Cucumber Mosaic Virus Replication in Cowpea Protoplasts: 
Time Course of Virus, Coat Protein and RNA Synthesis 

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SUMMARY

The time course of the synthesis of cucumber mosaic virus (CMV), CMV coat protein and the four CMV RNAs has been followed in cowpea protoplasts. CMV synthesis was estimated by analytical sucrose gradient centrifugation of extracts from protoplasts and by infectivity assay; coat protein synthesis was determined by incorporation of radioactive amino acids and analytical polyacrylamide slab gel electrophoresis, while the synthesis of CMV RNAs was determined by hybridization analysis with labelled specific complementary DNA (cDNA) probes. There was a co-ordinate synthesis of CMV, CMV coat protein and virus RNA up to 50 h p.i. whereas the rate of coat protein synthesis reached a peak at about 15 h p.i. Coat protein was the only virus-induced protein detected. Results on the relative synthesis of RNAs 3 and 4 indicated that RNA 4 was derived by nucleolytic cleavage of RNA 3 and not by transcription of a negative RNA 4 strand.

INTRODUCTION

Purified virions of cucumber mosaic virus (CMV) contain four major RNA species (designated RNAs 1 to 4, in order of decreasing mol. wt.), of which the largest three are required for infectivity (Peden & Symons, 1973; Lot et al. 1974). The coat protein is genetically specified by RNA 3 (Habili & Francki, 1974), and is the only functional gene product of CMV identified to date although it seems likely that there are virus-coded components in the CMV-induced RNA replicase found in infected plants (Peden et al. 1972; Clark et al. 1974; Kumarasamy & Symons, 1979b). In vitro translation studies have indicated that there are at least four virus gene products (Schwinghamer & Symons, 1977).

In order to characterize the in vivo gene products of CMV, we have initiated a study of the synthesis of virus RNA and virus-coded proteins in CMV-infected protoplasts of cowpea (Vigna unguiculata). CMV-infected tobacco protoplasts have been used by Takanami et al. (1977) to study the synthesis of single- and double-strand CMV RNAs, while the infection by CMV of protoplasts of cucumber and of Vigna sesquipedalis has been reported by Coutts & Wood (1976) and Koike et al. (1977), respectively.

We report here conditions for the infection of cowpea protoplasts by the Q-strain of CMV (Q-CMV) and results on the time course of virus, coat protein and virus RNA synthesis.

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METHODS

Virus. The Q-strain of CMV (Francki et al. 1966) was purified from tobacco plants (Nicotiana tabacum cv. White Burley) as described by Peden & Symons (1973). Brome mosaic virus (BMV) RNA was kindly provided by Dr A. O. Jackson.

Plants. Seeds of cowpea [Vigna unguiculata L. (Walpers) cv. Blackeye] were planted in vermiculite moistened with Long Ashton culture solution (Hewitt, 1952) and watered daily with the same solution. Plants were maintained in a growth room with a daily cycle of 14 h light (28 °C) and 10 h dark (22 °C). Illumination was supplied by a mixture of 65 W white fluorescent tubes and 60 W incandescent bulbs to give a total intensity of approx. 11000 lux at leaf height.

Isolation of protoplasts. Protoplasts were isolated essentially as described by Beier & Bruening (1975, 1976) from 9-day-old plants, except that the enzyme solution used was 1.0% cellulase Onozuka R-10, 0.1% Macerozyme R-10 (Kinki Yakult Manufacturing Co. Ltd., Japan) and 0.1% bovine serum albumin, and neither the leaves nor the enzyme solution was sterilized. After incubation of the leaf pieces (approx. 1 g per 10 ml) at 30 °C for 2 h, the flask was swirled to release the protoplasts, the suspension filtered through muslin to remove undigested material and the protoplasts washed three times with sterile 0.45 M mannitol by centrifuging at 1000 g for 2 min. This procedure yielded 8 x 10^6 to 12 x 10^6 protoplasts per gram of leaf tissue, 80 to 90%, of which were intact as assessed by phase contrast microscopy.

Inoculation of protoplasts. Protoplasts were pelleted by centrifugation at 100 g for 2 min and resuspended (10^6/ml) in the inoculum of sterile 0.025 M potassium phosphate buffer, pH 5.6, 0.45 M mannitol, 2 µg/ml poly-L-ornithine (Sigma, Type I-C, approx. mol. wt. 122000) and 2 µg/ml CMV (unless otherwise stated) which had been pre-incubated at 25 °C for 5 min just before use. After incubation at 25 °C for 15 min with occasional shaking to keep the protoplasts in suspension, the protoplasts were pelleted by centrifugation and washed twice with sterile 0.45 M mannitol. Mock infection was carried out in the same way but in the absence of CMV.

Incubation of protoplasts. Protoplasts were incubated at 25 °C and at a concentration of about 8 x 10^6/ml in a sterile medium of 0.45 M mannitol, 50 mM-MES (N-morpholinoethane sulphonic acid), pH 5.4, 10 mM-CaCl_2, 1.0 mM-MgSO_4, 1.0 mM-KNO_3, 0.2 mM-KH_2PO_4, 1.0 µM-KI, 0.01 µM-CuSO_4, 250 µg/ml carbenicillin, 10 µg/ml gentamycin and 25 units/ml nystatin. Illumination at 2000 lux was provided by a cool white fluorescent tube. The percentage of infected protoplasts was determined by fluorescent antibody staining essentially as described by Otsuki & Takebe (1969, 1973) using rabbit antiserum to formaldehyde-fixed Q-CMV.

Analysis of protoplast extracts on sucrose gradients. The dialysed extract (0.5 or 1.0 ml) was layered on to an 11 ml 5 to 30% (w/v) sucrose gradient in 5 mM-sodium borate, 0.5 mM-EDTA, pH 9.0. The dialysed solution was then used for local lesion infectivity assays on Vigna unguiculata (Peden & Symons, 1973) and for sucrose gradient centrifugation to determine virus yield as follows.

Analysis of protoplast extracts on sucrose gradients. The dialysed extract (0.5 or 1.0 ml) was layered on to an 11 ml 5 to 30% (w/v) sucrose gradient in 5 mM-sodium borate, 0.5 mM-EDTA, pH 9.0, and centrifuged at 40000 rev/min and 4 °C for 90 min in a Beckman SW41 rotor. Gradients were analysed using an Isco Model 640 density gradient fractionator on the
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0.1 absorbance scale and virus was quantified by comparing the areas of the peaks on the absorbance trace corresponding to CMV with the areas of peaks containing added known quantities of virus. Virion numbers were calculated using a particle weight for CMV of 5.5 x 10^6 (Van Regenmortel et al. 1972).

When uninoculated protoplasts were extracted as described above in the presence of a known quantity of CMV and the extract analysed on a sucrose gradient, 55% of the initial quantity of virus was recovered. All data in Results were corrected for this recovery.

Radioactive labelling of protoplast proteins in vivo and their analysis by SDS-polyacrylamide gel electrophoresis. Infected and mock infected protoplasts in incubation medium were labelled with either 3H-leucine (50 Ci/mm) or 14C-protein hydrolysate at about 20 µCi/ml, either continuously or for 2-h pulses at various times after infection (p.i.). Samples of 0.3 ml were centrifuged and the protoplast pellet stored at -80 °C. When required, pellets were resuspended in 80 µl of loading buffer (Laemmli, 1970) and 10 to 30 µl samples were analysed by electrophoresis on discontinuous polyacrylamide slab gels (Laemmli, 1970; Schwinhauer & Symons, 1977). Marker proteins were labelled with 3H-borohydride (Kumarasamy & Symons, 1979a). Radioactive proteins were detected by autoradiography using pre-flashed film (Bonner & Laskey, 1974) and fluorograms scanned on a Joyce-Loebl densitometer.

Isolation of single-stranded (ss) RNA from protoplasts. Protoplast samples were thawed in 2.0 ml of 0.05 M-NaCl, 0.01 M-tris-HCl, pH 8.5, 0.01 M-EDTA, 1% (w/v) SDS, and the aqueous phase extracted twice with an equal vol. of water-saturated phenol and then three times with an equal vol. of ether. The solution was made 0.25 M in NaCl and the nucleic acids precipitated with 2.5 vol. ethanol overnight at -15 °C. The precipitate was collected by centrifugation, dried and dissolved in 0.2 ml of 0.1 M-EDTA, pH 7, when total nucleic acid was required, or in 0.5 ml of 15:85 (v/v) ethanol:STE (0.1 M-NaCl, 0.05 M-tris-HCl, pH 6.85, 1 mM-EDTA) for further fractionation on CF-11 cellulose. The ssRNA, isolated by chromatography on CF-11 cellulose, essentially as described by Franklin (1966) and Jackson et al. (1971), was precipitated with ethanol as described above and dissolved in 0.2 ml of 0.1 M-EDTA, pH 7.

Preparation of 32P-cDNA probes. CMV RNAs 1 to 4 were purified by the method of Symons (1978) and 32P-cDNA to each RNA was prepared by the method of Taylor et al. (1976), essentially as described by Gould & Symons (1977).

For the preparation of 32P-cDNA to sequences unique to RNA 3 (cDNA 3U), the cDNA fragments in cDNA 3 which were complementary to RNA 4 sequences were removed as follows. To 30 to 60 ng of 32P-cDNA 3 (1.5 x 10^8 to 6 x 10^8 ct/min) was added 12 µg each of CMV RNAs 1 and 2 and 1 µg of CMV RNA 4 in a total volume of 250 µl of hybridization buffer (0.18 M-NaCl, 0.01 M-tris-HCl, pH 7.0, 1.0 mM-EDTA, 0.05% SDS). After overlaying with paraffin oil, the solution was heated at 100 °C for 3 min and then hybridization was allowed to occur for 30 min at 60 °C; thus, RNAs 1, 2 and 4 were hybridized to R0 values (initial RNA concentration in mol nucleotide residues/1 x time of hybridization in s) of 2.5 x 10^-1, 2.5 x 10^-1 and 2.0 x 10^-2 mol/s, respectively. After cooling to 0 °C, 23 µg BMV RNA 4 [purified from BMV RNA (Symons, 1978) and for use as a marker] was added and 200 µl of the reaction mix loaded on to an 11.6 ml, 10 to 40% (v/v) sucrose gradient in 0.1 M-NaCl, 10 mM-tris-HCl, pH 7.4, 1 mM-EDTA. The gradient was centrifuged at 38000 rev/min in a Beckman SW41 rotor for 16 h at 4 °C and then fractionated with an Isco model 640 density gradient fractionator into 0.5 ml fractions. Samples of 5 or 10 µl from each fraction were counted, appropriate fractions pooled (Fig. 4), made 0.3 M in NaOH and incubated at 37 °C for 2 h to degrade RNA. After neutralization with acetic acid and the addition of 100 µg/ml of carrier RNA, the 32P-cDNA was ethanol precipitated, dried and dissolved in hybridization buffer.
Hybridization procedures and analysis of hybridization kinetics. The hybridization of
\( ^{32}P \) cDNA to RNA in hybridization buffer and the nuclease S1 assay for the extent of
hybrid formation were essentially as described by Gould & Symons (1977). \( R_{0.14} \) values were
determined using the computer program of Pearson et al. (1977). To determine the pro-
portion of particular CMV RNA sequences in the total RNA, the \( R_{0.14} \) for the pure RNA
species (Gould & Symons, 1977) was divided by the measured \( R_{0.14} \). Since cDNA 3U
measured sequences unique to RNA 3, which comprise 59% of RNA 3, the standard \( R_{0.14} \)
for these sequences was taken at 59% of that for RNA 3.

RESULTS

Optimal conditions for infection of cowpea protoplasts with Q-CMV

The conditions given in Methods for the inoculation of cowpea protoplasts with Q-CMV
resulted in 40 to 80% of protoplasts becoming infected, as detected by fluorescent antibody
staining, whilst the proportion of viable protoplasts at 40 to 48 h p.i. varied from 50 to 70% (results not shown). Infection was completely dependent on poly-L-ornithine and was
reduced by increasing the virus concentration in the inoculum above 2 \( \mu g/ml \). These con-
ditions are very similar to those reported by Alblas & Bol (1977) for the infection of cowpea
protoplasts with alfalfa mosaic virus (AMV) but are very different to those of Koike et al.
(1977) for the infection of Vigna sesquipedalis protoplasts (called cowpea by the authors)
with CMV. The latter authors found that infection was not completely dependent on the
presence of poly-L-ornithine and used a 10:1 ratio by weight of virus to poly-L-ornithine.
We suspect that these differences in infection conditions are due to the different strains of
CMV used.

Quantification and time course of virus multiplication

Three methods were used to assess virus production in protoplasts harvested at 0, 16, 32
and 48 h p.i.: fluorescent antibody staining, local lesion assay of extracts on cowpea and
sucrose gradient sedimentation analysis of extracts. Results of three such experiments are
shown in Table 1.

Virus production could be detected at 16 h p.i. by both fluorescent antibody staining and
infectivity assay. (It is important to note that the CMV antibody only reacted against intact
virions and not against isolated coat protein in immunodiffusion tests; our unpublished
data.) As measured by these techniques, the level of virus increased appreciably between
0 and 32 h p.i. but very little, if at all, between 32 and 48 h p.i. It is unlikely that the increase
in proportion of protoplasts infected with time was due to secondary infection, since there
was no poly-L-ornithine in the incubation medium.

Protoplast extracts were analysed by sedimentation on a sucrose gradient (Fig. 1) in
order to estimate the average number of virions/cell (Table 1). Although an absorbance peak
due to virus was detected in extracts from protoplasts 32 and 48 h p.i. no peak was detectable
in the 16 h sample (Fig. 1). However, it was estimated that the smallest quantity of virus
detectable by this technique was about \( 10^6 \) virions/cell; hence, although infectious virus was
present at 16 h p.i. (Table 1), the level of virus must have been below \( 10^6 \) virions/cell.

Overall, therefore, the three methods employed in Table 1 provided reasonably consistent
results in showing an increase in virus production from 0 to 32 h p.i. with either further or
no increase for the next 16 h.
CMV replication in protoplasts

Table 1. Time course of virus synthesis in cowpea protoplasts infected with Q-CMV as determined by three different methods

<table>
<thead>
<tr>
<th>Time p.i. (h)</th>
<th>Expt. 1</th>
<th>Expt. 2</th>
<th>Expt. 3</th>
<th>Mean number of lesions/half leaf</th>
<th>Virions/cell (×10^{-9})</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>48</td>
<td>69</td>
<td>73</td>
<td>51</td>
<td>233</td>
<td>52</td>
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</table>

* Determined by fluorescent antibody staining.
† Produced by dialysed protoplast extracts prepared and assayed as described in Methods.
‡ Calculated from sucrose gradient analyses (Fig. 1) of dialysed protoplast extracts as described in Methods.

Fig. 1. Sucrose gradient analysis of protoplast extracts. (a) Standards of 3 and 10 μg of purified Q-CMV in the absence of protoplast extract. The same peak areas were obtained when virus was mixed with uninfected protoplast extract prior to centrifugation. (b) Extracts of infected protoplasts collected at 0, 16, 32 and 48 h p.i. Each gradient was loaded with extract from the equivalent of 1.3 to 1.8 × 10^6 infected protoplasts, as determined at 48 h p.i. Further details are given in Methods.

**Time course of coat protein synthesis**

Proteins synthesized in infected and mock-infected protoplasts were labelled with radioactive amino acids and analysed by SDS-polyacrylamide slab gel electrophoresis followed by fluorography. Both continuous and pulse-labelling were used in an attempt to identify the synthesis of virus-stimulated proteins.
Time p.i. (h)

Fig. 2. Fluorogram of a 15% polyacrylamide-SDS gel of proteins labelled with \(^{3}H\)-leucine (added at 1 h p.i.) in mock-infected (M) or infected (I) protoplasts. Protoplasts (78% infected) were collected at the indicated times p.i. Further details are given in Methods. Mol. wt. standards (S) were: pyruvate carboxylase (115,000); bovine serum albumin (68,000); glutamate dehydrogenase (53,000); glyceraldehyde-3-phosphate dehydrogenase (36,000); and CMV coat protein (24,500).

Table 2. Time course of labelling of CMV coat protein in infected cowpea protoplasts

<table>
<thead>
<tr>
<th>Continuous labelling</th>
<th>Pulse labelling</th>
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<td></td>
<td>Radioactivity in coat protein as % total radioactivity*</td>
</tr>
<tr>
<td>Time p.i. (h)</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>8</td>
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</table>

* Calculated from densitometer scan of fluorogram of Fig. 2.  † Calculated from densitometer scan of fluorogram of Fig. 3.

Continuous labelling of protoplasts

Proteins synthesized in protoplasts labelled continuously from 1 to 50 h p.i. were analysed on a 15% polyacrylamide slab gel (Fig. 2). Only one protein was detectable in CMV-infected protoplasts which was not present in mock-infected protoplasts; this co-migrated with the coat protein marker (M\(_r\) 24,500) and was shown to be coat protein by two-dimensional gel electrophoresis (A. R. Gould, T. J. Gonda and R. H. Symons, unpublished data). The same pattern as that of Fig. 2 was obtained when the same samples were electrophoresed on a 12% gel which gave better resolution of high mol. wt. proteins. Further analysis of proteins from the protoplasts labelled up to 30 or 43 h p.i. on 7.5% or 10% gels also failed to reveal virus-induced proteins other than coat protein (not shown).
**CMV replication in protoplasts**

**Fig. 3.** Fluorogram of a 12% polyacrylamide-SDS gel of proteins synthesized in mock-infected (M) or infected (I) protoplasts and pulse-labelled with 16 µCi/ml of 14C-protein hydrolysate for 2-h periods beginning at the indicated times p.i. At 46 h p.i., 96% of the protoplasts were infected. Mol. wt. standards (S) were as in Fig. 2; other details are given in Methods.

The coat protein was clearly detectable at 15 h but not 10 h p.i. (Fig. 2). Densitometer scanning of the fluorogram of Fig. 2 showed that the proportion of total radioactivity incorporated into coat protein increased rapidly between 15 and 40 h p.i. with little increase between 40 and 50 h p.i. (Table 2). The percentage of total radioactivity which was incorporated into coat protein is not necessarily proportional to the amount of coat protein synthesized, as the rate of incorporation of 3H-leucine was not constant over the labelling period (data not given). However, an increase in the proportion of label in coat protein after any time period clearly demonstrates synthesis of coat protein.

**Pulse labelling of protoplasts**

Rottier et al. (1978) reported that pulse labelling of cowpea protoplasts was essential for the detection of cowpea mosaic virus-specific polypeptides. Therefore, infected and mock-infected protoplasts were pulse labelled for 2-h periods at various times until 44 h p.i. and the labelled proteins were analysed on a 12% polyacrylamide-SDS gel (Fig. 3). The fluorogram shows that coat protein was still the only virus-induced protein detectable, although it could now be detected in the 8 to 10 h p.i. interval; coat protein was not detected at 10 h p.i. when continuous labelling was used (Fig. 2). Densitometer scanning of the fluorogram of Fig. 3 showed that coat protein synthesis, as a proportion of total protein synthesis, was maximal during the 16 to 18 h and 24 to 26 h p.i. pulses (Table 2).

**Synthesis of virus RNAs**

The synthesis of CMV RNAs in tobacco protoplasts has been followed by the incorporation of 3H-uracil (Takanami et al. 1977); a combination of actinomycin D treatment and u.v. light irradiation was needed to suppress host RNA synthesis. A more direct approach
has been used here which does not rely on the suppression of host RNA synthesis but which employs quantification of the four CMV RNAs by hybridization analysis with specific complementary $^{32}$P-DNA (cDNA) probes. The data provide quantitative estimates of total RNA present, something which cannot readily be achieved by in vivo labelling techniques.

**Preparation of $^{32}$P-cDNA probes**

Since RNA 4 is completely contained at the 3'-end of RNA 3 (Gould & Symons, 1977), it was necessary for the present work to prepare a cDNA probe for sequences unique to RNA 3 (cDNA 3U). An additional problem in the preparation of this probe was the presence in RNA 3 of breakdown products of the same size derived from RNAs 1 and 2 (Gould & Symons, 1977). The method developed (see Methods) was to hybridize $^{32}$P-cDNA to total RNA 3 with excess RNAs 1, 2 and 4 and to separate the hybridized cDNA from the unhybridized cDNA 3U by sucrose gradient centrifugation. This was feasible because cDNA 3 had an average mol. wt. of approx. 1·2 to 1·5×10^6 (Gould & Symons, 1977) whereas RNAs 1, 2 and 4 have mol. wt. of 1·35, 1·16 and 0·35×10^6, respectively (Peden & Symons, 1973).

Fig. 4 shows that, after hybridization to RNAs 1, 2 and 4, the majority of $^{32}$P-cDNA to RNA 3 (peak B) sedimented a little more slowly than brome mosaic virus RNA 4 (mol. wt. 0·28×10^6; Lane & Kaesberg, 1971). When $^{32}$P-cDNA from the slowest sedimenting peak A was hybridized to RNAs 3 and 4, the kinetics shown in Fig. 5 were obtained. The $R_0R_4$ for the hybridization of RNA 3 was 4·2×10^{-3} mol. s/l, a value essentially the same as that obtained by Gould & Symons (1977) for the hybridization of RNA 3 to cDNA 3 and the result expected for cDNA 3U. Hybridization of peak A to RNA 4 (Fig. 5) showed very little hybridization at the $R_0R_4$ of RNA 4 (2·2×10^{-3} mol. s/l; Gould & Symons, 1977) which indicates no more than about 5% contamination of cDNA 3U by cDNA 4 sequences. This

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**Fig. 4.** Distribution of radioactivity after sucrose gradient centrifugation of the hybridization reaction mixture of $^{32}$P-cDNA 3 to CMV RNAs 1, 2 and 4, as described in Methods. The vertical arrows indicate the positions of BMV RNA 4 (left) and CMV RNAs 1 and 2 (right), determined by their absorbance at 254 nm during fractionation of the gradient. Fractions A, B and C were pooled as shown.

**Fig. 5.** Hybridization kinetics of $^{32}$P-cDNA recovered from peak A of Fig. 4 to CMV RNAs 3 (■) and 4 (▲). Hybridization procedures were as described in Methods.
**CMV replication in protoplasts**

Fig. 6. Hybridization kinetics of $^{32}$P-cDNA 2 (prepared against CMV RNA 2) to RNA extracted from infected protoplasts at the indicated times in hours p.i. All procedures were as described in Methods.

**Table 3. Amounts of the four virus RNA sequence classes in CMV-infected protoplasts**

<table>
<thead>
<tr>
<th>Time p.i. (h)</th>
<th>RNA 1 Am- (µg)</th>
<th>RNA 1 s.d.</th>
<th>RNA 1 Mol-%</th>
<th>RNA 2 Am- (µg)</th>
<th>RNA 2 s.d.</th>
<th>RNA 2 Mol-%</th>
<th>RNA 3U Am- (µg)</th>
<th>RNA 3U s.d.</th>
<th>RNA 3U Mol-%</th>
<th>RNA 4 Am- (µg)</th>
<th>RNA 4 s.d.</th>
<th>RNA 4 Mol-%</th>
<th>RNA 4 pmol (%) ar%</th>
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* Each sample contained $1 \times 10^6$ infected protoplasts.

The curve had a $R_{R0}^4$ of $4·3 \times 10^{-2}$ mol. s/l which is about 10 times the $R_{R0}^4$ of RNA 3; this indicates that the RNA 4 used here contained about 10% of sequences derived from RNA 3U. Similar experiments on the hybridization of cDNA 3U to RNAs 1 and 2 showed that cDNA 3U contained less than 5% of sequences hybridizing to RNAs 1 and 2 (data not given). Overall, therefore, these results show that peak A cDNA hybridized to at least 90% to sequences unique to RNA 3 and was cDNA 3U.

Material from peaks B and C (Fig. 4) hybridized extensively to all four virus RNA species with peak C showing some enrichment for cDNA to RNAs 1 and 2 (data not given).

**Time course of virus RNA synthesis**

The approach used was to examine the hybridization kinetics of specific cDNA probes to ssRNA extracted at various times p.i. from CMV-infected protoplasts. Since, under RNA excess conditions, the rate of hybridization is directly proportional to the concentration of hybridizing RNA sequences (Birnstiel et al. 1972; Young & Paul, 1973), the proportion of each virus RNA sequence in the total RNA extracted could be determined by comparison of the observed rate with that obtained on hybridization to the purified virus RNA species to the cDNA probe (Gould & Symons, 1977, 1978). Any virus dsRNA present would contain + strand sequences and therefore it was removed by CF-11 cellulose chromatography prior to the hybridization reactions (see Methods).

Fig. 6 shows the kinetics of hybridization of $^{32}$P-cDNA 2 to ssRNA from infected proto-
plasts sampled at various times p.i.; corresponding curves for the other three cDNAs are not given. The $R_{d4}$ values plus standard deviations for all curves were determined using the computer programme of Pearson et al. (1977) and the data used to calculate the amounts of the four virus RNA sequence classes (Table 3). In general, the data are internally consistent, although some discrepancies are apparent. For example, there was a decrease in the amount of RNA 4 from 20 to 30 h p.i. while the doubling of the amount of RNA 3U from 40 to 50 h p.i. was not accompanied by a similar change in the other RNAs. Such variation is considered to be due to a combination of experimental errors, only some of which were reflected in the standard deviations.

In spite of these variations, the following features of the data of Table 3 are significant. The RNA from the virus particles taken up by or adsorbed to the protoplasts during the inoculation procedure could be detected as given by the data at 0 h p.i. For three of the four RNAs, there was a significant increase above this level by 10 h p.i. while all species showed at least a threefold increase by 15 h p.i. Little or no increase in the level of any of the virus RNAs was detected between 20 and 30 h p.i. Except for the 50 h sample, the level of RNA 3U sequences was approximately equimolar with RNA 4 sequences; the important implications of this are considered below. The relative molar amounts of the four sequence classes remained fairly constant for all time samples; certainly there was no large selective increase in the level of any one species.

**Proportion of the RNA species in virion RNA**

The observation that the molar ratio of RNA 3U to RNA 4 sequences was approximately 1:1 in the 0 h p.i. sample was unexpected since RNA 4 sequences are present in both RNA 3 and RNA 4 in virions. When the total virion RNA was fractionated by electrophoresis on a polyacrylamide tube gel (Peden & Symons, 1973), the molar ratio of full-length RNA 3 to full-length RNA 4 was 1:2.2. However, hybridization of this total virion RNA to cDNA 3U and to cDNA 4 gave a molar ratio of 1:0.93 (data not shown), in good agreement with the 0 h p.i. result of 1:0.92 (Table 3). From these data, it must be concluded that there were RNA 3U sequences in the virion RNA other than those in intact RNA3.

**Estimates of initial and final numbers of virions per infected cell**

Since all the virus RNA in the 0 time sample of Table 3 must have been derived from virus in the inoculum, it was calculated that $2.8 \times 10^4$ virions/infected protoplast were taken up or adsorbed. This value is roughly 10-fold higher than that reported for several other virus-protoplast systems (Motoyoshi et al. 1973; Hibi et al. 1975; Kubo et al. 1976; Shaw, 1978).

If all of the virus RNA in the infected protoplasts at 40 to 50 h p.i. was encapsidated, it was calculated that there should have been 2 to $3 \times 10^6$ virions per infected cell. This is four to six times higher than the values found in three different experiments shown in Table 1 and indicates that a considerable proportion of virus RNA synthesized in protoplasts was not encapsidated.

**Comparison of time courses of virus, RNA and coat protein synthesis**

From the data in Tables 1 to 3 and Fig. 2 and 3, the accumulations of virus, virus RNA and coat protein, as well as the rate of coat protein synthesis, as a function of time after infection were calculated as percentages of their maximum values and plotted in Fig. 7. For these calculations, the final level of RNA was assumed to be that at 40 h p.i., the proportion of total radioactivity incorporated into protein found in coat protein was taken as a measure of the amount of coat protein, the results of the three experiments in Table 1 on the numbers of virions/cell were averaged and at 16 h p.i. virion numbers/cell were taken as 20% of
CMV replication in protoplasts

Fig. 7. Comparison of the time courses of total virus RNA (●), virus (○) and coat protein (□) synthesis in CMV-infected protoplasts. Also shown is the rate of coat protein synthesis (△) as determined by pulse-labelling. The level of total virus RNA was determined by summing the amounts of each sequence class in Table 3. For further details, see text.

the maximum value on the basis of the lesions/half leaf (Table 1) and of considerations above on the sensitivity of the estimation of virion numbers/cell. In view of the origin of the data for Fig. 7, the overall pattern must be considered approximate.

Fig. 7 indicates that virus RNA and coat protein synthesis and the assembly of virions occurred co-ordinately while the rate of coat protein synthesis reached a relatively early peak at about 15 h p.i.

DISCUSSION

The results presented here have allowed the first comparative analysis of the time course of synthesis of CMV coat protein (both total and rate) of the four CMV RNAs and of intact virions in CMV-infected protoplasts. The only other system in which similar data are available is for TMV-infected tobacco protoplasts (Takebe et al. 1975). In contrast to the coordinate synthesis of virus, RNA and coat protein found with CMV, there was a lag between TMV RNA synthesis on the one hand and TMV and coat protein synthesis on the other in tobacco protoplasts. Further, the peak rate of CMV coat protein synthesis at about 15 h p.i. is to be compared with an increasing rate of TMV coat protein synthesis with time p.i. (Siegel et al. 1978). This difference between CMV and TMV may be due in part to different assembly mechanisms for rod-shaped and spherical viruses.

The only virus-induced protein detected here by one-dimensional polyacrylamide slab gel electrophoresis was CMV coat protein. It appears, therefore, that other virus-induced proteins are synthesized at much lower levels than that of coat protein and hence are obscured by protoplast proteins on the fluorograms. Further studies aimed at detecting other virus-induced proteins by the more sensitive technique of two-dimensional gel electrophoresis are in progress.

Single-strand CMV RNA synthesis was measured here by hybridization analysis with specific 32P-cDNA probes in RNA excess. Virus RNA present as replicative form or replicative intermediate was removed before hybridization analysis; synthesis of these will be followed at a later date as will the detection and quantification of any single-strand minus RNA sequences. It is important to note that the hybridization method used here determined total virus RNA sequences and did not distinguish between intact virion RNAs and lower mol. wt. fragments of them. Techniques for the hybridization analysis of RNAs on the basis of size are now available (Alwine et al. 1977; Stark & Williams, 1979).
The usual procedure of incubating infected protoplasts in the presence of radioactive precursors, usually $^{32}$P-phosphate or $^3$H-uridine, followed by gel electrophoresis in order to examine virus RNA synthesis has a number of disadvantages over the use of labelled cDNA probes as described here. One of these is that the virus RNAs may be close in size to cell-specific RNA species; thus, Takanami et al. (1977) needed to use a combination of u.v. irradiation and actinomycin D treatment to suppress completely ribosomal RNA synthesis in tobacco protoplasts infected with CMV as CMV RNAs 1 and 2 are very close in size to 25S ribosomal RNA. Further, the use of u.v.-irradiation in particular has disadvantages as it probably interferes with virus replication. Sakai et al. (1977) found that u.v.-irradiation of tobacco protoplasts altered the kinetics of cowpea chlorotic mottle virus (CCMV) synthesis while, in the present work, u.v.-irradiation severely inhibited CMV infection of cowpea protoplasts (data not given). In addition, interpretation of time course studies relies on the assumption that the specific radioactivities of the virus RNAs remain constant; Bancroft et al. (1975) found differences in the specific activities of the CCMV $^{32}$P-RNA species synthesized in tobacco protoplasts. The use of hybridization analysis with cDNA probes circumvents these problems and allows the actual amount of virus RNA to be determined.

From the observation that, at most times after infection, the molar ratio of RNA 3U to RNA 4 sequences was close to 1:1 (Table 3), it follows that all the RNA 4 sequences detected could be accounted for by those present as RNA 3. This conclusion is inconsistent with any model for the replication of RNA 4 which involves transcription from a negative RNA 4 strand or partial transcription from a negative RNA 3 strand but is consistent with RNA 4 being produced by the specific nucleolytic cleavage of RNA 3. Hence, RNA 3U sequences detected by hybridization would be a mixture of those present in intact RNA 3 and those cleaved off during the production of RNA 4. Since the results of Takanami et al. (1977) did not show any peak corresponding to full length RNA 3U in RNA isolated from tobacco protoplasts infected with CMV and labelled with $^3$H-uracil, it is possible that RNA 3U after cleavage is randomly degraded but to sizes still large enough to be detected by hybridization. It is of considerable interest that Takanami et al. (1977) found only trace amounts of dsRNA corresponding to CMV RNA 4 while Bancroft et al. (1975) obtained no evidence for a dsRNA form of RNA 4 of either BMV or CCMV in tobacco protoplasts; the latter data contrast with the reported isolation of the double-strand replicative form of BMV RNA 4 by Bastin & Kaesberg (1976).

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