1400, 1300, 395
	Eco RI	3900, 1300
	Eco RI + Pst I	3500, 1200, 740, 500
	Eco RI + Pvu II	2750, 2200, 1380
	Eco RI + Sph I	3500, 1300, 800, 550
pCE 62	Eco RI	4300, 780
	Bam HI	4500, 500
	Pst I + Bam HI	3200, 1400, 500
	Bam HI + Eco RI[b]	660, 115
pCB 125	Bam HI	4300, 870
	Bam HI + Eco RI	660, 385, 210
	Eco RI	580
pCYMV 171	Pst I	4300, 850
	Pvu II + Bam HI	2900, 2200
	Bam HI + Eco RI	600, 385, 115
	Bam HI + Pst I	640, 200
	Eco RI + Pst I	700, 82

Table 12. cont'd

pCE 56	Eco RI	4300, 900
	Sph I	4100, 1100
	HindIII	200
	Eco RI + Sph I	560, 540, 360
	Eco RI + HindIII	700, 170
	Sph I + HindIII	530, 370, 360, 200
pCYMV 60	Pst I	4300, 650
	HindIII + Pst I	3600, 1100
	HindIII + Pst I	337, 330
	Eco RI + Pst I	750, 500, 90, 70
	Eco RI + HindIII	170
	Eco RI + HindIII + Pst I	337, 170, 90, 70
pCYMV 176	Pst I	4300, 1100
	Sph I	4200, 1300
	HindIII	4300, 1200
	Eco RI	4200, 1300
	Pvu II	3500, 1900
	Sph I + Pst I	4300, 1100
	HindIII + Pst I	4300, 700
	Eco RI + Pst I	4300, 550 (d)
	Pvu II + Pst I	2800, 1550, 800
	Xba I + Pst I	4300, 800
	Eco RI + Pvu II	120, 70
	Eco RI + Xba I	310, 70
pCYMV 189	Pst I	210
	Pvu II + Pst I	118, 95
	Xba I + Pst I	133, 80

Table 12. cont'd

pCYMV 101	Pst I	4300, 1100
	Kpn I + Pst I	4300, 900
	Kpn I + Eco RI	4400, 900
	Pvu II	3700, 1600
	Pvu II + Eco RI	2000, 1650, 1600
	Xba I + Pst I	4300, 900
pCYMV 65	Pst I	4300, 2000
	Ava I	3400 (d)
	Ava I + Pst I	2200 (d), 1075, 960
	Ava I + Pvu II	3300, 2600, 650
	Kpn I	5800
	Kpn I + Pst I	4300, 1250, 700
	Kpn I + Pvu II	3600, 2900
	Kpn I + Ava I	3100, 2800, 340
pCYMV 69	Pst I	4300, 740, 570
pCP 43	Pst I	4300, 1100
	Ava I	4800, 650
	Ava I + Pst I	600, 400, 100
pCYMV 92	Pst I	440, 260, 340

a. DNA fragments which correspond to vector sequences are underlined with a solid line. Those which contain both vector and insert sequences are underlined with a broken line and those which correspond to insert sequences are not underlined.

Table 12. cont'd

b. Digestion products resolved on 1% agarose gels were determined using an Eco RI + HindIII digest of λ plac 5 as molecular size marked.
Digestions which produced fragments less than 600 bp were resolved on 6% polyacrylamide gels. Fragment sizes shown are only those resolved by the gel.

of reference within our library of CYMV sequences to adjacent sequences. The hybridization probes used in this case were cloned DNA inserts purified from either agrose or polyacrylamide gels. After purification, the inserts were ^{32}P-labeled by nick-translation (see Materials and Methods, Section 4.2.9.2.ii). The results are summarized in Table 13 and some representative colony hybridizations are shown in the appendix, Figure A1.

As noted in Table 13 the insert from pCYMV 21 hybridized only to the colony bearing the plasmid from which it was derived. Inserts derived from pCYMV 65, pCYMV 60 and pCYMV 101, however, hybridized to a number of colonies. In particular, all three inserts (65, 60 and 101) hybridized to the colony harbouring plasmid pCYMV 176. A preliminary screening with restriction enzymes (Table 11) had established that these four cloned inserts shared some restriction sites. The orientation of the restriction sites in these plasmids was determined (Table 12 and Figure 36). The results established that part of the sequences of pCYMV 101 was nested within that of pCYMV 65. The restriction map of pCYMV 101 established that the Pvu II and Xba I cleavage sites were to the left side of the insert as it is oriented in the vector. pCYMV 189 was found to hybridize with the insert from pCYMV 101 and was known to be a small (210 bp) insert (Table 10) containing the Xba I and Pvu II restriction sites (Table 11). This plasmid was used, therefore, to establish the

Table 13 Clones exhibiting a positive hybridization signal in response to ^{32}P-labeled DNA inserts.

Inserts Used in Hybridization [a]	Colonies Displaying a Positive Hybridization Signal [e]
pCYMV 21 [c,d]	21
pCYMV 65 [b,c]	60, 65, 70, 71, 99, 101, 113, 126, 128, 138, 140, 176
pCYMV 101 [b,c]	47, 48, 65, 70, 87, 94, 101, 162, 176, 189
Eco RI - HindIII fragment of pCYMV 60 (170 b.p.) [b,d]	60, 81, 100, 104, 114, 129, 139, 141, 176, pCE 56
pCE 56 [b,c]	25, 60, 70, 74, 75, 80, 81, 84, 90, 94, 95, 99, 100, 102, 103, 108, 113, 128, 131, 137, 138. 141, 144, 166, 176

Table 13. cont'd

Kpn I - Pst I fragment of pCYMV 65 [d] (700 b.p.)	65, 70, 94, 95, 162, 176
Ava I - Pst I fragment of pCYMV 65 [d] (960 b.p.)	65, 69, 74, 95, 162
pCB 125 [b,d]	61, 73, 91, pCE 62, pCE 81, 148, 169, 171, 175
Eco RI - Bam HI fragment of pCYMV 171 [b,d] (115 b.p.)	67, pCE 62, pCE 81, 122, 171, 175
pCP 43 [c]	69, 92, 209
99 [c]	47, 48, 49, 60, 65, 70, 71, 80, 89, 101, 103, 113, 126, 128, 138, 140, 176, 189, 192

a. Approximately 10 µg of CsCl purified plasmids bearing CYMV inserts were digested for 6 hours with a twofold excess of the appropriate restriction enzyme(s). The insert fragment(s) was then resolved from the vector

Table 13. cont'd

fragment(s) on either 1% (w/v) agarose gels or 6% (w/v) polyacrylamide gels. Fragments were eluted and purified free of gel matrix (Materials and Methods, Section 4.2.8) prior to labeling with ^{32}P.

b. In situ colony hybridization for the probes indicated (from which these data were obtained) are illustrated in Figure A1 of the appendix.

c. The colonies probed by these inserts are indicated by template a of Figure A1 of the appendix.

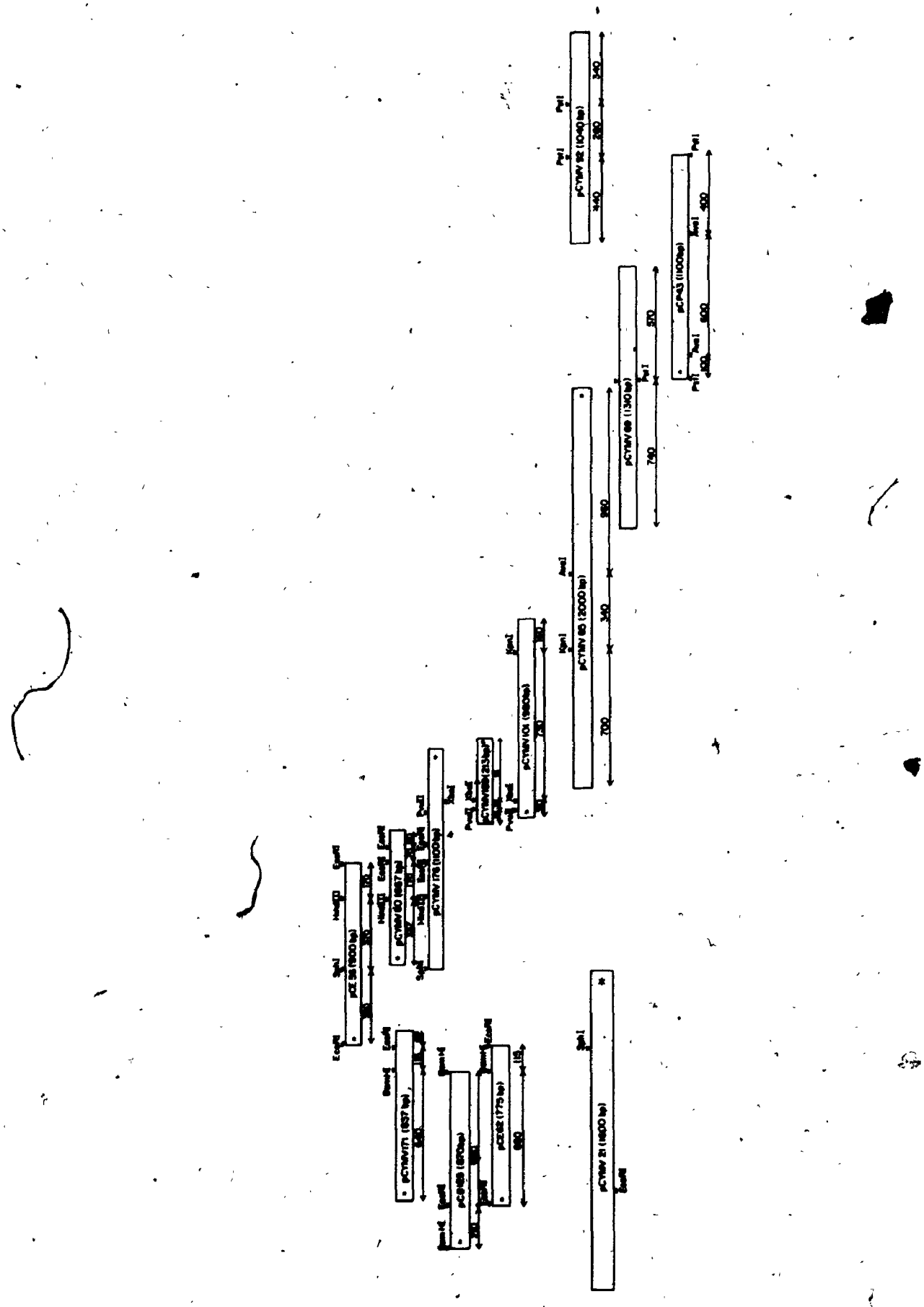
d. The colonies probed by these inserts are indicated by template b of Figure A1 of the appendix.

e. The criterion used to indicate a positive response was the indication that the intensity of the hybridization signal was greater than that of background hybridization.
Underlined numbers indicate pCYMV colonies whose hybridization signal was clearly more intense than all the others.

distance between these two sites. Similarly, the restriction digests of pCYMV 60 revealed a 70 bp Eco RI fragment which was initially missed in the digestions of pCYMV 176. The hybridization of the Eco RI - HindIII fragment of pCYMV 60 established that part of its sequence was nested within that of pCE56 which contains a 900 bp insert with unique Eco RI ends. Again, the orientation of restriction sites confirmed their relatedness using this technique the insert of pCYMV 65 has been overlapped some 1300 bp. The hybridization of the 960 bp Ava I-Pst I fragment of pCYMV 65 (i.e. from its 'right' end in Figure 36) to colonies lysed in situ revealed that plasmids pCYMV 69 and pCYMV 74 shared sequence homologies with this end. The size of pCYMV 74 (500 bp, Table 10) was smaller than the probe and therefore might have sequences nested with the Ava I-Pst I fragment. The insert of pCYMV 69 was, however, unique in two ways. First, it contained a Pst I site within the insert and secondly, the insert of pCYMV 69 annealed with a probe made from the insert of pCP43 which has unique Pst I ends. The insert of pCP43 also hybridized with plasmid pCYMV 92 which has two Pst I sites within its insert. Neither pCYMV 69 nor pCYMV 92 were subjected to further analysis due to problems we encountered with their stability. Thus, the inserts of pCYMV 69, pCP43 and pCYMV 92 have been oriented (Figure 36) to produce the maximum overlap of fragments. With maximum overlap, the

Figure 36 Partial restriction map and restriction site orientation of pCYMV inserts which span the CYMV RNA genome.

Restriction sites and orientation of fragments were derived from data in Table 12. The left hand end of the insert as orientated in the plasmid vector is indicated with an *.



insert of pCYMV 65 is overlapped 1770 bp in the right hand direction. Our tentative map at this point spans 5070 bp from the end of pCE56 to the end of pCYMV. 92, centred on pCYMV 65 (see Figure 36). Since none of the fragments analyzed thus far contained Bam HI sites, the insert from pCB 125 which has Bam HI termini was hybridized to colonies in situ. Two clones pCYMV 171 (thought to be 1200 bp at the time) and pCE62 (because it had unique Eco RI ends) were selected and further characterized by restriction digestion. The orientations in Figure 36 are consistent with the restriction data given in Table 12. As the data stand, however, these three plasmids, pCYMV 171, pCB 125 and pCE62, which comprise 1050 base pairs do not overlap with any others in our tentative map. We have arbitrarily assigned this insert group to the left side of our map (Figure 36). In total, the plasmids mapped in Figure 36, span roughly 6100 nucleotides or 90% of the 6800 nucleotide genome.

An unsolved problem is the assignment to our map of the 1600 bp insert in pCYMV 21. This plasmid (part of the series pCYMV 16 to pCYMV 49), was constructed in a separate experiment from a different preparation of cDNA. The disposition of restriction sites in pCYMV 21 is incompatible with the present map. We do not know, however, whether this is a cloning artifact (rearrangement) or a bona-fide product.

4.4 Discussion

The work presented in this chapter reports the cloning of PMV and CYMV RNAs. We were disappointed in both cases in not being able to construct single plasmids containing DNA insert representing the entire genome. In addition, none of the clones constructed from either PMV or CYMV RNA was found to express the respective coat protein in *E. coli* as determined by an *in situ* colony immunodetection assay which was sensitive to 1 ng of coat protein (data not shown; Kemp and Cowman, 1981). Also none of the recombinant plasmids tested would produce recognizable products in an *E. coli* cell-free system in which transcription and translation are coupled (data not shown; Mackie, 1977). Clone pCYMV 92, however, was found in preliminary experiments to inhibit coat protein synthesis in hybrid-arrested translation of CYMV RNA (data not shown; Patterson *et. al.*, 1977).

The terminal location of pCYMV 92 in our map (Figure 36) is fully consistent with our placement of the coat protein coding sequences towards the 3' end of the genome (Chapter 3). DNA sequencing experiments to be performed in collaboration with Dr. M. Abouhaidar (University of Toronto) should confirm this.

Two other approaches to identify plasmids containing 3' or 5' terminal viral sequences have failed. In one case (for 3' ends) we attempted to purify polyadenylated products of an RNAse III digest of CYMV RNA. The probe

was obviously not pure as all colonies tested responded positively. We attempted to construct a 5' probe by protecting CYMV RNA from partial nuclease digestion with coat protein. Again the resultant probe was apparently impure.

We had not anticipated our failure to generate nearly full length cDNA clones when we began this work. First, making full length second-strand cDNA on first strand templates of 6000 nucleotides or greater is difficult. This problem has also been encountered in attempts to prepare full length ds cDNA to TMV RNA (Goelet et. al., 1982, Meshi et. al., 1982) Secondly, the DNA nicking activity associated with the terminal transferase clearly illustrates that the quality of the enzymes used at all stages is crucial to such an undertaking. We feel, nonetheless, that the clones we have constructed will enormously facilitate the determination of the nucleotide sequence of CYMV and PMV RNAs. These clones should also be of considerable value in probing viral replication in vivo. Furthermore, this work has established some guidelines which may aid future attempts to clone larger full-length double-stranded CYMV cDNA. For example, Cla I was found not to cleave within any of the recombinant plasmids bearing CYMV inserts. Thus, attaching Cla I linkers to the ends of the double-stranded cDNA (Goodman and MacDonald, 1979) should provide a means of cloning without cleavage within the

insert. Secondly, the finding that full length first-strand synthesis can be accomplished using methylmercury hydroxide suggests that cDNA made to purified plus and minus strands (replicative intermediates) of CYMV RNA may be a successful alternative to create full length double-stranded cDNA. (Taniguchi et. al, 1978; Both et. al., 1982). Another alternative would be to employ a recent method (Okayama and Berg, 1982) which is claimed to be more efficient for cloning larger messenger RNAs.

The importance of eventually obtaining a full-length cDNA to a plant viral genome, is the possibility that it may be infectious in vivo. Such was recently found to be the case for a full-length cDNA copy of poliovirus RNA (Rancaniello and Baltimore, 1982). If this could be accomplished for plant virus genomes as well, it would allow the construction of defined mutants which would enhance our understanding of expression and replication of plant viruses.

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SUMMARY

As noted in Chapter 1, the technique of *in vitro* translation as applied to plant viral RNA genomes has provided a means for determining what proteins are encoded by plant viral genomes as well as a means of localizing the regions of the genome which encode each protein. Furthermore, *in vitro* translation has provided insight into the translational strategy or how the information within the genome is converted into that of discrete protein. At the time that this study was initiated, information of this nature was unavailable for a group of morphologically related plant viruses known collectively as the potexvirus group. To characterize further the physical and functional properties of potexviral RNA genomes we have applied the techniques of *in vitro* translation and cDNA-RNA hybridization to the genomes of potexvirus members whose morphology and architecture were well characterized, namely, papaya mosaic virus (PMV), clover yellow mosaic virus (CYMV), barrel cactus virus (BCV), foxtail mosaic virus (FTMV) and viola mottle virus (VMV).

The major findings described in this report are as follows:

1. The RNA genome of each potexvirus member tested consisted of a single component of approximately 2.2 x 10^6 daltons which within the limits of detection were devoid of any low molecular weight components.
2. PMV and CYMV RNAs direct the translation of numerous polypeptide products in either wheat germ extracts of rabbit reticulocyte lysates. In each case, three major products are reproducibly encountered. The major products directed by PMV RNA have apparent molecular weights of 155 000, 73 000 and 22 000 daltons whereas those directed by CYMV RNA have apparent molecular weights of 182 000, 76 000 and 20 000 daltons. The 22 000 and 20 000 dalton polypeptides directed by PMV and CYMV RNA respectively, comigrated on SDS-polyacrylamide with their respective coat proteins.
3. Peptide mapping has revealed that the high molecular weight polypeptides (i.e. 155 000 and 73 000 dalton) as well as many of the minor products of PMV RNA translation are related. The sequences of the 73 000 dalton protein are most likely nested within that of the 155 000 dalton protein. These high molecular weight polypeptides are, however, unrelated to that of the 22 000 dalton protein. Similarly, the 182 000 and 76 000 dalton products specified by CYMV RNA

shared peptides in common and as such are related. However, the peptides of the 20 000 dalton product were unique.

4. The 22 000 and 20 000 dalton *in vitro* polypeptides specified by PMV and CYMV RNA respectively were immunoprecipitated by affinity purified antisera raised against their respective coat proteins and thus share antigenic determinants with their respective coat proteins. Such antisera did not react with the high molecular weight polypeptides in either case. Furthermore, comparison of peptides of the CYMV RNA directed 20 000 dalton product with that of CYMV coat protein again suggested that the two were related.

5. Subviral particles generated by partial encapsidation of either PMV or CYMV RNAs were found to be templates for *in vitro* translation. Partial encapsidation led to a substantial enhancement in the yield of the respective *in vitro* coat protein and a concomitant reduction in other products. By correlating the disappearance of the *in vitro* products with the extent of encapsidation of the RNA it was possible to construct a tentative map for each genome. In each case, the high molecular weight products were localized towards the 5' end of the genome whereas the coat protein was localized towards the 3' end.

6. The genomic RNAs of BCV, FTMV and VMV directed the synthesis of numerous polypeptides *in vitro*, none of which comigrated with their respective coat proteins.

7. Our finding that the morphological relatedness of members of this group appeared not to be a reliable indication of the functional behavior of the corresponding RNAs led us to examine the relationship of these viral genomes at the level of nucleotide sequence. Using RNA-cDNA hybridization we found that no homology exists between the genomes of all members of the potexvirus group tested, with other unrelated viruses.

It is no surprise that this work has created more questions than it has answered. There are many directions this work could proceed. Perhaps, the most important direction would be to resolve the mechanism by which coat protein is expressed from either PMV or CYMV genomic RNA. This could be achieved in several ways; by using *in vitro* translation systems composed of purified components to minimize degradation of the RNA during translation, or by analyzing the nature and translatability of RNA found in polysomes. For this latter method, it would be helpful to have a cDNA clone to the region of the genome which specifies the coat protein. Another direction would be to determine the mechanism by which coat protein is expressed from the genomes of BCV, VMV and FTMV. Since

their genomic RNAs did not direct the synthesis of coat protein during translation *in vitro* it seems likely that they may produce an LMC RNA *in vivo* in a manner analogous to that described for TMV. Detection of such an LMC RNA could be accomplished by using cDNA probes to analyze viral RNA sequences made *in vivo* using the method used to analyze CYMV RNA sequences *in vivo* described in Chapter 2.

Finally, as described in Chapter 4, the construction of full length cDNA clones to potexviral genomes would facilitate answering questions with regards to the structure and expression of their genomic RNAs and provide a means of analyzing the relationship and function of the genome specified products.

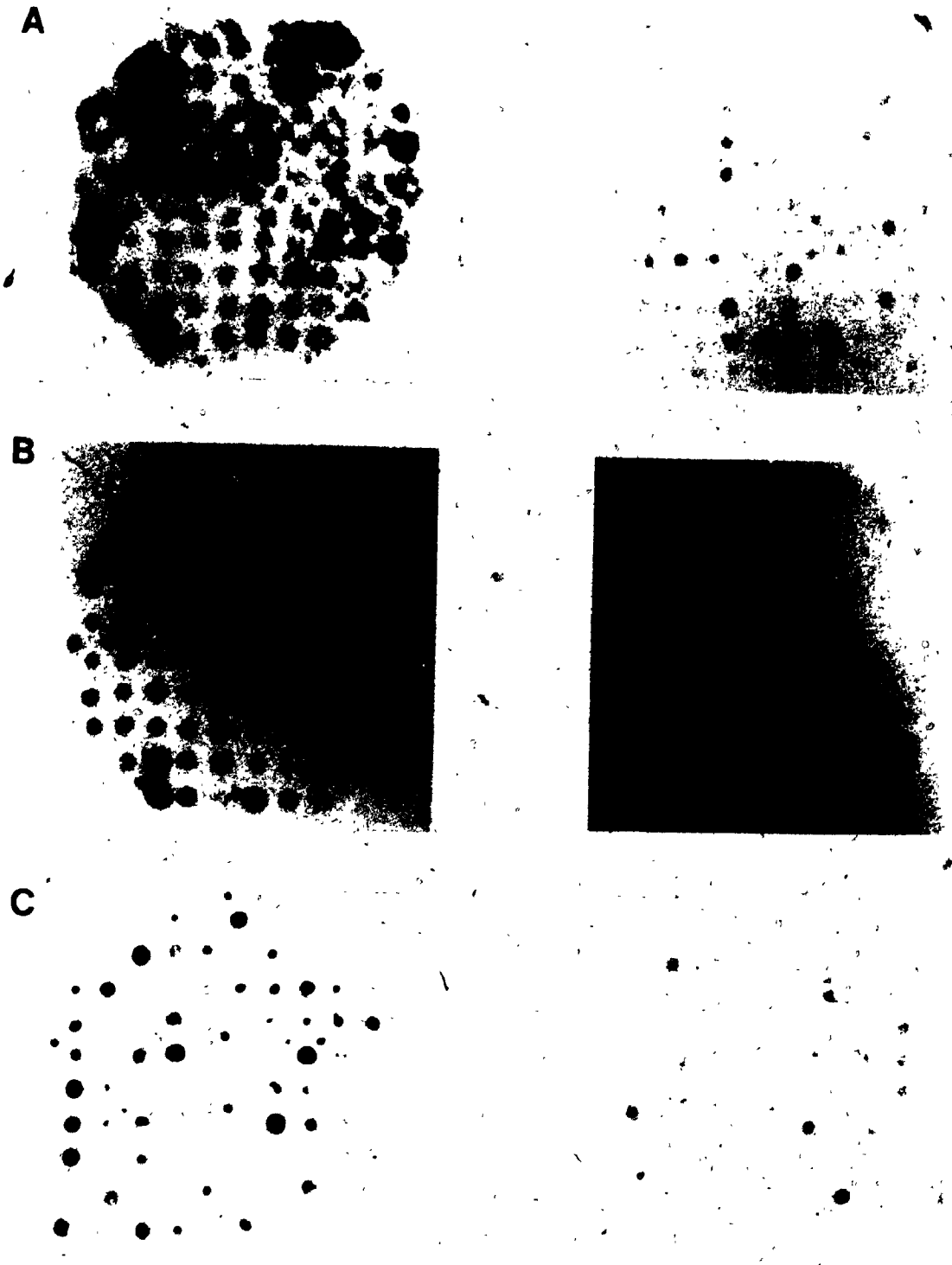
APPENDIX

Figure A1 In situ colony hybridization of pCYMV clones using labeled CYMV ds DNA sequences as probe.

DNA inserts were released and purified from their plasmid vector as described in Table 13. Inserts were labeled by nick-translation (Materials and Methods, Section 4.2.9.2.ii) then hybridized to the DNA of bacterial colonies which had been immobilized on nitrocellulose filters as described in Materials and Methods, Section 4.2.9.3. The inserts used as probe are as follows:

a. pCYMV 65, b. pCYMV 101, c. pCE 56, d. pCB 125, e. Eco RI - Bam HI fragment of pCYMV 171, f. Eco RI - HindIII fragment of pCYMV 60.

The colonies probed in a, b and c were those of template a; colonies probed in d, e and f were those of template b.


A
D
B
E
C
F

Template A.

								305				306								
					16		17		21		25		47		48					
			49		60		61		64		65		66		67		68			
	69		70		71		72		73		74		75		80		81		82	
	84		87		89		90		91		92		93		94		95		99	
196		205		207		209		218		221		231		266		267		268		269
	100		101		102		103		104		105		107		108		109		111	
270		276		280		281		282		283		285		286		287		292		304
	113		114		117		118		120		121		122		123		126		127	
	128		129		131		133		134		136		137		138		139		140	
	141		142		144		146		148		151		154		155		156		160	
			161		162		163		166		169		171		172		175			
					176		177		187		189		192		194					

Template B.

		16	17	21	25	47	48		
	49	60	61	64	65	66	67	68	
69	70	71	73	74	75	80	81	82	84
87	89	90	91	92	93	94	95	99	100
pCP9	pCP10	pCP16	pCP17	pCP26	pCP43	pCP46	pCP52	pCP98	
101	103	104	105	107	108	109	111	113	114
117	118	120	121	122	123	126	127	128	129
	pCE13	pCE56	pCE62	pCE81	pCE91	pCE118	pCE167	pCE169	
131	133	134	136	137	138	139	140	141	142
144	146	148	151	154	155	156	161	162	163
	166	169	171	172	175	176	177	187	
	189	192	194	196	205	207			

Table A1

Properties of restriction enzymes used in this study

Restriction Enzyme	Source	Number of Cleavage Sites in pBR322	Cleavage Coordinate in pBR322	Sequence Recognized [a]
Ava I	Anabaena viriabilis	1	1424	C ↓ PyCGPuG
Bam HI	Bacillus amyloliquefaciens H	1	375	G ↓ GATCC
Cla I	Caryophanon latum L	1	23	AT ↓ CGAT
Eco RI	Escherichia coli RY 13	1	4360	G ↓ AATTC
HindIII	Haemophilus influenzae Rd	1	29	A ↓ AGCTT
Kpn I	Klebsiella pneumoniae OK 8	0	–	GGTAC ↓ C
Pst I	Providencia Stuartii 164	1	3612	CTGCA ↓ G
Pvu II	Proteus vulgaris	1	2065	CAG ↓ CTG
Sal I	Streptomyces albus G	1	650	G ↓ TCGAC
Sma I	Serratia marcescens S_b	0	–	CCC ↓ GGG
Sph I	Streptomyces phaeochromogenes	1	561	GCATG ↓ C
Xba I	Xanthomonas badrii	0	–	T ↓ CTAGA

a. Pu = purine, Py = pyrimidine

END

1	1	H	0	9	H	8	4

FIN