AN ABSTRACT OF THE THESIS OF

Jeffrey L. Ried for the degree of Master of Science in Botany and Plant Pathology presented on April 18, 1983.

Title: Genomic Comparisons of Seed-transmissible and Non-seed-transmissible Strains of Cucumber Mosaic Virus

Approved: _____
Richard O. Hampton

A sensitive assay for the analysis of contaminating segments in pseudorecombinants constructed from a seed-transmissible (CVM-Pg) and a non-seed-transmissible (CMV-Le) strain of cucumber mosaic virus was developed using partial RNase T_1 digestion. Isolated genomic RNA segments produced unique digestion patterns when 3'-end labeled, treated with RNase T_1, and subjected to polyacrylamide gel electrophoresis. Although unique bands were present in these patterns, an overall similarity existed, resulting from putative insertions and deletions in the RNA of each strain.

Northern blot hybridization studies of the homology between CMV-Pg and CMV-Le indicated that all 4 heterologous RNA segments contained sequences that were greater than 83% homologous. Homology between the total RNA from each strain was estimated at 70% when assayed by S_1 nuclease digestion following hybridization of RNA to a complementary DNA probe under stringent conditions.

A comparison of the electrophoretic mobility of each RNA of both strains in agarose, glyoxal and polyacrylamide gels suggested that each segment was identical in size. RNA segments 1 and 2 when compared to the heterologous strain, however, had discernable differences in the secondary structure.

A 5th RNA was detected in both strains. It migrated as two distinct bands when subjected to polyacrylamide gel electrophoresis, and was 3'-end labeled preferentially when present with segments 1-4. Complementary DNA synthesized from RNA 5 of one strain hybridized to segments 1-4 of both strains as determined by northern blot hybridization. RNA 5 of both strains produced identical patterns when subjected to partial RNase T_1 digestion, yet they differed significantly when compared to the digestion patterns from RNA 4 of the homologous strain. These results indicated that RNA 5 of both strains contained virtually identical nucleotide sequences and that they originated from RNA's 1-4, but not from the extreme 3'-terminus.

Total CMV RNA, subjected to RNase H treatment after hybridization to a specific deoxyribohexanucleotide, produced two novel bands which were visualized by ethidium bromide staining. Double-stranded cDNA synthesized from RNA of each strain, cloned by blunt-end ligation, produced recombinant clones that contained only inserts of insignificant size.

Genomic Comparisons of Seed-transmissible and Non-seed-transmissible Strains of Cucumber Mosaic Virus

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INTRODUCTION

Interactions between certain viruses and their plant hosts result in virus transmission through seeds. Thus, infected seed can serve as a virus inoculum reservoir and as a means of viral perennation. Even low rates of seed transmission facilitate plant disease epidemics when appropriate vectors are numerous and active. Cucumber mosaic virus (CMV), the type member of the cucumovirus group, is known to be seed transmitted in 20 species of plants (34). In addition to several species of weeds, seed transmission occurs in such agriculturally important crops as tomato, pumpkin and southern pea.

Until 1974, CMV was not known to be seed-borne in beans (Phaseolus vulgaris). Since that time, 5 isolates of CMV have been reported to be seed transmissible in this host (4,8,44,46, 52). The isolate of Davis and co-workers was termed CMV-Pg since its source was Phaseolus germplasm. This isolate was recovered from plants of plant introduction bean accession number 271998, from Spain. Davis compared properties of CMV-Pg to properties of a non-seed transmissible legume strain, CMV-Le (7). He determined that they were not distinguishable by the molecular weights of their coat protein subunits or by gel immunodiffusion.

Since CMV contains a functionally divided genome (51,39, literature review), pseudorecombinants can be constructed by

mixing isolated RNA segments from different isolates of CMV. This can provide genetic information concerning each RNA segment. Pseudorecombinants of CMV have demonstrated that RNA 3 contains the genes for coat protein (25) and aphid transmissibility (48), while RNA 2 determines host response in several legume species (14). As part of his thesis work, Davis constructed pseudorecombinants between CMV-Pg (seed transmissible in beans) and CMV-Le (non-seed transmissible in beans) in order to identify which RNA segment encoded the seed transmissibility trait. He isolated RNA segments from sucrose density gradients by fractionation. Because RNA's 1 and 2 could not be separated by gel electrophoresis they were treated as a unit. Infectivity assays on local lesion host plants indicated contamination of the isolated segments had occurred by no more than 12% for RNA's 1 and 2 (designated RNA 1/2) from each strain, and less than 3% each for RNA's 3 and 4 from each strain. These partially isolated RNA's were mixed in various combinations to generate pseudorecombinants, then inoculated onto tobacco plants. Sap from infected tobacco plants was then inoculated onto P. vulgaris 'Bountiful' plants, the plants were allowed to mature, and the seeds collected.

The seeds were not germinated and tested for the seed transmissibility of pseudorecombinants because strain markers were unavailable to monitor purity of pseudocombined RNA components. Thus, should a pseudorecombinant consisting of Pg 1/2 and Le 3

RNAs have been found to be seed transmissible, contaminating Pg 3 RNA could not be ruled out as the facilitator of seed tranmissibility.

The development of a sensitive analytical assay of these pseudorecombinants for the presence of a contaminating RNA was the basis for this thesis. Approaches used to develop an assay involved comparing RNA isolated from each strain to identify any exploitable differences. These approaches included RNA segment comparisons by 1) gel electrophoresis, 2) direct nucleotide sequencing of the 3'-terminus, 3) cloning cDNA synthesized from CMV RNA primed the 3'-terminus, 4) northern blot and liquid hybridization, 5) RNase H treatment, and 6) partial RNase T_1 digestion. Most of these approaches provided information about the relatedness of these two isolates, but only partial RNase T_1 digestion provided information about the relatedness of these two isolates, but only partial RNase T_1 digestion provided practical methodology to distinguish RNA segments of the two CMV isolates.

A large portion of this thesis is devoted to Materials and Methods because of the variety and complexity of the techniques utilized. All the approaches used to develop a sensitive analytical assay of contaminating RNA segments in CMV pseudorecombinants are included, although several of these techniques provided little useful information.

Literature Review

General Information. Cucumber mosaic virus (CMV) is the type member of the cucumovirus group. Also included in this group are

tomato aspermy and peanut stunt viruses, but CMV is the most common and most studied member. CMV was first described by Doolittle (1916) and Jagger (1916). This virus is distributed worldwide and is found especially in temperate regions (18). It has an extremely broad range of natural hosts comprising 470 species representing 67 families (26), and infects diverse agricultural crops ranging from almond to Zea mays. CMV causes a variety of disease symptoms such as mosaic, blight, and fern leaf depending upon the virus strain and host cultivar infected (18). The virus also infects numerous weed species, which are believed to play a major role in the survival of CMV when agricultural crops are not present (3,34). CMV is also transmitted through the seed of at least four weed species, which greatly aids its long term persistence (56). The virus is transmitted both mechanically and by over 60 species of aphids in a non-persistent manner (18). Nearly 60 isolates of CMV have been reported in the literature, yet because of the lack of standardized tests, the number of discrete strains is unknown (34). Several purification procedures have been used for CMV and follow two general schemes (18). The first involves initial extraction from infected leaves in citrate buffer with sodium thioglycollate, followed by polyethylene glycol precipitation and differential centrifugation. The second procedure involves tissue extraction in a sodium phosphate buffer containing thioglycollic acid and diethyldithiocarbamate. Crude extracts are subjected to differential centrifugation followed by sucrose density centrifugation.

Biochemical Information. CMV has been the subject of many biochemical studies. The virions are 28-30 mm in diameter (19) and contain 180 identical protein subunits in pentamer-hexamer clusters with T = 3 surface lattice symmetry (29). The multicomponent nature of the RNA was first discovered in 1965 (35). This was followed by the demonstration that the RNA components constituted a functionally divided genome (39,51). At least 5 major and several minor RNA components have been identified in CMV RNA preparations (39,51,54). The sizes of the four major RNA species are 1.35×10^6 d, 1.16×10^6 d, 0.85×10^6, and 0.35×10^6 (51) and are designated RNA 1, 2, 3, and 4 in order of decreasing molecular weight. The relative proportions of these RNA components vary considerably between virus strain and host plant (34). Analysis of the distribution of these components within the virions is difficult because of the instability of the virion in salt solutions. A partially salt-stable strain, CMV-D, has been analyzed by bouyant density centrifugation (38). It was concluded that virions can encapsidate only one molecule each of RNA 1, RNA 2, or both RNA's 3 and 4.

Despite multiple CMV RNA components, only the 3 largest are required for infection (39,51). RNA 4 is recoverable from plants infected with RNA 1+2+3. By comparing RNA 3 and 4 through nucleotide fingerprinting (40) and nucleic acid hybridization (22), RNA 4 was found to be contained entirely within the 3'-terminus of RNA 3. In addition, all 4 CMV RNA's were found to have identical or nearly identical sequences at their 3'-termini (55). A comparison of the

3'-termini of the 4 major RNAs from 4 different strains of CMV (Q, P, M, and T) showed a high degree of sequence conservation (57). Interrelationships between the RNA segments are represented in Figure 1.

The 3'-terminus of these RNAs can be aminoacylated with tyrosine and the proper plant aminoacyl-synthetase, as is true with other types of plant virus RNAs (37). This 3'-terminus can be represented as a tRNA-like structure that exhibits a high degree of base pairing (1,24). The significance of this highly conserved sequence and the ability to bind amino acids is unknown, but it is suggested that this region could play a role in RNA-polymerase recognition.

The entire sequence of RNA 3 from CMV-Q has been determined by analysis of cDNA clones made from this RNA (23). This also revealed the sequence of RNA 4 since it is contained entirely in RNA 3 and the amino acid sequence of the 236 residues which make up the CMV coat protein.

CMV supports the replication of a satellite-like RNA, designated CARNA 5 for CMV - associated RNA 5 (36). CARNA 5 has a molecular weight of about 0.1×10^6 d and lacks sequence homology with genomic RNA as determined by competition hybridization (10) and oligonucleotide fingerprinting (40). It is dependent upon genomic RNA for its replication and can cause a severe necrosis disease in tomatoes when accompanied by CMV RNA (34). A more common characteristic of these satellite RNAs, however, is their

Figure 1. Relationship between genomic CMV RNA segments. Each RNA segment contains areas of nearly identical sequence homology at the 3'-terminus indicated by (▭). RNA 4 is subgenomic to the 3'-terminus of RNA 3 indicated by the dashed lines linking the two molecules.

RNA segment	length (bases)
1	4100
2	3500
3	2300
4	1000

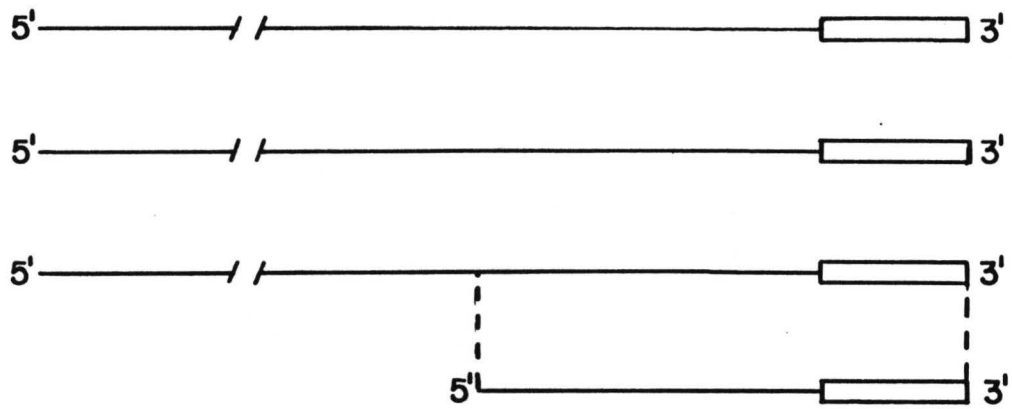

Figure 1.

ability to attenuate symptoms normally produced by CMV (32,33). Evidence indicates that attenuation occurs because CARNA 5 disrupts viral RNA synthesis (30).

MATERIALS AND METHODS

Sources, Propagation and Isolation of Virus

CMV-Pg was originally isolated by Dr. R. F. Davis from a plant introduction accession (No. 271998) of Phaseolus vulgaris. CMV-Le, a Japanese legume-infecting isolate, was recovered by Dr. T. Inouye. Both isolates were propagated in P. vulgaris 'Bountiful' using desiccated material as a standard inoculum source.

Each virus was propagated by grinding 0.1 g of desiccated virus-infected bean-leaf tissue in a mortar and pestle in 10 ml 0.02 M sodium phosphate buffer, pH 7.0. This crude virus preparation was inoculated onto carborundum-dusted, primary leaves of P. vulgaris 'Bountiful' bean plants approximately 3 weeks after planting. Plants inoculated with CMV-Pg or CMV-Le were grown in separate growth chambers on a temperature/light regime of 24 C/16 hours light and 20 C/8 hours dark. Plant tissues were harvested for virus purification 12-14 days after inoculation.

CMV was purified essentially as described by R. Davis (7) and stored at 4 C. The procedure is outlined in Table 1.

Isolation of RNA

Genomic RNA was isolated from purified virus following treatment with 1% sodium dodecyl sulfate (SDS) and 0.1 mg/ml Proteinase K at 37 C for 30 minutes (51). Protease treatment

TABLE 1. Purification for cucumber mosaic virus.[a]

1. Homogenize tissue in 2 volumes each of 0.5 M citrate pH 6.5 with 0.1% sodium thioglycollate and chloroform.
2. Low speed 10 min at 3000-4000 g.
3. Remove supernatant, then add polyethylene glycol 6000 to final concentration of 10% (w/v).
4. Stir 30-45 min, centrifuge 10 min at 12,000-16,000 g.
5. Resuspend pellet in 0.05 M citrate pH 7.0 with 0.2% Triton X-100, stir 30-45 min.
6. Centrifuge 1.5 hr at 105,000 g.
7. Resuspend pellet overnight in 0.02 M EDTA (ethylene-diamine-tetracetic acid), pH 7.0.
8. Centrifuge 15 min at 12,000 g.
9. Centrifuge 1.5 hr at 105,000 g.
10. Resuspend pellet overnight in EDTA buffer.
11. Layer 0.5-1.0 ml virus onto 10-40% linear sucrose gradients prepared in EDTA buffer, centrifuge 2 hr at 25,000 rpm in Spinco SW 28.
12. Collect band manually with syringe, centrifuge 1.5 hr at 105,000 g.
13. Resuspend pellet in EDTA buffer and store at 4 C.

[a]All glassware and reagents are kept at 0-4 C.

was followed by phenol extraction and ether extraction. The RNA was precipitated with ethanol, lyophilized to dryness and resuspended in 20 mM tris (hydroxymethyl) aminomethane hydrochloride (TRIS·HCl) pH 7.0, and 5 mM EDTA.

RNA Glyoxalation

RNA was denatured by glyoxal treatment (6). Single strain RNA samples of 2 ug or combined strain samples of 1.5 ug of RNA were glyoxalated in 15 ul reaction mixtures. The mixtures consisted of RNA, 10 mM sodium phosphate pH 6.9, 50% DMSO, and 1 M glyoxal, and were heated at 50 C for 60 minutes. Two microliters of tracking dye (7 M urea, 10 mM TRIS·HCl pH 8.0, 1 mM EDTA, 0.025% each of bromephenol blue and xylene cyanol) were added and samples were electrophoresed in horizontal 1% agarose gels in 10 mM sodium phosphate pH 6.9 for 3-5 hours at 50 mA constant current. Buffer was recirculated constantly and changed after 2.5 hours. The gel was stained in 30 ug/ml acridine orange for 15 minutes, followed by destaining in H_2O overnight at 4 C.

Polyacrylamide Gel Electrophoresis

Polyacrylamide gels were made from an acrylamide:bisacrylamide 38%:2% stock solution (Sigma) which had been deionized with a mixed bed resin, AG-501-X8 (Bio-Rad). These gels consisted of 4.5%, 8.0%, or 10% acrylamide, 50 mM TRIS base, 50 mM boric acid, 1.25 mM EDTA,

pH 8.3 (TBE buffer), 7 M urea, 0.08% $(NH_4)_2S_2O_8$ (Sigma), and 0.035% N,N,N',N'-tetramethylenediame (Bio-RAD) (45). Electrophoresis was performed at various voltages depending upon the gel concentrations and bed dimensions.

End Labeling with ^{32}pCp

Radiolabeled [5'-^{32}P] cytidine 3',5'-bisphosphate (pCp) was prepared by the transfer of the γ-phosphate of [γ-^{32}P-ATP (New England Nuclear, 5000 Ci/mmole) to 3'cytidine monophosphate using the procedures described by England et al (16). A 24 ul reaction mixture consisted of 50-200 uCi γ-ATP, 20 mM 2[N-cyclohexylamino] ethanesulfonic acid pH 9.3, 1 mM 3'cytidine monophosphate, 5 mM $MgCl_2$, 3 mM dithiothreitol (DTT), 50 ug/ml bovine serum albumin (BSA), and 100 U/ml T_4 polynucleotide kinase (PNK) (Bethesda Research Laboratories). The reaction mixture was incubated at 37 C for 90 minutes and then terminated by heating at 100 C for 60 seconds. The efficiency of the transfer of the terminal phosphate group was monitored using phosphoethyleneimine (PEI) thin layer chromatography (Van Waters and Rogers). Samples of less than 0.5 ul were spotted on the PEI plate before the addition of PNK and following the 90 minute incubation. The PEI thin layer plates were developed in 0.8 M $(NH_4)_2SO_4$.

Varying concentrations of RNA from 50-500 ug/ml were 3'-end labeled with pCp (16) in reactions of 20-30 ul consisting of

50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid pH 8.3, 10 mM $MgCl_2$, 3.3 mM DTT, 10 ug/ml BSA, 5.0 uM ATP, 10% dimethyl sulfoxide, 200-2500 uCi/ml pCp, and 135 U/ml T_4 RNA Ligase (P. L. Biochemicals). Samples were incubated at -15 C for at least 40 hours.

RNA Segment Isolation

RNA segments were isolated preparatively from 1% low-melting-temperature agarose gels (Bio-Rad) in TBE buffer and ethidium bromide (Sigma) at 0.5 ug/ml (20). Up to 30 ug total RNA was loaded onto the gel and electrophoresed at 150 volts for about 45 minutes. Bands, visualized by ultraviolet light, were sectioned from the gel and melted at 65 C in 20 mM TRIS·HCl pH 7.0, 5 mM EDTA. The solution was phenol extracted and the phases separated by centrifugation at 10,000 rpm in an SS34 rotor (12,000 g) for 10 minutes. The aqueous phase was extracted with 1-butanol until all cloudiness disappeared. This was followed by diethyl ether extraction and ethanol precipitation. The RNA precipitate was recovered by a 10 minute centrifugation at 12,000 g. The pellet was lyophilized to dryness, resuspended in H_2O and stored at -60 C.

Sequencing 3'-End Labeled RNA

3'-end labeled RNA segments were isolated from low-melting-temperature agarose gels and chemically sequenced by the method of Peattie (50). Digested RNA was then run on polyacryalmide gels. See Appendix I for details of the methodlogy.

RNase H Digestion

Oligodeoxyribonucleotides of defined sequence were hybridized to total RNA. The resulting RNA:DNA hybrid was digested with RNase H by the method of Donis-Keller (11), and either 3'-end labeled with pCp followed by electrophoresis on acrylamide gels, or run directly on acrylamide gels. See Appendix II for detailed methodology.

RNase T_1 Digestion

End labeled RNA was partially digested by RNase T_1 in 16 ul reaction mixtures as described by Donis-Keller et al. (12). Approximately 75,000 cpm of end labeled RNA (less than 5.0 ul) was added to a solution of 7 M urea, 2 mM EDTA, 2.5 mM TRIS·HCl pH 7.9, 0.25 mg/ml bovine tRNA, 0.02% each of bromephenol blue and xylene cyanol, 10 mM citrate pH 4.8, and 0.008 U or 0.01 U RNase T_1 (Sankyo Co. Ltd.). Samples were incubated at 50 C for 8 or 10 minutes, immediately cooled to 4 C and loaded onto pre-electrophoresed polyacrylamide gels. Electrophoresis was performed in TBE buffer at 600 or 1250 volts for 8% and 10% polyacrylamide gels, respectively.

Synthesis of cDNA

Randomly Primed Synthesis. Complementary RNA (cDNA) was synthesized as described by Buell et al. (5) in 40 ul reaction mixtures. About 2 ug RNA was added to 1.5 ug nicked calf thymus

DNA, boiled 3 minutes, then allowed to cool to room temperature. The solution was made 40 mM TRIS·HCl pH 8.0, 60 mM KCl, 8 mM $MgCl_2$, 20 mM DTT, 2 mM each of dATP, dGTP, and dTTP (P. L. Biochemicals), 100-600 uCi/ml α-^{32}-P-dCTP (New England Nuclear, 200-800 Ci/mmole) and 400-625 U/ml reverse transcriptase (J. Beard, Life Sciences, Inc.). The preparation was incubated at 42 C for 60 minutes. cDNA for use in liquid hybridization was synthesized in the presence of 100 ug/ml actinomycin D (Sigma).

RNA was digested by making the solution 0.1 N NaOH followed by incubation at 65 C for 60 minutes or at room temperature for 16 hours. This solution was neutralized by the addition of an equivalent amount of HCl. Nucleotides and salts were removed by Sephadex G-100 or G-50 column chromatography. cDNA was used directly at this point for either northern blot hybridization or liquid hybridzation.

Oligo dT Primed Synthesis. 35-675 ug/ml RNA was polyadenylated by the method of Emtage et al. (15) in a reaction mixture containing 50 mM TRIS·HCl pH 8.0, 10 mM $MgCl_2$, 0.25 M NaCl, 1.0 mg/ml BSA, 5 mM $MnCl_2$, 0.2 mM ATP, 15-150 uCi/ml ^{3}H-ATP (ICN, 26 Ci/mmole) and 20-200 U/ml poly (A) polymerase (Bethesda Research Laboratories or P. L. Biochemicals). The mixture was incubated at 37 C for 10-15 minutes, phenol extracted, ether extracted, ethanol precipitated and lyophilized. Oligonucleotides consisting of dT_8A, dT_8G, and dT_8C (P. L. Biochemicals) were added to the lyophilized RNA to produce a concentration of 26 ug/ml in the final cDNA reaction

mix. cDNA was then synthesized as described previously except cold dCTP was included at 0.1 mM while no nicked calf thymus DNA was present. The preparation was incubated at 42 C for 90 to 120 minutes. RNA was digested and the cDNA was isolated as previously described.

Cloning of cDNA to CMV-Pg RNA and CMV-Le

cDNA synthesized from polyadenylated CMV RNA was used for cloning experiments (47). Following first strand synthesis, the cDNA was polyadenylated with dATP using terminal deoxynucleotidyl transferase. dT_8X primers were hybridized to the d(A) tails of the cDNA, functioning as primers for the second strand synthesis of cDNA using Klenow fragment. The double stranded cDNA (ds cDNA) was then cloned by blunt-end ligation into the plasmid pUC9, which was used to transform E. coli (JM 103). Sizing of the clones indicated only small inserts were present. No further analysis was performed. See Appendix III for more detailed methodology.

Northern Blot Hybridization

Northern blot hybridization was performed as described by Alwine et al. (2). Aminobenzyloxymethyl (ABM) paper prepared according to Alwine et al. (2) was activated to diazobenzyloxymethyl (DBM) paper by wetting with ice cold 1.2 M HCl followed by a 35 minute immersion in a freshly prepared, ice cold solution of 1.2 M HCl/28 ug/ml $NaNO_2$. $NaNO_2$ at 10 mg/ml was added dropwise to

the HCl just prior to ABM paper immersion. The paper was then rinsed two times with ice cold double-distilled H_2O for 1 minute, followed by two 1 minute rinses in 200 mM NaOAc pH 4.0. At this point, the paper was fully activated and ready for blotting.

The gel to be blotted was soaked for 10 minutes at room temperature in 75 mM NaOH and twice rinsed for 15 minutes in 200 mM NaOAc pH 4.0. The mechanics of blotting is shown in Figure 2. Whatman 3 MM paper was wetted with 3M NaCl, 0.3 M sodium citrate pH 7.0 (20X SSC) prior to use. The gel was blotted at 4 C for 16 hours.

Blots were removed and neutralized at 42 C for 12-16 hours in the following buffer: 50% formamide, 0.9 M NaCl 50 mM sodium phosphate pH 6.9, 4 mM EDTA, 0.1% SDS, 1X Denhardt's solution (9) (0.02% each of ficoll, BSA, and polyvinylpyrrolidine in 0.15X SSC), 1% glycine, and 250 ug/ml sonicated salmon testes DNA. Approximately 0.1 ml neutralization buffer was used for each square centimeter of DBM paper.

The blots were prehybridized, just prior to hybridization, in the neturalization buffer except the DNA concentration was reduced to 100 ug/ml, the formamide was reduced to 20%, and the glycine was eliminated. Blots were incubated at 42 C for 3 to 12 hours. The prehybridization buffer was removed and replaced with fresh prehybridization buffer containing about 1 X 10^6 cpm of randomly primed cDNA. Blots were then incubated at 42 C for 40 hours, washed two times, 15 minutes each in washing buffer consisting of 20% formamide, 50 mM sodium phosphate pH 6.9,

Figure 2. Diagram of the procedure for transferring RNA onto DBM paper.

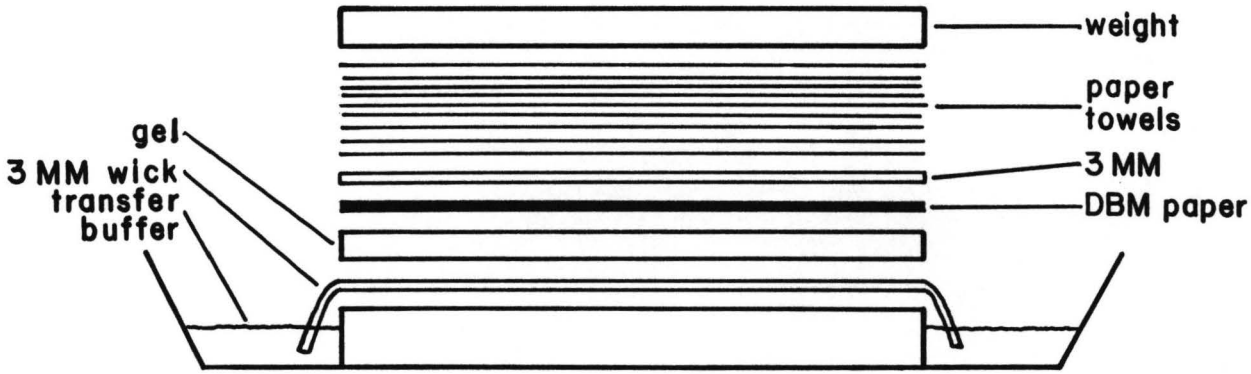
weight
paper towels
3 MM
DBM paper
gel
3 MM wick
transfer buffer

Figure 2.

4 mM EDTA, 0.1% SDS, and autoradiographed without drying.

For stringency variation, blots were washed twice for at least 30 minutes each at 42 C in the washing buffer at either 35% or 50% formamide. Hybridized probe was removed from the blots by washing in a buffer of 50% formamide, 50 mM sodium phosphate pH 6.9, 4 mM EDTA, and 0.1% SDS at 65 C for 18 to 36 hours. Removal of the probe was determined by autoradiography.

Liquid Hybridization

Randomly primed cDNA probes to total CMV RNA were hybridized to various RNAs in 50 ul reactions (27,53). Initially, about 100,000 cpm of the cDNA probe was boiled for 3 minutes either alone or with various RNA species, then immediately cooled on ice. The reaction was made either 20% or 50% formamide, 0.75 M NaCl, 0.15 M TRIS·HCl pH 8.0, and 5 mM EDTA. The final concentration of the RNA was 2 or 20 ug/ml. The reaction was incubated at 42 C for 18 or 24 hours.

S_1 Nuclease Assay

Liquid hybridization reactions were assayed for nuclease S_1 sensitivity by the method of Favolaro et al. (17). The reactions were placed on ice and 5 ul aliquots were assayed for S_1 resistance in triplicate in 200 ul reactions consisting of 30 mM NaOAc pH 4.5, 4.5 mM ZnOAc, 0.5 M NaCl, 500 ug/ml nicked calf thymus DNA, and 150

U/ml S_1 nuclease (New England Nuclear) and incubated at 42 C for 30 minutes. Controls, lacking the S_1 nuclease, were run in identical fashion. Reactions were terminated by the addition of 200 ul of a solution consisting of 100% trichloroacetic acid, 100% NaH_2PO_4, 100% sodium pyrophosphate (1:1:1), and incubation at 4 C for 10 minutes. Precipitable material was collected on 0.45 um nitrocellulose filters (Schleicher and Schuell) rinsed with 10% TCA and dried at 80 C for 10 minutes. The amount of radioactivity remaining on the filters was quantified by liquid scintillation counting.

RESULTS

RNA Isolation From Virions

CMV RNA of both CMV-Pg and CMV-Le, isolated from virions purified by the method outlined in Table 1, comprised four major electrophoretic bands corresponding to four major RNA segments, RNA 1, 2, 3 and 4 (Figure 3). This RNA, when isolated and stored at -15 C, became degraded with time (Figure 4). RNA samples stored at -60 C did not become degraded.

Electrophoretic Mobilities of CMV-Pg and CMV-Le

The component mobilities of genomic RNA electrophoresed in agarose gels were indistinguishable (Figure 3). Mobility differences between the RNA 2 of CMV-Pg and CMV-Le were discernable, however, by electrophoresis in 4.5% polyacrylamide-urea gels (Figure 5). No such differentiation occurred in 8% polyacrylamideurea gels. Segment 1 of both strains were repeatedly degraded in 4.5% gels. Electrophoresing glyoxalated RNA, in which RNA secondary structure was removed, resulted in co-electrophoresis of all RNA segments (Figure 6). Segments 1 and 2 from unglyoxalated RNA samples, in which secondary structure was maintained, had different electrophoretic mobilities (Figure 6). These results indicate that all the RNA segments of each isolate are identical in size, but segments 1 and 2 possess different secondary structures.

Figure 3. Comparison of the electrophoretic mobilities of CMV-Le and CMV-Pg RNA in 1% agarose gels in TBE buffer.

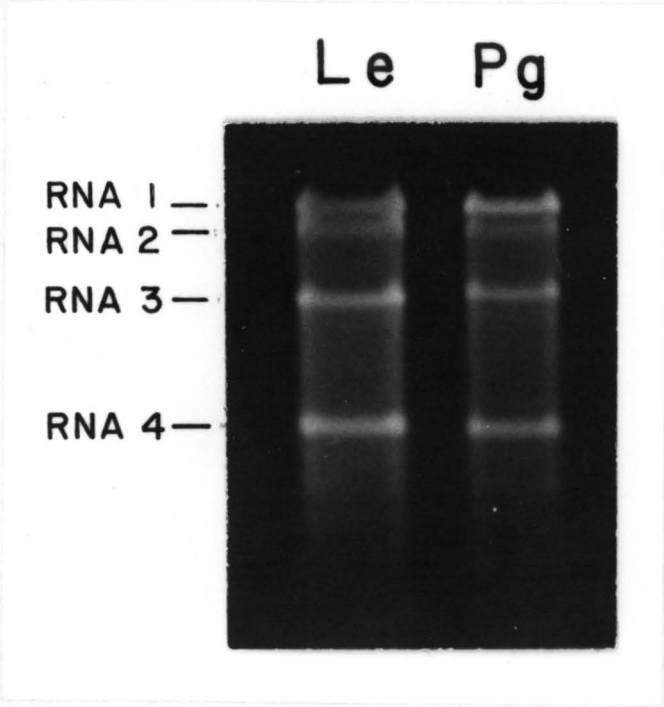

Figure 3.

Figure 4. Degradation of isolated RNA. The origin of the RNA is indicated above the bar with the age of the sample in months indicated directly above each lane. Note the relative intensities of the 4 major bands from fresh samples of both strains compared to that of the bands from 3-month-old samples stored at -15 C. CMV-Le segments 1 and 2 became degraded, while only segment 2 of CMV-Pg became degraded during storage.

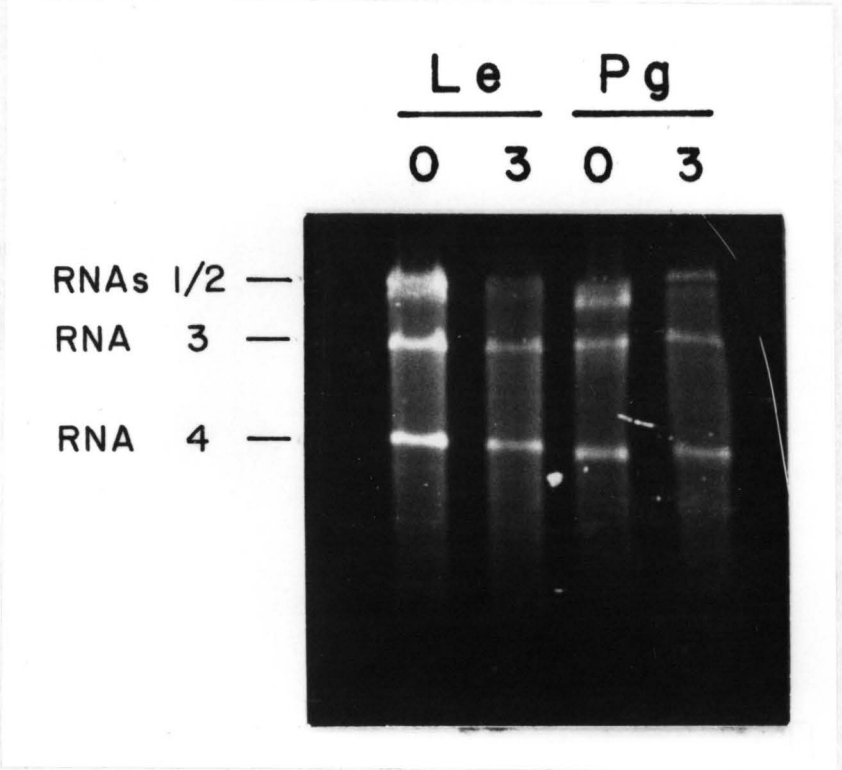

Figure 4.

Figure 5. Comparison of the electrophoretic mobilities of RNA segments from strains CMV-Pg and CMV-Le by polyacrylamide gel electophoresis in 4.5% and 8.0% gels. In 4.5% gels (A), CMV-Pg RNA 2 electrophoresed faster than CMV-Le RNA 2, whereas RNA segments 3 and 4 of each strain co-electrophoresed. RNA 1 of both strains was partially degraded, but intact CMV-Pg RNA 1 electrophoresed slower than that of CMV-Le RNA 1. In 8% gels (B), the four RNA segments from each strain co-electrophoresed. A band below RNA segment 4 in lanes Pg and Pg + Le, indicating CMV-Pg origin, commonly occurred during electrophoresis of CMV-Pg RNA preparations.

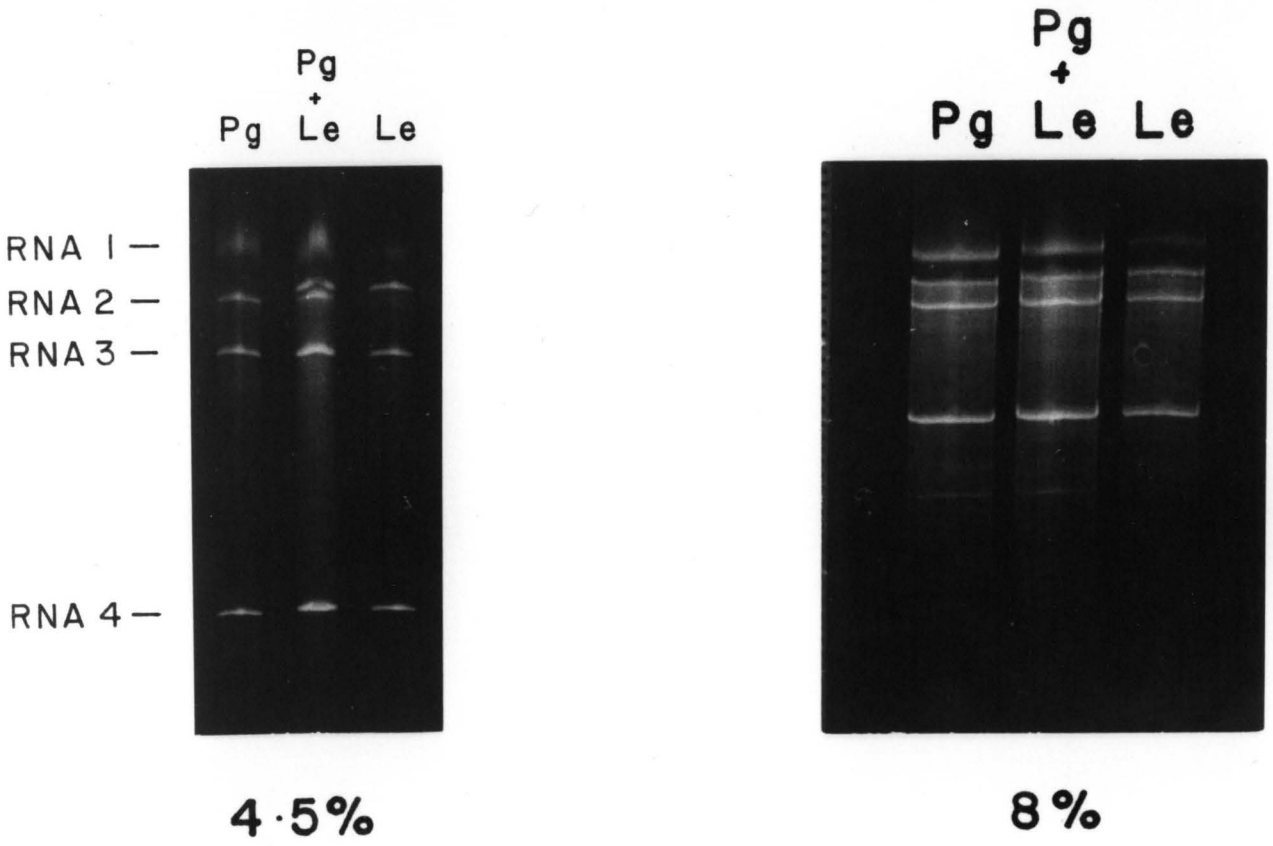

Figure 5.

Figure 6. Comparison of the mobilities of unglyoxalated and glyoxalated RNA segments by glyoxal gel electrophoresis. Lanes 1) unglyoxalated CMV-Le RNA, 2) unglyoxalated CMV-Pg RNA, 3) glyoxalated CMV-Pg RNA, 4) glyoxalated CMV-Le and CMV-Pg RNA, 5) glyoxalated CMV-Pg RNA. Note the greater mobility of CMV-Pg RNA's 1 and 2 (lane 2) than CMV-Le RNA's 1 and 2 (lane 1), and in unglyoxalated samples (lanes 3-5), the co-electrophoresis of all RNA segments. (Electrophoretic conditions as described in Materials and Methods.)

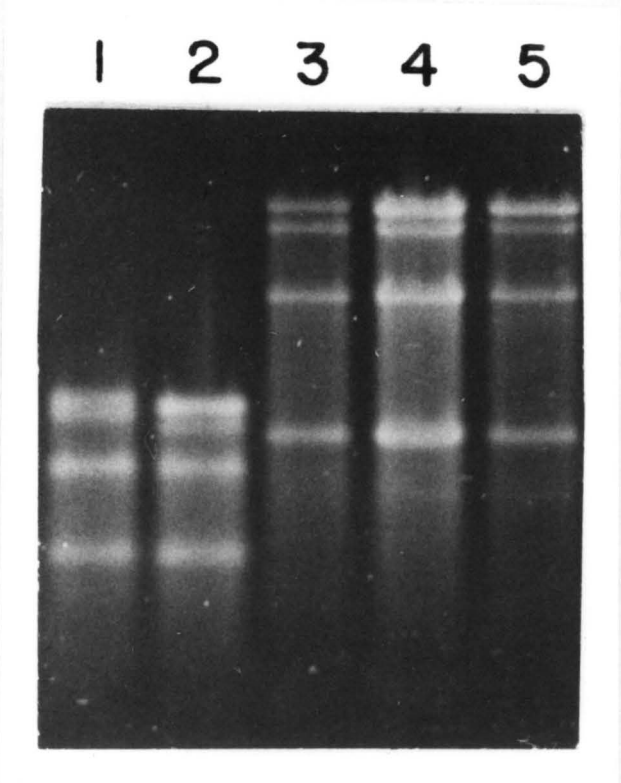

Figure 6.

End Labeling RNA

Thin layer chromatography of initial and final aliquots from the pCp formation reaction produced one area of radioactivity for each aliquot which differed in R_f values (Figure 7). This demonstrated that the transfer of the radioactive phosphate from ATP to cytidine 3'-monophosphate was nearly 100% complete. The highest percentage incorporation of radioactive pCp at the 3'-terminus of CMV RNA from both isolates occurred at -15 C (Figure 8). Autoradiographs of 3'-end labeled total RNA from either strain, electrophoresed in agarose gels, resulted in 4 distinct bands (Figure 9). These corresponded to RNA segments 1 and 2, 3, 4, and a 5th RNA.

Two approaches were taken to maximize incorporation of pCp into RNA segments for biochemical analyses. First, segments 1-4 were isolated from total RNA and 3'-end labeled, and second, the end labeling reaction was incubated at 37 C, instead of -15 C. Neither of these manipulations increased pCp incorporation.

CMV RNA Segment Isoation

CMV RNA segments, electrophoresed in low-melting-temperature agarose gels, were located with ultraviolet light prior to sectioning the gel (Figure 10). Autoradiographs of this gel resulted in four major bands of various intensities (Figure 9). This indicated that

Figure 7. Assay of the transfer of radioactivity to form pCp. Lanes 1) sample of reaction mixture prior to the addition of T_4 polynucleotide kinase (PNK), 2) sample of reaction mixture 90 minutes after the addition of PNK. Lane 1 is entirely [γ-^{32}P]-ATP since the PNK had not been added. Lane 2 has a single radioactive area corresponding to pCp with no area corresponding to the [γ-^{32}P]-ATP.

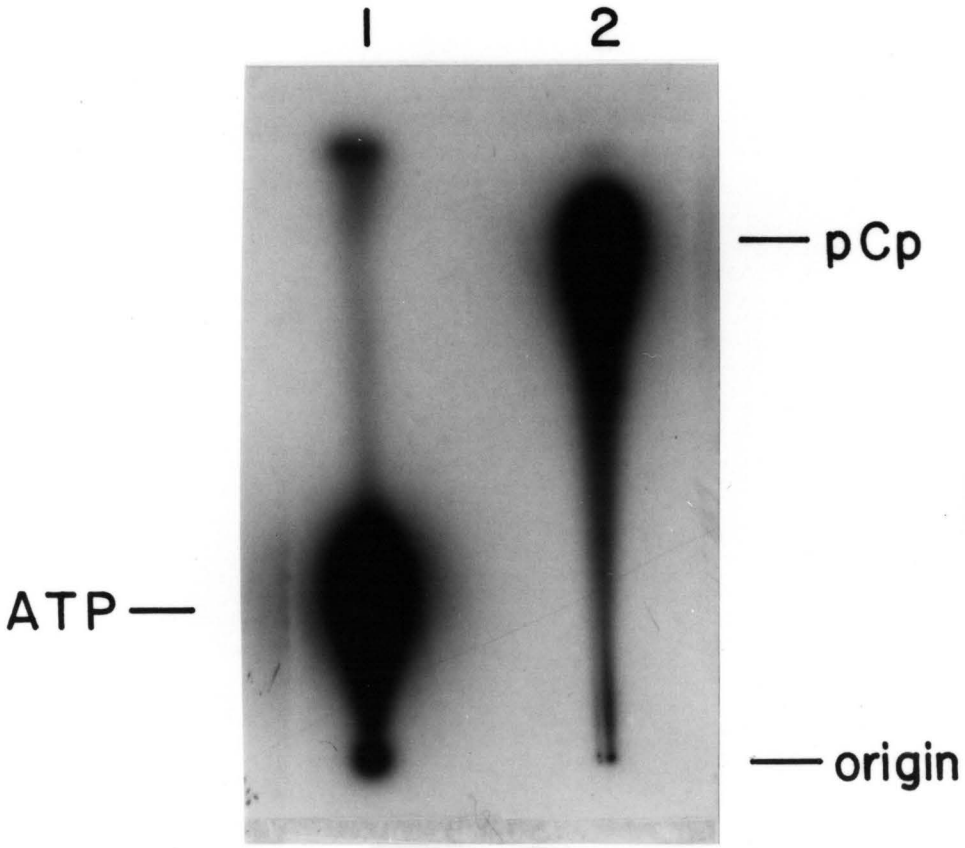

Figure 7.

Figure 8. Incorporation of pCp at the 3'-terminus of total RNA. CMV-Pg total RNA was 3'-end labeled (as described in Materials and Methods) and incubated at 4 C (o———o) or -15 C (o----o). Percentage incorporation was determined as the fraction of the total radioactivity which was acid precipitable. Note that when a portion of the sample was removed from 4 C after 18 hr and placed at -15 C, the percentage incorporation increased to 49%. Similar results were observed for CMV-Le.

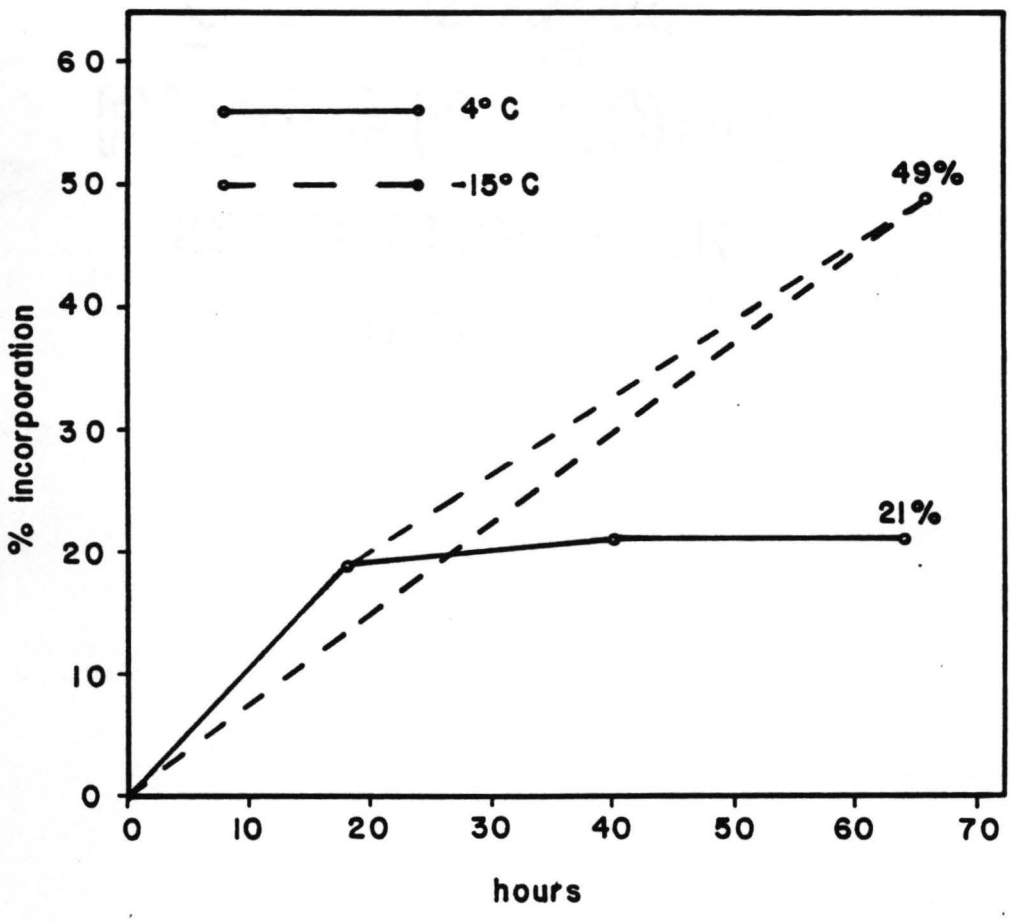
60
50
40
30
20
10
0
% incorporation
4° C
-15° C
49%
21%
0
10
20
30
40
50
60
70
hours

Figure 8.

Figure 9. Distribution of radioactivity in 3'-end labeled RNA. CMV-Le RNA, 3'-end labeled (as described in Materials and Methods) and subjected to agarose gel electrophoresis, produced 4 bands of radioactivity. The three slower migrating bands corresponded to the 4 major genomic RNAs, based on comparisons of unlabeled RNA electrophoresed in continguous lanes (data not shown). Tests with CMV-Pg RNA produced similar results.

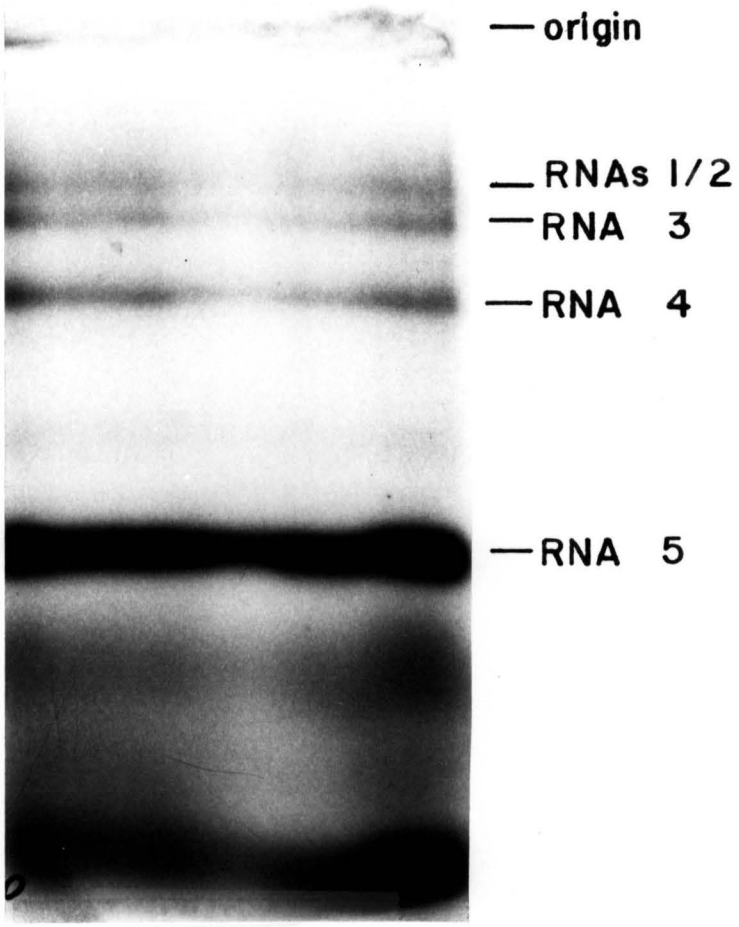

Figure 9.

the incorporation of the end label into the segments was not uniform, but appeared inversely proportional to their molecular weights. A band of high molecular weight material, which could be visualized by ultraviolet light (Figure 10) and end labeled by pCp and RNA ligase (data not shown), was observed in RNA preparations from both viruses. Its relationship to the genomic segments, however, is unknown. RNA segments 1 and 2 from both strains were isolated as a unit since they migrate very closely together. The yield of the RNA segments from low-melting-temperature agarose approached 90% for all segments.

The purity and intactness of the isolated RNA segments were determined electrophoretically. Agarose electrophoresis of unlabeled RNA segments (Figure 11) and glyoxal gel electrophoresis of 3'-end labeled RNA segments resulted in individual bands (Figure 12). Generally, separated RNA segments seemed quite pure, but degradation of segments 1 and 2 sometimes occurred.

Nucleotide Sequencing

Sequencing was initiated on 3' end labeled, isolated CMV RNA segments in order to compare the 3'-termini of each strain. All attempts at determining a sequence proved unsuccessful because of the low incorporation percentage of pCp.

RNase H Digestion

RNase H cleaves RNA at RNA:DNA hybrids and could theoretically produce a digestion pattern analagous to restriction endonuclease

Figure 10. Separation of total RNA into specific segments in preparative agarose gels. Total RNA from each strain was subjected to electrophoresis. Note the presence of a high molecular weight RNA (X) present in both strains (not observable for CMV-Pg in photograph.) and the presence of small amounts of RNA 5.

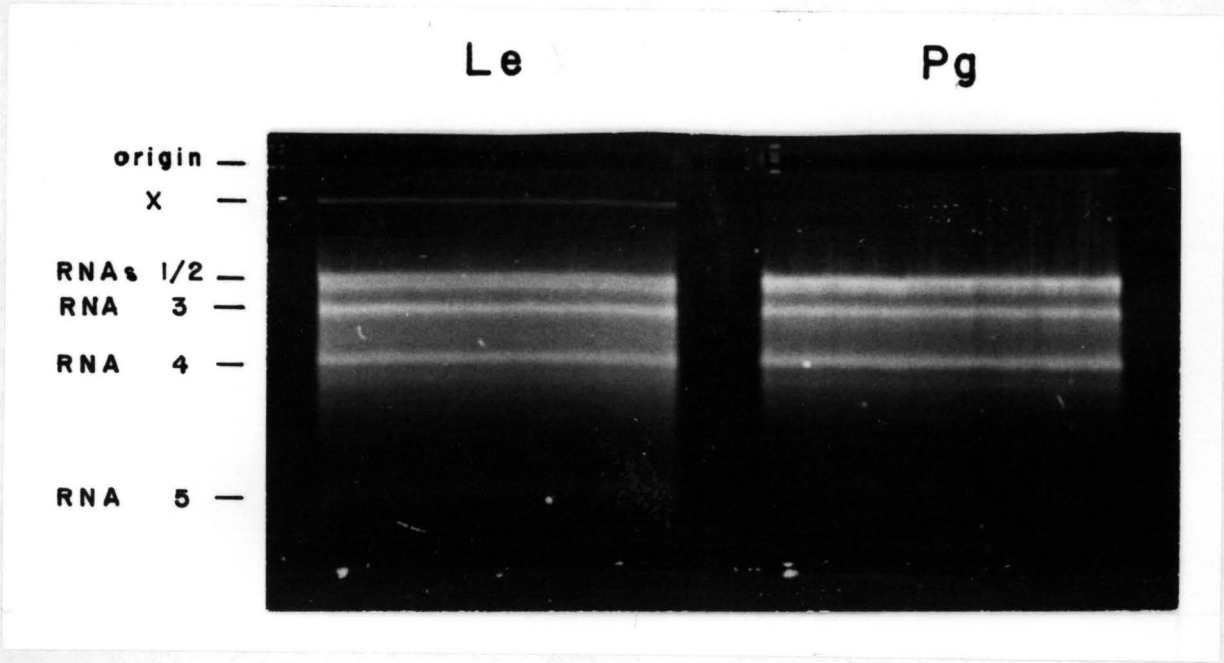

Figure 10.

Figure 11. Purity and intactness of unlabeled, isolated RNA segments. CMV-Le RNA segments, isolated from low-melting temperature agarose, were compared to total RNA by agarose gel electrophoresis. Lanes 1 and 5) total CMV-Le RNA, 2) RNAs 1 and 2, 3) RNA 3 and 4) RNA 4. Note the co-electrophoresis of the isolated segments and the corresponding RNA segments in total RNA, and the lack of contaminating RNA segments in the isolated RNA samples.

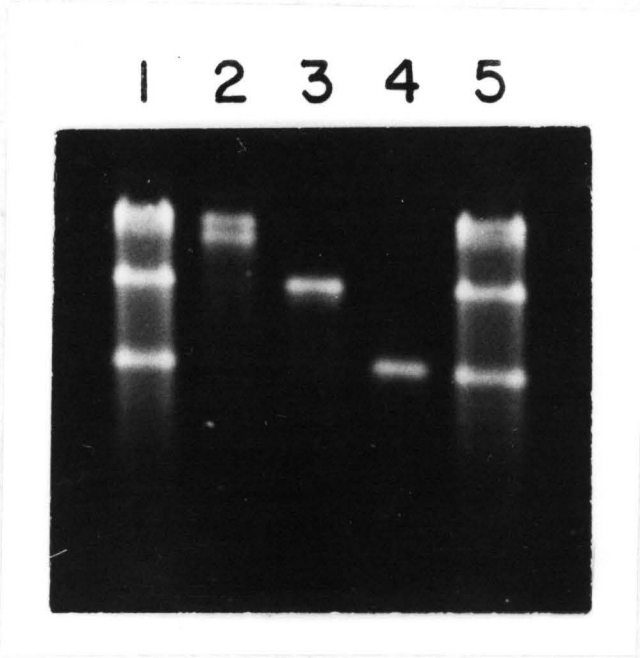

Figure 11.

Figure 12. Purity and intactness of 3'-end labeled, isolated RNA segments. 3'-end labeled RNA segments from both strains, isolated from low-melting-temperature agarose, were glyoxalated and electrophoresed in glyoxal gels (as described in Materials and Methods). The source of the RNA is listed above the bar, and the specific isolated RNA segment(s) are indicated above each lane. The isolated RNA segments electrophoresed as discrete bands which indicated that they were intact and not degraded. Trace contamination of several isolated RNA segments occurred (Le 1-2 and Pg 1-2), but this amount was insignificant when compared to the desired, isolated RNA segment(s).

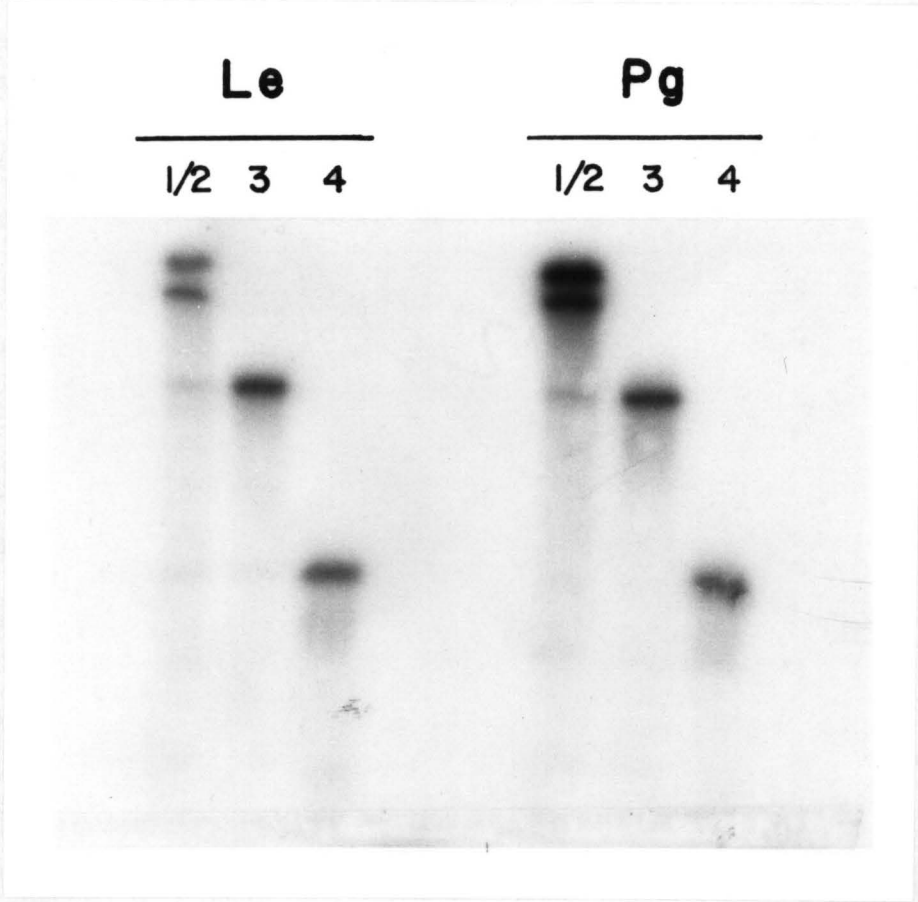

Figure 12.

Figure 13. RNase H digestion pattern of CMV-Le RNA:DNA hybrids. Hpa I DNA hexanucleotide or nicked calf thymus DNA was hybridized to total CMV-Le RNA, treated with RNase H, and subjected to polyacryalmide gel electrophoresis in 4.5% gels. The bands were visualized by staining the gel in ethidium bromide at 0.5 ug/ml for 15' at room temperature. Lanes 1) Hpa I DNA, 2) nicked calf thymus DNA, 3) no DNA oligonucleotide for hybridization, not treated with RNase H, 4 and 5) no DNA oligonucleotide for hybridization, treated with RNase H. Note the two novel bands (lane 1) produced by RNase H treatment of the Hpa I DNA:RNA, and the absence of these bands in untreated controls (lanes 3, 5, 5). The three bands in the control lanes (3, 4, 5), corresponding to RNA's 1 + 2, 3 and 4 respectively, show little or no degradation.

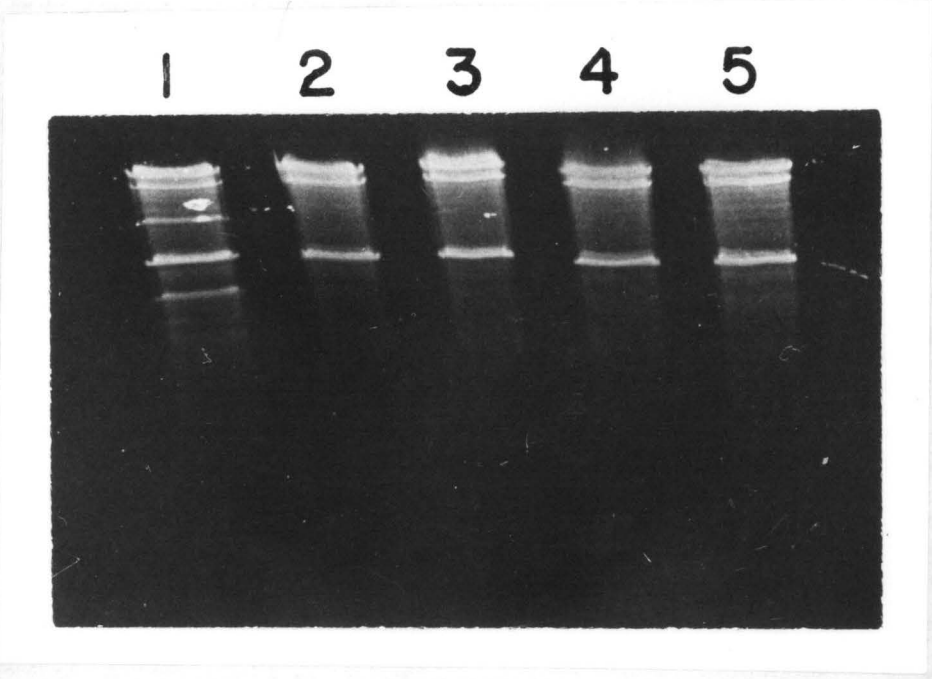

Figure 13.

mapping of DNA. Initial experiments produced altered RNA profiles in polyacrylamide gels (Figure 13). Total CMV-Le RNA, hybridized to Hpa I oligonucleotides and treated with RNase H, produced two distinct and reproducible bands. The reason nicked calf thymus DNA sample was not completely digested is unknown, but the possibility exists that hybridized RNA had numerous mismatched bases, inhibiting action of RNase H. Products which resulted from treatment of RNA:DNA hybrids by RNase H were 3'-end labeled and electrophoresed on polyacrylamide gels. No novel bands were present when compared to end labeled, RNase H treated RNA. Attempts to determine which RNA segment produced the two novel RNA bands by using isolated RNA segments proved unsuccessful because insufficient amounts of RNA were available for RNase H treatment.

Partial RNase T_1 Digestion

Isolated 3'-end labeled RNA segments from each strain were partially digested with RNase T_1 to produce a characteristic banding pattern when electrophoresed in polyacrylamide gels. Prior to this, isolated, end labeled RNA segments, electrophoresed in glyoxal gels, produced bands (Figure 12). This indicated the RNA segments were both pure and intact. An optimal concentration of RNase T_1, 2.7×10^{-3} U/ug RNA, was determined by treatment of 3'-end labeled CMV-Le segment 4 with varying concentrations of RNase T_1 (Figure 14). RNA segments from both strains, partially digested with T_1, produced fragments of varying size which were resolved

Figure 14. Determination of the optimal concentration of RNase T_1 for digestion of 3'-end labeled CMV RNA segments. Lanes 1) no RNase T_1, 2) 2.7×10^{-2} U RNase T_1/ug RNA, 3) the optimal, 2.7×10^{-3} U RNase T_1/ug RNA, 4) 2.7×10^{-4} U RNase T_1/ug RNA, 5) 2.7×10^{-5} U RNase T_1/ug RNA.

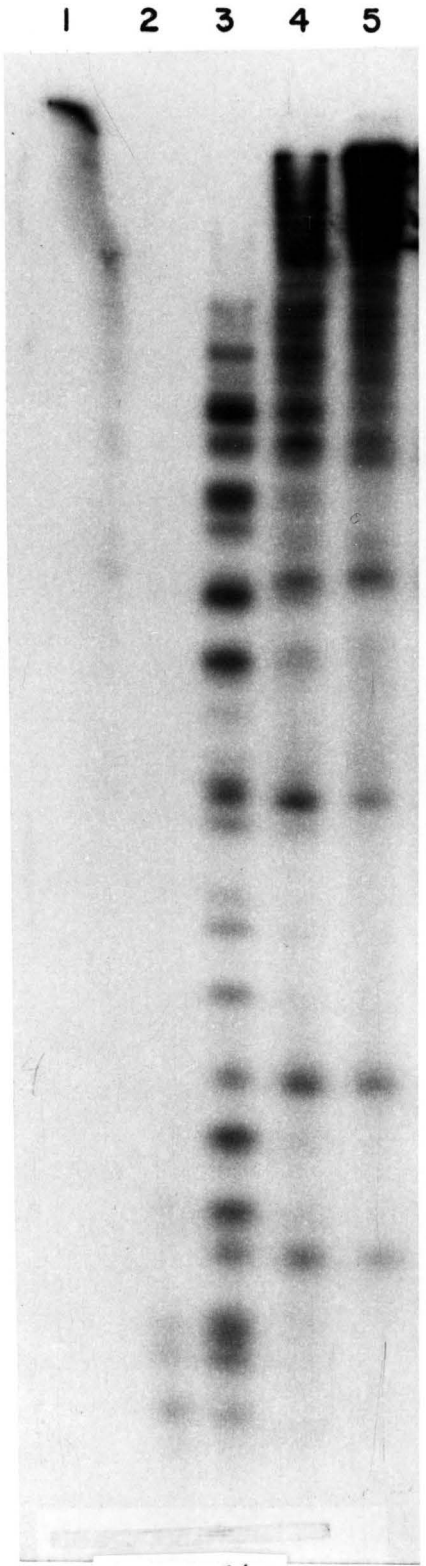

Figure 14.

on 10% polyacrylamide gels (Figure 15). Segment 4 of each strain produced a digestion pattern that was identical to the digestion pattern of segment 3 from the homologous strain because of the subgenomic property of segment 4 (Figures 1, 15, and 16). Within the same strain, the patterns produced by partially T_1 digestion of the RNA segments were nearly identical (Figures 15 and 16). When the digestion pattern from specific segments of each strain were compared, unique bands were found (Figures 15 and 16). Similarities were found, however, in the overall digestion patterns of each segment in both strains (Figures 15 and 16). Shifts in the banding patterns when the two strains were compared indicate that insertions and deletions were present at the 3'-terminus of each RNA.

RNA 5 was subjected to partial T_1 digestion to determine whether it had originated from the 3'-terminus of genomic RNAs. This required 7.5 to 10 times the amount of T_1 used to digest segments 1 through 4. This RNA from both strains produced identical digestion patterns when compared to each other, but when compared to segment 4 from each strain, the digestion patterns were dissimilar (Figure 17). This suggested that RNA 5 did not originate from the 3'-terminus of RNA's 1-4. Undigested RNA 5 of each strain consisted of two molecules that differed by only a few bases in size as determined by its electrophoretic mobility in polyacrylamide gels (Figure 17).

Complementary DNA

Randomly Primed cDNA. Complementary DNA was synthesized from total RNA based on acid precipitable radioactivity. This cDNA

Figure 15. Digestion patterns of 3'-end labeled, isolated RNA segments partially digested with RNase T_1, and subjected to polyacrylamide gel electrophoresis. Three different preparations of RNase T_1 treated samples (A, B, C) were electrophoresed on 10% gels for three different times (9 h, 6 h, 3 h, respectively). Lanes 1) markers (5'-^{32}P-labeled Hind III digested lambda DNA and Hae III digested ØX174 replicative form DNA), 2) CMV-Pg RNA 4, 3) CMV-Le RNA 4, 4) CMV-Pg RNA 3, 5) CMV-Le RNA 3, 6) CMV-Pg RNAs 1 and 2, 7) CMV-Le RNAs 1 and 2. When comparing the digestion patterns, the markers (lane 1) are useful to coordinate the three sets. Note similarities of patterns of homologous RNA's 3 and 4, indicating subgenomic redundancy.

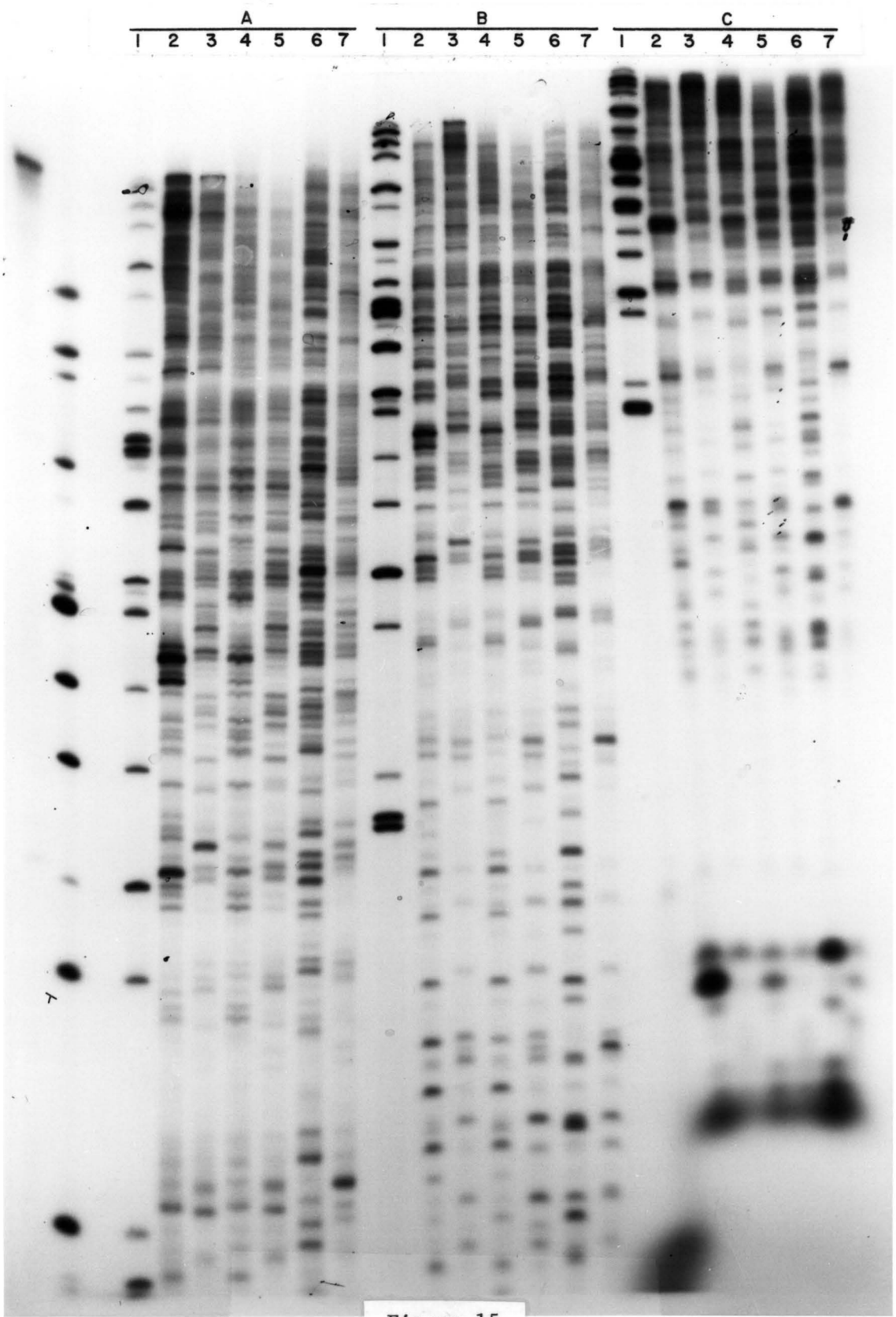

Figure 15.

Figure 16. Digestion patterns of 3'-end labeled, isolated RNA segments 3 and 4 partially digested with RNase T_1 and subjected to polyacrylamide gel electrophoresis. Lane 1 and 4) RNA markers.[a] 2) CMV-Pg RNA 4 -T_1.[b] 3 and 10) CMV-Pg RNA 4 + T_1.[c] 4 and 11) CMV-Le RNA 4 + T_1. 5) CMV-Le RNA 4 - T_1. 6) CMV-Pg RNA 3 - T_1. 7 and 12) CMV-Pg RNA 3 + T_1. 8 and 13) CMV-Le RNA 3 + T_1. 9) CMV-Le RNA 3 - T_1. 10) CMV-Pg RNA 4. Lanes 3, 4, 7 and 8 incubated with 0.01 U RNase T_1 for 8 minutes. Lanes 10 and 11 incubated with 0.008 U RNase T_1 for 10 minutes. Lanes 12 and 13 incubated with 0.01 U RNase T_1 for 10 minutes. Note especially the similarities in the overall digestion pattern of CMV-Pg RNA 4 and CMV-Le RNA 4 (lanes 3 and 4, respectively).

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[a] RNA markers; 3'-end labeled CMV-Pg RNA was incubated at 100 C for 40 minutes in 50% formamide, 1 mM $MgCl_2$, and 50 ug/ml unlabeled tRNA.

[b] Minus RNase T_1; samples were treated in identical fashion to the samples that contained RNase T_1.

[c] Plus RNase T_1; samples were treated with RNase T_1 as described in Materials and Methods.

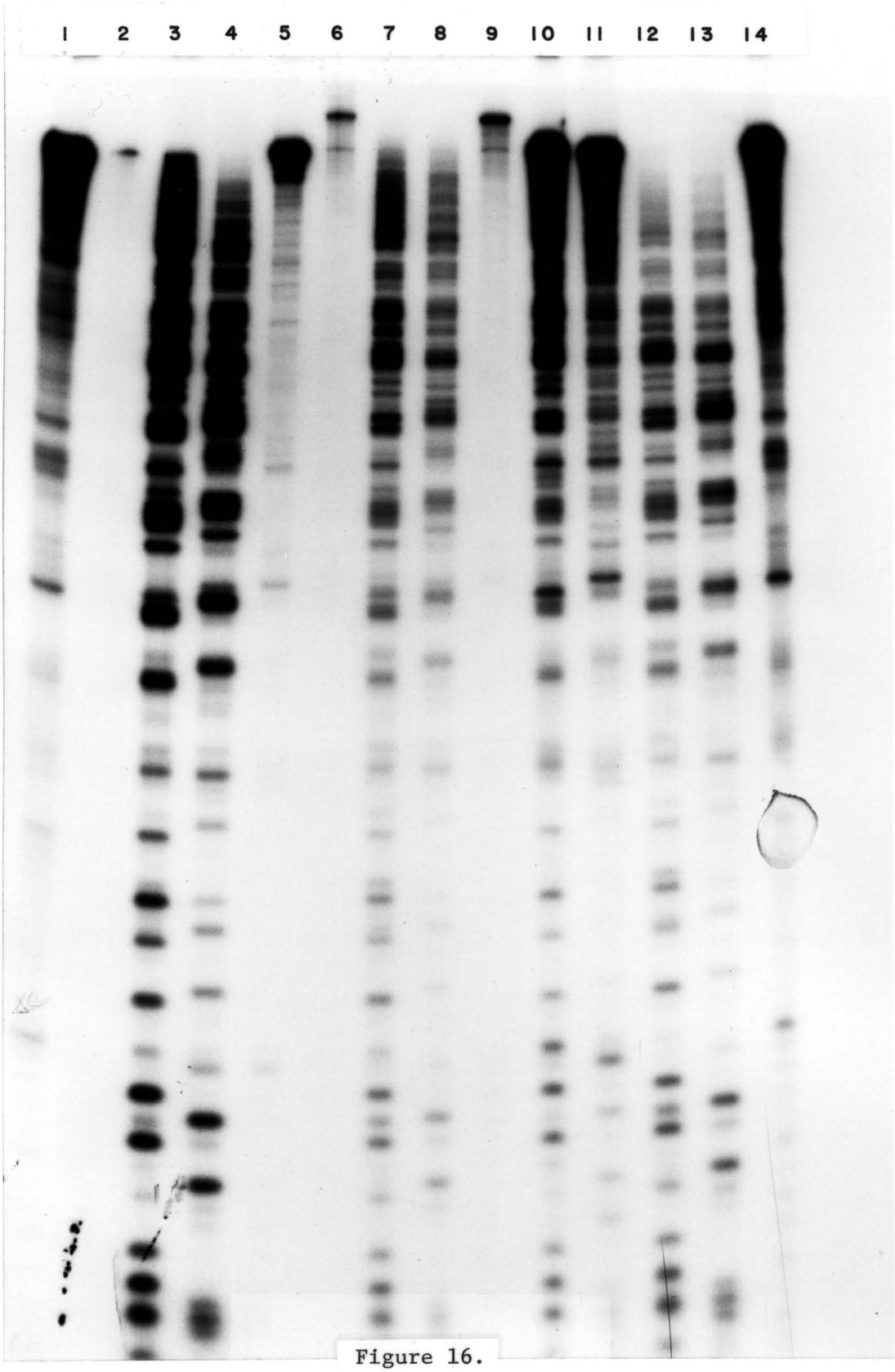

Figure 16.

Figure 17. Comparison of the partial digestion patterns of RNA 5 and RNA 4 of both strains. Lanes 1 and 10) RNA markers, formed as described in Figure 16. 2) CMV-Pg RNA 5 - T_1.[a] 3 and 5) CMV-Pg RNA 5 + T_1.[b] 4) CMV-Pg RNA 4 + T_1. 6 and 8) CMV-Le RNA 5 + T_1. Note the identical patterns formed from CMV-Pg RNA 5 (lane 5) and CMV-Le RNA 5 (lane 6), and the lack of similarity of these patterns with those of RNA 4 from the homologous strain (Pg: lanes 5 and 4; Le: lanes 6 and 7).

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[a] Minus RNase T_1; samples were treated in identical fashion as samples containing RNase T_1.

[b] Plus RNase T_1; samples treated with RNase T_1, as described in Materials and Methods.

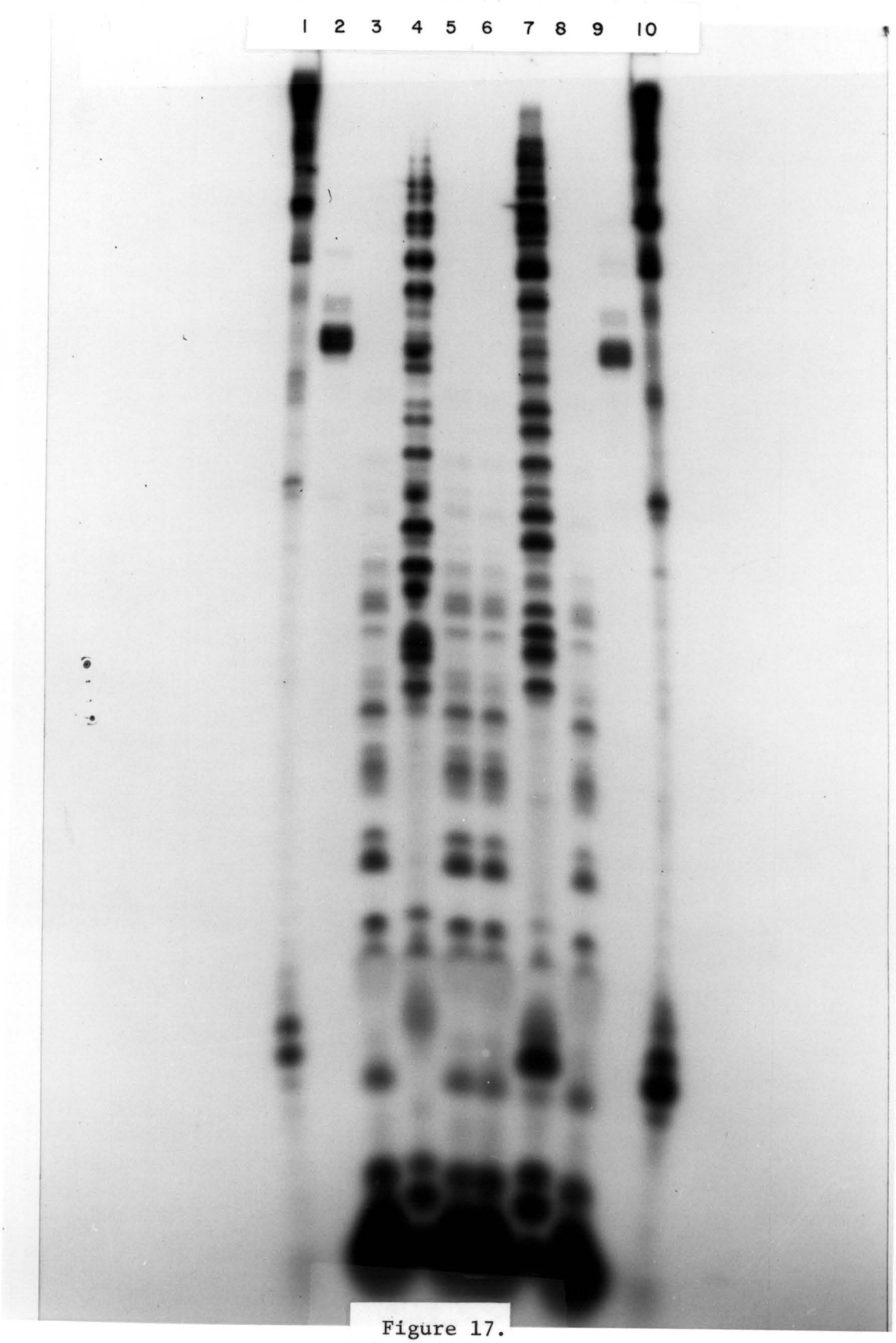

Figure 17.

produced a smear of fragments ranging in size from approximately 100 base pairs to 900 base pairs when compared by agarose gel electrophoresis with known DNA size markers (Figure 18). Attempts to synthesize randomly primed cDNA to RNA 5 from each strain were not successful.

Northern Blot Hybridization

Blots of CMV RNA from both strains, prepared as in Figure 18, were probed with cDNA synthesized from both CMV-Le and CMV-Pg to determine whether gross differences were present in the sequence homology. Autoradiographs of RNA:cDNA hybrids showed no loss of intensity of the bands under the increased stringencies for hybridization of the probe for either the homologous or heterologous strain (Figure 19). CMV-Pg probe more strongly hybridized to the homologous RNA than to the heterologous RNA primarily because of a mass difference in the amount of the RNA originally blotted.

Complementary DNA synthesized from polyadenylated CMV-Le segment 5 hybridized to all RNA segments of both strains (Figure 20).

S_1 Nuclease Digestion

S_1 nuclease, an endonuclease which digests both single-stranded RNA and DNA, was used to quantify the homology between the two CMV strains. The data is presented in Table 2. The percentage sequence homology between each strain is 70% when probe from each strain is hybridized to RNA in 50% formamide.

Figure 18. Size determination of randomly primed cDNA probe. Complementary DNA synthesized from CMV-Le total RNA was electrophoresed in a 1% agarose gel with size markers for Hae III digested ØX174 replicative form DNA and Hind III digested lambda DNA. Lanes 1) cDNA. 2) DNA size markers; the sizes of the specific bands are indicated in kilobases. cDNA probe size ranged from approximately 100 to 900 base pairs.

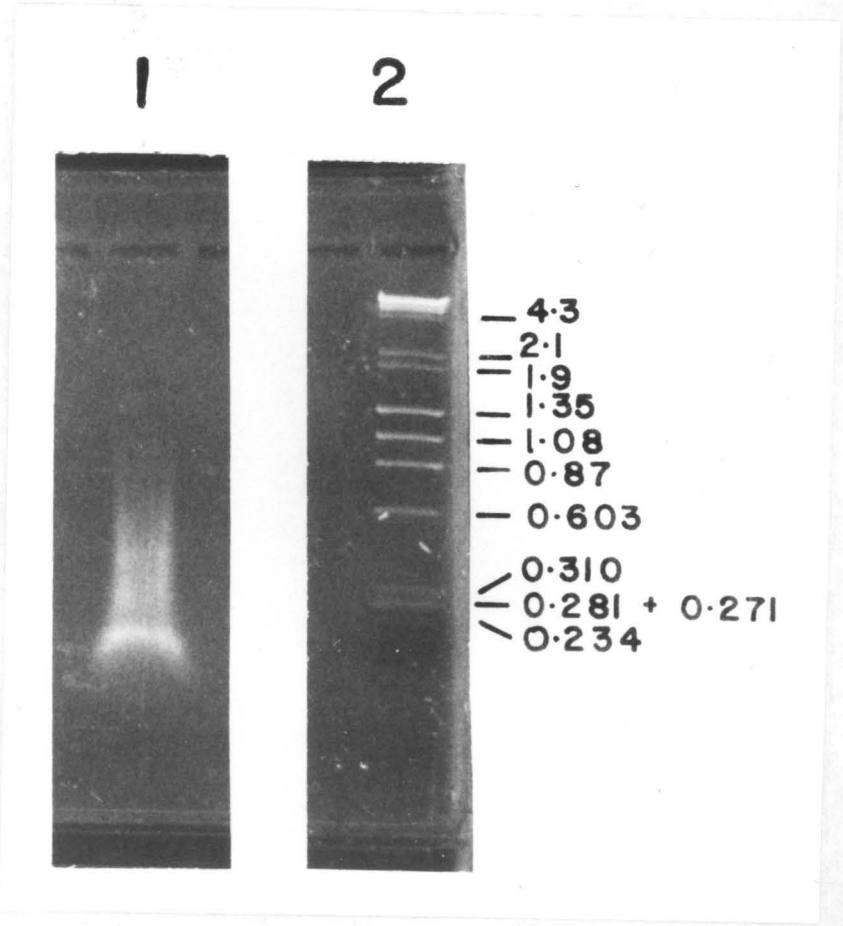

Figure 18.

Figure 19. Sequence homology between CMV-Le and CMV-Pg determined by northern blot analysis. Complementary DNA synthesized from CMV-Le total RNA (A) and CMV-Pg total RNA (B) was hybridized to RNA blots of total RNA in 20% formamide. These hybridized blots were then washed in either 35% or 50% formamide to increase the stringency of hybridization. No differences in the intensity of the hybridization to the hterologous blots were observed among 20%, 35% and 50% formamide hybridization conditions.

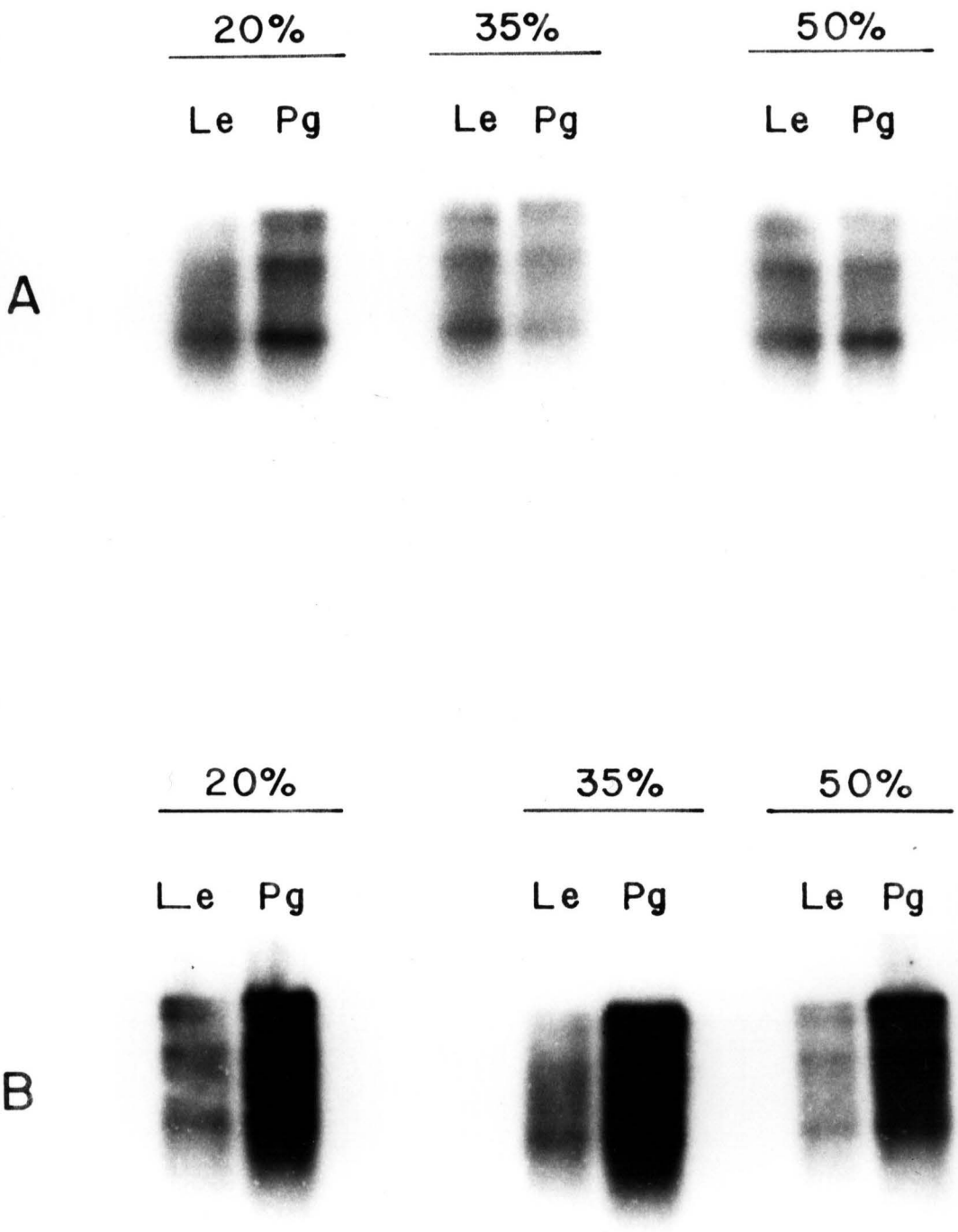

Figure 19.

Figure 20. Sequence homology between CMV-Le RNA 5 and total RNA from both CMV-Le and CMV-Pg as determined by northern blot hybridization. Complementary DNA synthesized from CMV-Le RNA 5 was hybridized to RNA blots of total RNA from CMV-Le (lane 1) and CMV-Pg (lane 2). Note hybridization of cDNA to all segments of both strains.

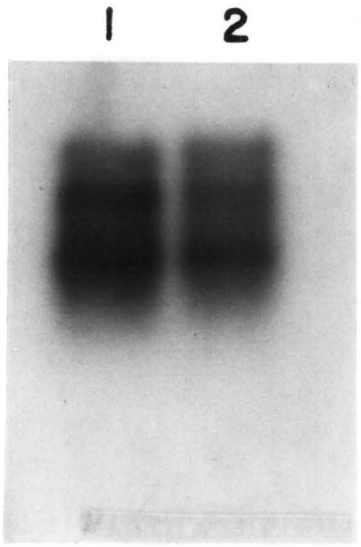

Figure 20.

TABLE 2. Calculation of the percentage homology between CMV-Le and CMV-Pg using stringent conditions for hybridization.[a]

	CMV-Le Probe			
	$-S_1$[b]	$-S_1$[c]	% S_1 Resistance[d]	% CMV-Le[e]
CMV-Le cDNA	17540±681	867±172	4.9	-
CMV-Le RNA	18352±880	13887±753	75.7	100
CMV-Pg RNA	16194±1376	8906±91	55.0	70.8

	CMV-Pg Probe			
	$-S_1$[b]	$-S_1$[c]	% S_1 Resistance[d]	% CMV-Pg[e]
CMV-Pg cDNA	12996±466	1522±194	11.7	-
CMV-Pg RNA	9999±265	7927±60	79.3	100
CMV-Le RNA	12806±361	7403±846	57.8	68.2

[a] For experimental details see Materials and Methods.

[b] Minus S_1 nuclease reaction; the results are expressed as the $\bar{X} \pm$ S.D. where n = 3.

[c] Plus S_1, nuclease reaction; the results are expressed as the $\bar{X} \pm$ S.D. where n = 3.

[d] Percentage S_1 nuclease resistance was determined using the means of the $+S_1$ and $-S_1$ nuclease reactions by the following formula:
$[(+S_1 \text{ resistance})/(-S_1 \text{ resistance})] \times 100$.

[e] The percentage homology to either CMV-Le or CMV-Pg was determined following normalization of the hybridization reaction by the following formula (21):

$$\frac{(\%\ S_1 \text{ resistance heterologous RNA}) - (\%\ S_1 \text{ resistance probe})}{(\%\ S_1 \text{ resistance homologous RNA}) - (\%\ S_1 \text{ resistance probe})} \times 100$$

Cloning CMV-Pg and CMV-Le

Double-stranded cDNA was blunt-end lignited into pUC9 and cloned into E. coli JM 103. None of the clones contained inserts of significant size, based on the results that migrational positions of the plasmid supercoils and the nicked circles of the clones were either indistinguishable or only slightly different from the isolated pUC9 and the plasmid forms from a non-transformed blue colony (Figure 21).

Figure 21. Insert size of cDNA clones. The insert size of each recombinant plasmid was compared to the size of the purified plasmid and a non-transformed colony (no insert) by electrophoretic mobility. Lanes 1) DNA size markers (lambda DNA digested with Hind III). 2) Purified plasmid, pUC9. 3 and 9) CMV-Pg recombinant plasmid. 4-8, 10 and 11) CMV-Le recombinant plasmids. 12) Non-transformed plasmid from a blue colony. No significant migrational differences were observed when the nicked circle and the super coiled forms of the plasmid from the clones were compared to pUC9 and the non-transformed plasmid.

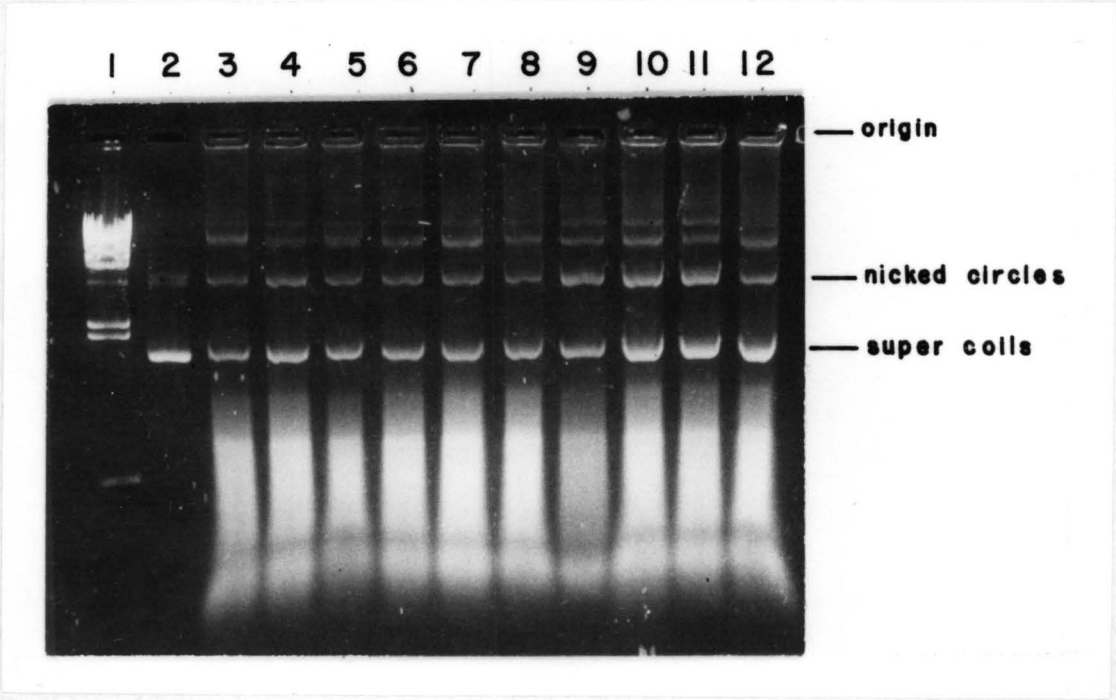

Figure 21.

DISCUSSION

Analytical Distinction of CMV-strain RNAs

In this work, several techniques were evaluated to distinguish the RNAs of CMV strains Pg and Le. Only one technique, polyacrylamide gel electrophoresis (PAGE) of partial RNase T_1 digests of strain-RNAs, provided a precise analytical distinction between CMV-Pg and CMV-Le. PAGE patterns of these partial digests were clearly strain specific and revealed extreme similarities among the RNAs of individual strains (Figure 15). Such within-strain RNA similarities are explainable by means of known interrelationships among the 3'-termini of CMV RNAs 1, 2, 3, and 4 (22,40). Subgenomic CMV RNA 4, contained entirely within the 3'-terminus of RNA 3, would thus be expected to produce identical PAGE patterns after subjection to RNase T_1.

These data therefore appear to provide a plausible means for detecting and identifying RNA contaminants in pseudorecombinants.

Sequence Homology

Northern blot hybridization results demonstrated a high degree of homology in CMV-strain nucleotide sequences. None of the four RNAs was uniquely heterologous, precluding strain distinction. Heterologous cDNA probe remained hybridized to the blot even under the most stringent conditions (50% formamide), indicating at least 83% nucleotide homology (27). Short sequences of less than 83% homology, however, could conceivably be masked by regions of high

homology, as assessed by this type of analysis. The probe would most likely contain regions of both homologous and heterologous sequences. Hybridization across short heterologous sequences could thus cause overestimation of RNA-relatedness between strains.

To assess the extent of intersticial short-sequence heterology between strains, hybridized cDNA was subjected to S_1 nuclease. Unmatched tails or loops should be attacked by this enzyme, leaving only thermodynamically stable portions of the RNA:DNA heteroduplex. The results presented in Table 2 indicate that CMV-Pg and CMV-Le RNAs are at least 70% homologous. These results combined with those from northern blot hybridization suggest that nucleotide homology is uniformly distributed among CMV-Pg and CMV-Le RNAs.

Electrophoretic Mobility

PAGE and agarose gel electrophoresis of intact and denatured CMV RNAs provided data about the secondary structures and lengths of the RNA segments. Denatured (glyoxal-treated) RNAs of CMV-Pg and CMV-Le co-electrophoresed, whereas native RNA's 1 and 2 electrophoresed differentially in the same gels (Figure 6). This information suggests that there is no strain difference in RNA lengths, but that the secondary structure of CMV-Pg RNA's 1 and 2 is measurably different from that of CMV-Le. This difference in glyoxal gels was verified in 4.5% polyacryalmide-urea gels, but was not discernable in 8.0% polyacryalmide-urea gels. Differentiation

in 4.5% gels could indicate non-denaturation of RNAs at room temperature (8.0% gels were run at about 40 C).

RNase H Digestion

RNase H experiments indicate that this technique could potentially distinguish virus isolates by differential digestion patterns (11). Nucleotide insertions or deletions and overall similarities should be discernable by comparative digestive patterns. Hybridization of DNA oligonucleotides containing the same recognition sequence as restriction enzymes should allow viral RNA to be mapped analagously to endonuclease restriction of DNA. This information could facilitate analysis of cDNA clones by correlating insert cDNA with specific regions of RNA.

Analysis of a 5th RNA

In this study, a low molecular weight RNA into which radioactive pCp was selectively incorporated was found in both CMV isolates. Since it formed a discrete band during electrophoresis of RNA's 1-4 in agarose gels (Figure 17), it was studied to determine whether it was a satellite RNA as had been described in other CMV isolates (36), or was simply a breakdown product from segments 1 throug 4. For this purpose cDNA synthesized from this RNA of CMV-Le (CMV-Le-5) was hybridized to genomic RNA from both strains. Hybridization results suggested that at least some portion of the RNA was derived from RNA's 1 through 4 (Figure 19). The

identity of T_1 digests of end labeled CMV-Le-5 and CMV-Pg-5 (Figure 17) and the progressive development of RNA 5 was aging of RNA preparations further argue against an exogenous origin of this RNA.

Peden and Symons suggested that the origin of this RNA is the 3'-terminus of RNA segments 1 through 4 (51). Electrophoresed T_1 digests of RNA 4 and the 5th RNA from each strain do not support this suggestion (Figure 17). It must be noted, however, that digestion patterns for segment 4 were incomplete and pattern similarity indicating 3'-terminus origin could have been obscured.

By examining the RNA segments from serologically indistinguishable strains of CMV, their similarities and differences have been characterized. The original problem of developing a method to identify a contaminating RNA segment from a mixture of RNAs is actually part of a larger question: how much change in the RNA sequence is required to produce change in seed-transmissibility? The extent of RNA modification (adaptation) associated with acquired CMV seed-transmissibility cannot yet be discerned. Ultimately, this or other traits may be most directly defined by altering specific regions of viral RNA and fulfilling Koch's Postulates with altered, reconstituted isolates.

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APPENDICES

APPENDIX I

Chemical Sequencing of 3' End Labeled RNA

RNA segments were labeled at their 3'-ends with pCp and isolated from low-melting-temperature agarose as previously described. RNA segments 4 and 5 from CMV-Le were then sequenced by the method of Peattie (50). This procedure involves preferential modification of each of the four bases followed by analine cleavage at the specifically modified base. Conditions that were used favored modification of only one base per molecule. This was essential for generating a set of radioactive, end labeled molecules of various lengths.

Guanosine Modification Reaction. Two microliters of carrier tRNA (5mg/ml) was added to 3'-end labeled RNA and lyophilized to dryness. The residue was redissolved in 300 ul 50 mM sodium cacodylate pH 5.5, 1 mM EDTA and chilled to 0 C. One half microliter dimethyl sulfate was added and samples were incubated at 90 C for 1 min, and ice chilled immediately. This was followed by the addition of 75 ul cold 1.0 M TRIS·acetate pH 7.5, 1.0 M 2-mercaptoethanol, 1.5 M sodium acetate and 900 ul cold ethanol. The solution was chilled at -60 C for 30 min, the RNA pelleted by centrifugation and resuspended in 200 ul 0.3 M sodium acetate. The RNA was reprecipitated with ethanol and the pellet was dried. The RNA was dissolved in 10 ul in 1.0 M TRIS·HCl (pH 8.2) followed by 10 ul of fresh 0.2 M $NaBH_4$. The solution was incubated on ice,

in the dark, for 30 minutes. Two hundred microliters of cold 0.6 M sodium acetate, 0.6 M acetic acid (pH 4.5), 25 ug/ml carrier tRNA were added, followed by 800 ul cold ethanol. RNA was precipitated as previously described. The lyophilized pellet was treated with analine.

Adenosine Modification Reaction. Two microliters of carrier tRNA (5 mg/ml) was added to 3'-end labeled RNA and lyophilized. The residue was redissolved in 200 ul 50 mM sodium acetate, pH 4.5, 1 mM EDTA and chilled to 0 C. One microliter of diethyl pyrocarbonate was added, incubated at 90 C for 10 minutes and ice chilled immediately. Fifty microliters of cold 1.5 M sodium acetate and 750 ul ethanol were added. The solution was chilled and the RNA pelleted by centrifugation. The RNA was reprecipitated by the addition of 200 ul 0.3 M sodium acetate, 800 ul of ethanol and incubated at -60 C. RNA was pelleted by centrifugation, lyophilized, then treated with analine.

Cytidine Modification Reaction. Two microliters of carrier tRNA (5 mg/ml) was added to 3'-end labeled RNA and lyophilized. The residue was redissolved in 10 ul of 3.0 M NaCl, hydrazine, at 0 C and incubated on ice for 30 minutes. One millilter of 80% ethanol: 20% H_2O (v/v) at -20 C was added, and the RNA was precipitated, pelleted, lyophilized, and treated with analine.

Uridine Modification Reaction. Two microliters of carrier tRNA (5 mg/ml) were added to 3'-end labeled RNA and lyophilized. The residue was redissolved in 10 ul of a 50% hydrazine: 50%

H_2O (v/v) at 0 C and incubated on ice for 15 minutes. Two hundred microliters of 0.3 M sodium acetate, 1 mM EDTA and 750 ul cold ethanol were added and the solution was chilled. The RNA was pelleted by centrifugation and resuspended in 200 ul 0.3 M sodium acetate followed by 800 ul ethanol. This solution was chilled and the RNA pelleted, lyophilized, and analine treated.

Analine Cleavage Reaction. The chemically modified RNA was dissolved in 20 ul of 1.0 M analine/acetate buffer (pH 4.5) and incubated in the dark at 60 C for 20 minutes. Reactions were terminated by freezing at -60 C and then lyophilized. The residue was resuspended in 20 ul H_2O and lyophilized. This last step was repeated. Samples were dissolved in 3-5 ul tracking dye and electrophoresed on 10% polyacrylamide gels.

APPENDIX II

RNase H Digestion

CMV RNA was digested using RNase H in the presence of specific oligonucleotide primers using the procedures of Donis-Keller (11). In separate 10 ul reactions, 0.5 ug hexamer or tetramer DNA linkers of Eco RI, Xho I, Hpa I (restriction endonuclease sites from Bethesda Research Laboratories) or $pd(AT)_2$ (P. L. Biochemicals), or nicked calf thymus DNA were boiled for 60 seconds with either 1.0-2.4 ug total RNA or 0.07-0.14 ug isolated RNA segments from CMV-Le. The solution was allowed to slowly cool to room temperature and made 20 mM TRIS·HCl pH 7.5, 10 mM $MgCl_2$, 0.1 mM DTT, 5% glycerol, 0.1 M KCl, and 42.5 U/ml RNase H (Bethesda Research Laboratories). Reactions were incubated at 37 C for 15 minutes. The products were then examined either directly by polyacrylamide gel electrophoresis, or 3'-end labeled and then electrophoresed on polyacrylamide gels.

APPENDIX III

Cloning CMV-Pg and CMV-Le RNA

Total RNA was polyadenylated, and the first strand of cDNA synthesized and isolated as described previously in Materials and Methods (5,15). Single stranded cDNA (ss cDNA) was homopolymer tailed with dATP in a 32 ul reaction (49). Lyophilized cDNA was resuspended in a solution consisting of 0.18 M cacodylic acid pH 7.0, 2 mM DTT, 10 mM $CaCl_2$, 0.1 mM dATP, 8.0 uCi of α-^{32}P-dATP (New England Nuclear, 200-800 Ci/mmole), 500 U/ml *E. coli* terminal deoxynucleotidyl transferase (New England Nuclear) and incubated at 37 C for 5 minutes. Samples were chromatographed in a Sephadex G-100 column. The void volume was collected and the tailed cDNA recovered by ethanol precipitation.

Double stranded cDNA (ds cDNA) was synthesized by the procedures of Maniatis et al. (41) in 40 ul reaction volumes consisting of a 33.5 ug/ml mixture of pdT_8A, pdT_8C, pdT_8G, 0.1 mM dATP, 2.0 mM each of dCTP, dGTP, dTTP, 6.2 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 10 ug/ml BSA, 13 uCi of α-^{32}P-dATP (New England Nuclear, 200-800 Ci/mmole), and 150 U/ml Klenow fragment (P. L. Biochemicals). Incubation was at 37 C for 2 1/2 hours. Low-melting-temperature agarose was used to isolate high molecular weight ds cDNA. Specific areas on the gel were cut out and the ds cDNA was isolated as described under "RNA Segment Isolation" in the Materials and Methods section.

Isolated ds cDNA was allowed to reanneal for 8 minutes, then 65 C for 10 minutes, and finally allowed to cool to room temperature. In a 10 ul reaction, ds cDNA was synthesized again by Klenow fragment as previously described. This ds cDNA was blunt-end ligated to a plasmid, pUC9, in 10.5 ul reactions. This plasmid had been digested with the restriction endonuclease Hinc II and phosphatase treated (a gift from M. Galinski). The ligation reaction consisted of 1.8 ug/ml pUC9, 20 mM TRIS·HCl pH 7.5, 7.5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM ATP, 20 ug/ml BSA, 0.5 ul, 2 ul, or 8 ul ds cDNA, and 71 U/ml T_4 DNA ligase (Collaborative Research). Incubation was at 14 C for 30 hours.

E. coli JM 103 cells were transformed with the ligation reaction by the following procedure (42). A single colony of JM 103 was removed from glucose minimal media plates and inoculated into 3 ml of a 1% tryptone, 0.5% yeast extract, and 0.5% NaCl (all w/w) nutrient broth (YT media). Cells were cultured overnight at 37 C. One milliliter of this culture was added to 100 ml of YT media and incubated at 37 C with agitation. When the optical density of the culture at 660 nm reached 0.30-0.40, the cells were harvested and cooled to 4 C, then centrifuged for 5 minutes at 12,000 xg. They were resuspended in 50 mM $CaCl_2$, 10 mM TRIS·HCl pH 8.0 to 1/2 the original starting volume and incubated at 4 C for 20 minutes. The cells were centrifuged as before and resuspended in the $CaCl_2$, TRIS·HCl buffer to 1/10 the original starting volume. After the

the cells had begun to flocculate (about 40 minutes), 0.1 ml of the cell suspension was mixed with 2 ul of the ligation reaction and incubated at 4 C for 40 minutes, followed by a heat shock of 42 C for 2 minutes. One milliliter of YT media was added to this mixture and incubated at 37 C for 60 minutes. Fifty microliters of 2% X-galactose (Sigma) in dimethyl formamide, 10 ul of 100 mM isopropyl β-D-thiogalactoside (IPTG) (Sigma), and 3 ml of YT with 0.8% agarose tempered at 50 C were added and the entire solution overlayed onto 1% agarose-YT plates containing 50 ug/ml ampicillin. Plates were incubated at 37 C for 18-24 hours. White colonies were streaked onto YT - amp plates containing X-galactose and IPTG. The plates were incubated at 37 C and white colonies were analyzed by mini-plasmid isolation to estimate the size of the insert (43). A scrapping of each screened colony was mixed with 22 ul of 8% sucrose, 0.5% triton X-100, 50 mM EDTA, 10 mM TRIS·HCl pH 8.0, and 1 mg/ml freshly prepared lysozyme. Samples were incubated at 100 C for 60 seconds, briefly chilled on ice, then centrifuged at room temperature for 10 minutes. Two microliters of 0.025% xylene cyanol and bromephenol blue were added and the samples were electrophoresed on 1% TBE agarose gels. The migrational position of the plasmid supercoils and nicked circles were compared to determine which recombinants contained inserts of significant size.