Kinetics of Accumulation of the Three Non-structural Proteins of Alfalfa Mosaic Virus in Tobacco Plants

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(Accepted 21 February 1986)

SUMMARY

Antisera to synthetic peptides corresponding to the C-termini of two non-structural proteins (NSP) of alfalfa mosaic virus (AIMV), P1 (126K) and P3 (32K), were prepared and characterized. These antisera, together with one which had previously been made against the C-terminus of P2 (90K), enabled us to detect the three NSPs in a crude membrane fraction from AIMV-infected tobacco leaves. The accumulation of these proteins at 25 °C and at 10 °C was followed as a function of time after inoculation, and their amounts were compared with viral replicase activity. All three proteins accumulated at the beginning of the infection period and then disappeared, P3 more rapidly than the other two. There was a good correlation between replicase activity and the amounts of P1 and P2, but not the amount of P3. These results are consistent with the notion that P1 and P2 are part of the replication complex. Although much less coat protein was made in inoculated leaves at 10 °C than at 25 °C, the maximum amounts of the three NSPs and the maximum replicase activity were at least as high at 10 °C as at 25 °C. Thus, 10 °C is not a restrictive temperature for the assembly of a functional replication complex.

INTRODUCTION

The genome of alfalfa mosaic virus (AIMV) encodes four proteins, only one of which, the coat protein (mol. wt. 24500, 24K) is produced in large quantity in infected plants. The other three (P1, 126K; P2, 90K; P3, 32K) are non-structural. Although they are readily obtained by in vitro translation of the genomic RNAs, RNA 1, RNA 2 and RNA 3 (van Tol & van Vloten-Doting, 1979, and references therein; Godefroy-Colburn et al., 1985), they do not accumulate to any great extent in vivo.

The role of the non-structural proteins (NSPs) is still open to speculation. P1 and P2 are almost certainly involved in replication because, in protoplasts, inoculation with a mixture of RNA 1, RNA 2 and coat protein is necessary and sufficient to obtain replicative forms (RF) (Nassuth et al., 1981; Nassuth & Bol, 1983). The study of mutations in RNA 1 and RNA 2 also suggests that P1 and P2 function in close interaction with each other (Sarachu et al., 1983), and it is thought that both are part of the replicase. However, RNA 3 is required for the switch from symmetric plus-strand and minus-strand synthesis (production of RF) to asymmetric plus-strand synthesis (production of viral RNA) to occur (Nassuth & Bol, 1983). This function may therefore involve P3 and/or the coat protein, both of which are encoded by RNA 3. The detection of P2 and P3 (Berna et al., 1984, 1985) in a membrane fraction of tobacco leaves, which is thought to contain the viral replicase (Weening & Bol, 1975; Le Roy et al., 1977), is consistent with this view. In addition, there are strong similarities between three regions of P2 and homologous regions of the polymerases of poliovirus and cowpea mosaic virus (Kamer & Argos, 1984). It is thus tempting to speculate that P2 is the equivalent of the picornavirus or comovirus replicase.
The direct functional study of the NSPs of AlMV has not been possible because sensitive and reliable detection methods have not been available. Proteins with the electrophoretic mobilities of the full-length AlMV translation products were detected in pulse-labelled protoplasts when host protein synthesis was inhibited by prior u.v. irradiation (Samac et al., 1983) and a protein with the electrophoretic mobility of P3 was detected in infected tobacco plants by pulse-labelling in the presence of actinomycin D and chloramphenicol (Joshi et al., 1984). However, positive identification of P2 and P3 in infected plants was possible only with specific antisera (Berna et al., 1984, 1985), although the methods did not permit a quantitative study to be undertaken.

In this paper, we report the immunodetection of P1, P2 and P3 using a much improved method, the kinetics of accumulation of the three NSPs of AlMV in tobacco plants, and a comparison of the amount of each of these proteins in a crude membrane fraction with the viral replicase activity of the same fraction throughout the infection period.

Some early steps of tobacco mosaic virus (TMV) replication take place at low temperature although no virus is synthesized (Dawson & Schlegel, 1976) and preliminary observations (Berna et al., 1984) suggested that the same was true of AlMV. Therefore, we compared the accumulation of NSPs and replicase activity at 25 °C, at which the infection is systemic and yields large amounts of virus, and at 10 °C, at which little virus is made.

**METHODS**

**Virus propagation and purification.** The Strasbourg strain (S-strain) of AlMV was propagated in *Nicotiana tabacum* (cv. Xanthi nc) according to Pinck & Hirth (1972). Six-week-old plants were inoculated and kept at 25 °C or 10 °C for the indicated times under 16 h illumination.

For preparation of the coat protein, the virus was purified according to Franck & Hirth (1976).

**Preparation of subcellular fractions.** The 1000 g pellet (Pe-1), the 30000 g pellet (Pe-30) and the supernatant fraction (S-30) were prepared from inoculated leaves and from similar leaves of healthy plants as described by Berna et al. (1985). Pe-1 and Pe-30 were resuspended in extraction buffer (100 mM-Tris–HCl pH 8.1 at 4 °C, 10 mM-KCl, 5 mM-MgCl₂, 0·4 M-sucrose, 10% glycerol, 10 mM-2-mercaptoethanol) and diluted with an equal volume of glycerol. The final samples were concentrated 20-fold with respect to the original extract. In order to keep the amount of supernatant protein to be tested within manageable limits, the S-30 was fractionated by ammonium sulphate precipitation from 0 to 35%, from 35 to 45%, from 45 to 55% and from 55 to 75% saturation. The fractions were redissolved in extraction buffer (1/30 the original volume of S-30) and dialysed against the same buffer.

**Preparation of the wheat germ translation products of AlMV RNA.** The [³⁵S]methionine-labelled translation products of AlMV RNA were synthesized in wheat germ extracts and partially purified by centrifugation through a sucrose gradient (Berna et al., 1985). Three preparations were made, with 60 μg/ml, 120 μg/ml and 200 μg/ml of AlMV RNA, concentrations found to be optimum for the synthesis of P3, P1 and P2 respectively.

**Peptide synthesis.** Two peptides were synthesized by the solid phase method (Barany & Merrifield, 1980) and had the sequences of the C-terminal 21 amino acids of P1 (pep1, Fig. 1a) and the C-terminal 26 amino acids of P3, minus the terminal histidine (pep3, Fig. 1b). The peptides were purified by filtration through a Sephadex G-25 column (1·20 m × 1·4 cm for 150 mg of peptide) in 8% acetic acid. Their amino acid composition was verified using a 'Durrum DS500' analyser.

**Preparation of antisera to the synthetic peptides.** Peptides were injected subcutaneously into rabbits at 3- to 4-week intervals. Each injection contained 150 μg of peptide in 0·5 ml phosphate-buffered saline (0·15 M-NaCl in 10 mM-phosphate pH 7·2; PBS) emulsified with 0·5 ml Freund's adjuvant, either complete (first injection) or incomplete (boosters). Sera were prepared from blood collected 10 days after each booster and were kept at −20 °C in 50% glycerol.

**Preparation of an antiserum to the coat protein of AlMV.** The coat protein was prepared from the purified virus by SDS-PAGE in 13% gels. The band containing the coat protein was excised and injected into a rabbit as described for P3 by Berna et al. (1985). The rabbit received three injections, each containing 200 μg of coat protein. The antiserum was collected 10 days after the last injection.

**Electrophoretic analysis of proteins.** SDS–PAGE was in the buffer system of Laemmli (1970). The gels (1·5 mm thick) were made with a ratio of bisacrylamide to acrylamide of 1:36 (w/w). They contained 6% acrylamide for the detection of P1 and P2, 9% for P3 and 12% for the coat protein. The samples were prepared as described in Berna et al. (1985).

**Immunoblotting procedure.** After SDS–PAGE, the proteins were immediately blotted onto nitrocellulose (NC) sheets essentially as described by Towbin et al. (1979), at a constant current of 2 mA/cm². The coat protein was electroblotted for 45 min. The transfer of the NSPs was monitored by the recovery of [³⁵S]-labelled translation proteins.
AIMV non-structural proteins in plants

Fig. 1. Hydrophilicity of the C-terminal regions of (a) P1 and (b) P3, and sequences of the synthetic peptides. The sequences of P1 and P3 were derived from those of RNA 1 (strain 425, Cornelissen et al., 1983a) and RNA 3 (strain S, Ravelonandro et al., 1984) and the hydrophilicity diagrams of these proteins were established according to Hopp & Woods (1981) with a scan size of four amino acids. For each protein, P1 or P3, the part of the diagram corresponding to 100 C-terminal residues is shown with the sequence of the peptide which was synthesized (pep1 or pep3). The C-terminal amino acid of P3 (histidine) was omitted from pep3.

The preparation and characterization of an antiserum against a synthetic peptide corresponding to the C-terminus of P2 (pep2) have been described (Berna et al., 1984). The new antisera, made against the C-terminal peptides of P1 and P3, were similarly tested against the wheat germ translation products of AIMV RNA.

RESULTS

Characterization of antisera to the C-terminal peptides of the AIMV non-structural proteins

The preparation and characterization of an antiserum against a synthetic peptide corresponding to the C-terminus of P2 (pep2) have been described (Berna et al., 1984). The new antisera, made against the C-terminal peptides of P1 and P3, were similarly tested against the wheat germ translation products of AIMV RNA.
Fig. 2. Characterization of the antisera to pep1 and pep3 with AlMV translation products. Partially purified preparations of the 35S-labelled AlMV translation products (or equivalent blank preparations made without mRNA) were immunoblotted with the antisera to be tested and with a non-immune serum. Lane 1 is an autoradiogram and lanes 2 to 11 are immunoblots. (a) Characterization of the anti-pep1 serum. Products of translation of 120 ng/ml AlMV RNA, containing about 10 ng P1 (lanes 1 to 3 and 5 to 8), or an equivalent amount of the blank preparation (lane 4), were separated in a 6% gel and immunoblotted with a non-immune serum (lane 2) or with the anti-pep1 serum (lanes 3 to 8), both diluted 5000-fold. In lanes 6 to 8, the antiserum (4 gl in 50% glycerol) had been preincubated with pep1 for 30 min at room temperature in 200 gl PBS, then diluted to 10 ml for incubation with the blot. The final concentrations of pep1 were 0.01 g/ml (lane 6), 0.1 g/ml (lane 7) or 2.0 g/ml (lane 8). (b) Characterization of the anti-pep3 serum and comparison with the anti-P3 serum. Products of translation of 60 g/ml AlMV RNA, containing about 11 ng P3 (lanes 1 to 3 and 6 to 11), or equivalent
Of four rabbits injected with pep1, only two made antibodies which reacted with electroblotted P1. Fig. 2(a) presents the reaction of the best antiserum (obtained after four injections) with a partially purified preparation of the AlMV translation products. The immunoblot (lane 3) showed one band which corresponded to radioactive P1 (lane 1) and did not appear if the translation products were reacted with a non-immune serum (lane 2). The anti-pep1 serum did not react with a blank translation medium (lane 4). The specificity of the reaction observed in lane 3 was further demonstrated by preabsorbing the anti-pep1 serum with increasing amounts of the peptide: the reaction with P1 was progressively eliminated and disappeared completely in the presence of 2 μg/ml pep1 (lanes 5 to 8). The anti-pep1 serum also detected a fainter band (marked with a triangle in lane 3) which was competed out by the peptide (lanes 5 to 8) and which was absent from the immunoblot of the blank translation medium (lane 4). The corresponding protein therefore seemed to be related to P1. Since it migrated with an apparent mol. wt. of 200K, it could have been a minor aggregated form of this protein. An overexposed autoradiogram (not presented) showed radioactive material in this region of the gel.

Pep3 was injected into one rabbit and sera obtained throughout the immunization period were assayed on electroblots of the partially purified translation products. The best antiserum was obtained after five injections. It reacted strongly with P3 and with P'3 (the minor wheat germ translation product of RNA 3; Godefroy-Colburn et al., 1985; Berna et al., 1985), as shown by comparison with the autoradiogram (Fig. 2b, lanes 1 and 3). No reaction was observed with a non-immune serum (lane 2). The coloured bands corresponding to P3 and P'3 were also absent when the anti-pep3 serum was assayed against a blank translation medium (lane 4), and they were eliminated by preabsorption of the antiserum with the peptide, further proving their specificity (lanes 7 to 11). Their elimination was complete at 2 μg/ml pep3. A faint band, marked with a triangle in lane 3, was visible on all the immunoblots which had reacted with the anti-pep3 serum. However, this reaction was probably not due to the anti-pep3 antibodies since it took place with the blank sample as well as with the translation medium, and, furthermore, was not competed out by the peptide.

The anti-pep3 serum was compared with the anti-P3 serum which had previously been prepared against the gel-purified wheat germ translation product of RNA 3 (Berna et al., 1985). Although the anti-P3 serum specifically reacted with P3 and P'3 after preabsorption with the wheat germ extract (Fig. 2b, lanes 5 and 6), it was fivefold less efficient than the anti-pep3 serum when used at the same dilution. An additional, poorly reproducible reaction of the preabsorbed anti-P3 serum (diamond in lane 5) may correspond to a gel artefact (Tasheva & Dessev, 1983).

Quantification of the immunoblots

As a test of the proportionality between the intensity of staining of immunoblots (measured by reflection densitometry) and the amount of viral protein present, 35S-labelled translation products were assayed by scintillation counting and immunoblotting. Fig. 3 shows that the intensity of colour obtained after 2 h of peroxidase reaction was nearly proportional to the amount of viral protein, especially when the integrated absorbance was less than 1-5 arbitrary units. In our assay conditions, this absorbance corresponded to 0-2 pmol of P1, or 0-18 pmol of P2, or 0-12 pmol of P3. The anti-pep3 serum detected P3 and P'3 with the same efficiency, which suggests that the epitopes of pep3 which were accessible in blotted P3 were equally accessible in P'3. A similar observation was made with the anti-P3 serum (Berna et al., 1985).
Fig. 3. Quantification of NSPs by immunoblotting. Partially purified $^{35}$S-labelled translation products were separated in a 9% gel (P3) or in a 6% gel (P1 and P2), and immunoblotted with the anti-pep3 serum or a mixture of anti-pep1 and anti-pep2 respectively. Each serum was diluted 5000-fold. The peroxidase reaction was for 2 h. The absorbance at 460 nm of the viral protein bands was measured by reflection and the amount of protein was calculated from the amount of radioactivity in the band. ●, P1; △, P2; ■, P3; ☐, P3.

Time course of accumulation of the viral proteins at 25 °C

A batch of plants was inoculated with AlMV and kept at 25 °C. Symptoms characteristic of systemic infection appeared on the inoculated leaves after 2 days, and on the upper leaves after 3 days. Inoculated leaves from four to six plants were harvested at different times after inoculation and extracted immediately, to obtain the S-30 and the Pe-30 fractions. S-30 contains most of the coat protein (mainly in virus particles) which was assayed by immunoblotting with anti-coat-protein serum. As shown in Fig. 4(a), coat protein synthesis proceeded at maximum rate after a lag period of 20 to 30 h. After 9 days its concentration in the S-30 stabilized at 370 µg/ml (measured on immunoblots, not shown, by comparison with purified virus). This was equivalent to 740 µg of coat protein per g of leaves.

The Pe-30, which had previously been shown to contain P2 and P3, was immunoblotted with the anti-pep1 serum (Fig. 4b). In infected extracts (lanes 1 to 8), the antiserum detected a polypeptide which was absent from the healthy extract (lane H) and which had the same mobility as P1 synthesized in vitro (lane T). The reactions, both with the Pe-30 and with the translation medium, were abolished by preabsorbing the antiserum with pep1 (lanes 5' and T'), proving their specificity. P1 first appeared at 45 h post-infection (lane 2). Its concentration was maximum in the Pe-30 at 69 h post-infection (lane 3) and seemed to decrease towards the end of the infection period (lane 8).

The anti-pep2 serum clearly revealed P2 in the same samples which also contained P1 (Fig. 4c, lanes 2 to 8). Several other bands (marked with filled triangles), which were visible in all the samples, whether healthy or infected, were certainly due to cellular contaminants. However, this immunoblot was much cleaner than those obtained in preliminary experiments (Berna et al.,...
Fig. 5. Accumulation of the NSPs and replicase activity as a function of time at 25 °C. (a) The experiment of Fig. 4 and another similar experiment done on the same plant extracts were quantified and the mean results normalized to the maximum amount of each protein: 15 ng P1, 9 ng P2 and 2 ng P3 in 30 µl Pe-30. ●, P1; △, P2; ○, P3. (b) The replicase activity of the Pe-30 was normalized to the maximum activity, i.e. 0.51 pmol UMP incorporated in 10 µl Pe-30 in 1 h.

1984), which demonstrates the improvement of the immunoblotting method. Preabsorption with the peptide abolished the reaction with P2 from the Pe-30 as well as from the translation medium (lanes 5' and T'). The kinetics of P2 accumulation seemed to follow rather closely that of P1.

P3 was similarly assayed with the anti-pep3 serum (Fig. 4d). In spite of a significant background (particularly the bands marked with arrows, on either side of P3), a band with the same mobility as P3 synthesized \textit{in vitro} (lanes T) appeared in the infected extracts at 45 h post-infection (lane 2). This band was absent from the healthy samples (lanes H) and was eliminated by serum preabsorption with the peptide (lane 4'), as was the reaction with bona fide P3 from the translation medium (lane T'). The reactions with cellular contaminants were insensitive to serum preincubation (lanes 4' and H'). Unlike the other two NSPs, P3 reached its maximum concentration in the Pe-30 very early in infection (lane 2) and disappeared rapidly thereafter (lanes 3 to 8).
Table 1. Absolute amounts of viral proteins and replicase activity at 25 °C and at 10 °C*

<table>
<thead>
<tr>
<th>Protein assayed</th>
<th>Amounts of indicated viral protein after infection at</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>25 °C</td>
</tr>
<tr>
<td>Replicase (unit/g)</td>
<td>7.0</td>
</tr>
<tr>
<td>P1 (ng/g)</td>
<td>18</td>
</tr>
<tr>
<td>(pmol/g)</td>
<td>0.14</td>
</tr>
<tr>
<td>P2 (ng/g)</td>
<td>10.5</td>
</tr>
<tr>
<td>(pmol/g)</td>
<td>0.12</td>
</tr>
<tr>
<td>P3 (ng/g)</td>
<td>7</td>
</tr>
<tr>
<td>(pmol/g)</td>
<td>0.22</td>
</tr>
<tr>
<td>CP (~tg/g)</td>
<td>440</td>
</tr>
</tbody>
</table>

* Plants of uniform size were inoculated and kept either at 25 °C for 48 h or at 10 °C for 170 h. The NSPs were assayed in 30 μl of Pe-30 and the coat protein (CP) was assayed in 2 μl of crude extract (before 30000 g centrifugation), essentially as in Fig. 4 to 7. Replicase activity was assayed in 10 μl of Pe-30 and is expressed as pmol UMP incorporated in 1 h. The results are normalized to 1 g of leaves.

Fig. 5 shows the results of quantifying the immunoblots, and presents the kinetics of accumulation of the NSPs compared with the replicase activity of the immunoblotted samples (Fig. 5b; see also Le Roy et al., 1977). The curves are plotted as percentage of the maximum to facilitate comparison, and it is evident that the accumulation of both P1 and P2 closely followed the replicase activity whereas the accumulation of P3 did not. As late as 200 h post-infection leaves still contained a significant amount of P1, P2 and replicase, but very little P3 remained. P2 seemed to be synthesized slightly earlier than P1. The difference, although small, was also observed at 10 °C, as shown later.

From calibrations such as those shown in Fig. 3, the maximum amounts of P1, P2 and P3 in a volume of Pe-30 corresponding to 1 g of leaves were about 50 ng of P1 (0.4 pmol) and 30 ng of P2 (0.33 pmol) after 70 to 80 h of infection, and about 6 ng of P3 (0.2 pmol) after 45 h. The accuracy of these determinations was about 15% (Fig. 5) and the results obtained with different batches of plants were within a factor of two. Variations seemed to be related to the age of the plant: infection of very young leaves proceeded slightly faster and yielded larger amounts of the NSPs than that of fully-grown leaves.

**Time course of accumulation of the viral proteins at 10 °C**

When the inoculated plants were kept at 10 °C, small white lesions appeared on the inoculated leaves 7 days after infection but no symptoms were seen on the upper leaves at 15 days post-infection. Coat protein was undetectable in the S-30 of the upper leaves (not shown) although it was made in the inoculated leaves. It took about 10 times as long at 10 °C as at 25 °C for a given amount of coat protein to be made (Fig. 6a).

The NSPs were assayed in the Pe-30 as in the preceding experiment. As shown in Fig. 6(b, c), all three were synthesized and their accumulation proceeded much faster than expected from the rate of coat protein synthesis. P2 was first detectable at 66 h post-infection, whereas P1 and P3 appeared at 96 h. They accumulated for the following 4 to 5 days and reached their maximum at about 200 h after infection (Fig. 7a). The decay of the NSPs was much slower at 10 °C than at 25 °C, but the difference seen at 25 °C between P1 and P2 on the one hand, and P3 on the other, was also apparent at 10 °C.

Replicase activity (Fig. 7b) followed closely the amount of P1 throughout infection, and that of P2 after 150 h. (A significant amount of P2 was found as early as 66 h post-infection without detectable replicase.) As at 25 °C, there was no correlation between replicase activity and the amount of P3.

To compare the absolute amounts of replicase and NSPs at the two temperatures, plants from one inoculated batch were kept either at 10 °C or at 25 °C. The leaves were harvested when replicase activity was 50 to 60% of the maximum, i.e. after 48 h at 25 °C or 170 h at 10 °C. Table 1 shows that the absolute amount of replicase was nearly the same at the two temperatures and
Fig. 6. Detection of AlMV proteins in infected plants kept at 10 °C. Inoculated leaves were collected after different times at 10 °C (66, 96, 120, 152, 200, 224, 272, 296 and 345 h post-infection respectively in lanes 1 to 9), as well as comparable leaves from healthy controls (lane H). They were processed as in Fig. 4. (a) is equivalent to Fig. 4(a). In (b) the Pe-30 samples were separated in a 6% gel and immunoblotted with a mixture of the anti-pep1 and anti-pep2 sera, both diluted 5000-fold. In (c) the Pe-30 samples were separated in a 9% gel and immunoblotted with a mixture of the anti-pep2 serum (diluted 10000-fold) and anti-pep3 serum (diluted 2500-fold).
that the amounts of the three NSPs were slightly higher at 10 °C than at 25 °C whereas the amount of coat protein was sevenfold less at the lower temperature than at the higher temperature.

**Distribution of P1 and P2 among the subcellular fractions**

In the preceding experiments, accumulation of the NSPs was examined in a crude membrane fraction (Pe-30) from which large organelles had been eliminated. We verified that this fraction, which contains most of the viral replicase (Le Roy et al., 1977), also contains the major quantities of P1 and P2, by testing amounts of the low-speed pellet (Pe-1) and of the ammonium sulphate-fractionated S-30 equivalent to 400 μl of S-30. Neither protein could be detected in any of the supernatant fractions, but 20 to 30% of P1 and P2 were consistently found in the Pe-1 throughout infection, both at 25 °C and at 10 °C. This could probably be accounted for by cross-contamination of the different types of membranes.
DISCUSSION

This work shows that antibodies raised against synthetic C-terminal peptides can detect minute amounts of the homologous protein containing that peptide by immunoblotting. As pointed out by Westhof et al. (1984), the hydrophilicity of the peptide may not be very important and indeed, of the three that we have used, only one, pep2, was strongly hydrophilic (compare Fig. 1 of Berna et al., 1984, and Fig. 1 of the present paper). Nevertheless, all three peptides were strongly immunogenic and the antisera obtained detected P1, P2 and P3 with similar efficiencies (Fig. 3). It was fortunate for us, in fact, that the antisera made against peptides derived from the sequences of RNAs 1 and 2 of strain 425 (Cornelissen et al., 1983a, b) recognized P1 and P2 of strain S.

The relative amounts of a given viral protein in the different lanes of an immunoblot were easy to quantify. However, the absolute amounts were more difficult to evaluate. The estimations relied on the use of radioactive translation products as standards, and it was implicitly assumed that these products had exactly the same C-termini as the natural proteins. We observed, however, that the reaction of the P1 standard with the anti-pep1 serum weakened significantly over a period of a few months without loss of radioactivity other than that due to the decay of $^{35}$S. This could have resulted from the breakdown of a few amino acids at the C-terminus. Even though freshly prepared standards were used for calibration, the amount of P1 in leaves would have been overestimated if such breakdown occurred during in vitro protein synthesis.

Our major finding is that the amounts of P1 and P2 in a crude membrane fraction containing the replication complex were correlated with replicase activity throughout infection, both at 25 °C and 10 °C, but that the amount of P3 was not. This lends support to the idea that P1 and P2 (but not necessarily P3) are part of the replication complex. The fact that P2 was found early after inoculation at 10 °C, when neither replicase nor P1 could be detected, does not contradict this statement: it is conceivable that both P1 and P2 are required for replicase activity, and P1 may have been limiting. Maximum replicase activity, as well as the maximum amounts of P1 and P2, were nearly the same at the two temperatures (possibly a little higher at 10 °C). Thus, 10 °C is not a restrictive temperature for the assembly of a functional replication complex. This step of AIMV replication may be equivalent to the 2-thiouridine-sensitive (or 10 mM-guanidine-sensitive) step of TMV replication (Dawson & Schlegel, 1976; Dawson, 1976) which is known to take place at 12 °C. The viral protein which is most affected by temperature is the coat protein: it takes seven to ten times as long at 10 °C as at 25 °C to obtain a given amount of coat protein in the inoculated leaves, whereas the same temperature drop only slows down the accumulation of NSPs by three- to fourfold. This could reflect the smaller proportion of RNA 4 in the viral RNA mixture synthesized at the lower temperature (Franck & Hirth, 1976). In addition, the lower amount of coat protein may indirectly favour the synthesis of NSPs by slowing down encapsidation of the genomic RNAs.

The infections that we have followed were obviously non-synchronous and therefore cannot be interpreted in terms of a single infection cycle. What we measured was the balance between the synthesis and the decay of viral proteins. Synthesis of the NSPs predominated only for a limited period, during which the infection was spreading from cell to cell as evidenced by the enlargement of the symptoms. Later (presumably after all the competent cells had been reached by infection), decay took over and the NSPs gradually disappeared from the crude membrane fraction, P3 more rapidly than the other two. Our finding that P3 reaches its maximum concentration sooner than P1 and P2 at 25 °C may be explained in two ways: either P3 is synthesized very transiently (this is the case for the 30K protein of TMV; Watanabe et al., 1984) whereas the cell keeps making P1 and P2 for several days, or P3 decays faster than the other two.

Several explanations can be proposed to account for the apparent decay of the NSPs. They could be cleaved by proteases or otherwise become unrecognizable to our antibodies. If proteolytic degradation were taking place, we would only be able to detect the pieces with an intact C-terminus, and indeed some of our immunobLOTS show faint bands which could correspond to a C-terminal fragment of P2 (Fig. 6c, open arrowhead). Alternatively, some NSPs could migrate to a different subcellular compartment. Our search for P1 and P2 in the low-speed
pellet and in the high-speed supernatant did not give any evidence for this. The fate of P3 will be dealt with in a future report.

We thank M.-J. Gagey for excellent technical assistance, M. Meyer for taking care of the rabbits, M. Le Ret for amino acid analysis and C. Hubert for photography. This work was supported in part by grant 84V 0813 from the Ministère de la Recherche et de la Technologie (Biotechnology Program).

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(Received 23 December 1985)