Synthesis of Proteins in Tobacco Protoplasts Infected with Brome Mosaic Virus

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SUMMARY

The proteins synthesized in protoplasts infected with strain V5 of brome mosaic virus have been studied. Four new proteins of mol.wt. 2, 3.5, 10 and 10.7 × 10^4 were observed. These account for over 90% of the virus genome. The three proteins with lowest mol.wt. corresponded closely in size to those observed in protoplasts infected with cowpea chlorotic mottle virus.

INTRODUCTION

Synchronously infected protoplasts are useful in the study of the appearance of virus-specific proteins during infection. Sakai & Takebe (1972, 1974) and Paterson & Knight (1975) have, for example, described three proteins, including coat protein, which are produced specifically in tobacco protoplasts infected with tobacco mosaic virus (TMV). Sakai et al. (1977) have reported three new proteins, including coat protein, synthesized in tobacco protoplasts infected with cowpea chlorotic mottle virus (CCMV), one of the bromoviruses. The bromoviruses are multicomponent systems (Lane, 1974), typically having four major RNA species of which the three largest are essential for infection (Lane & Kaesberg, 1971; Bancroft & Flack, 1972). The fourth, smallest RNA codes in vitro for coat protein and may be the intracellular coat messenger, although the gene for the coat is on RNA 3 (Lane & Kaesberg, 1971; Davies & Kaesberg, 1974). The three proteins described by Sakai et al. (1977) would account for all the RNA in the CCMV genome except for an amount equivalent to the largest component (RNA 1). We report here some experiments with brome mosaic virus (BMV) which show that with this virus four proteins are synthesized in infected protoplasts, so accounting for virtually all the virus genome.

METHODS

Protoplasts were prepared from leaves of Nicotiana tabacum cv. White Burley (Motoyoshi et al. 1974b). They were irradiated with u.v. light before inoculation, to reduce the level of host protein synthesis, using a 7W Hanovia model 16 lamp; 5 min exposure at a distance of 10 cm from the lamp (about 2 × 10^4 erg/mm^2) was normally given (Sakai et al. 1977). The protoplasts were then inoculated with a strain of BMV (V5) which infects tobacco (Motoyoshi et al. 1974a) using an inoculum of 10 μg BMV and 1 μg poly-L-ornithine (mol. wt. 120000; Sigma London Chemical Co. Ltd) per ml of 0.7 M-mannitol containing 0.01 M-potassium citrate (pH 5.2). After inoculation, the protoplasts were washed with 0.7 M-mannitol and cultured by the method of Motoyoshi et al. (1974b).

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G-\(^{14}\)C-L-leucine (specific radioactivity 300 mCi/mmol) or \(4,5\)-H-L-leucine (specific radioactivity 50 Ci/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks. and used at 1 and 20 μCi, respectively, per ml medium.

Typically, 5 ml of culture containing \(10^6\) protoplasts was incubated at 25 °C in a 25 ml Erlenmeyer flask. The percentage infection was determined after 24 or 48 h using fluorescent antibody staining (Motoyoshi et al. 1973). When time-course experiments were done 0.5 ml samples were taken at suitable times, the protoplasts were spun down (600 rev/min for 2 min) and dispersed by heating for 2 to 3 min at 100 °C in 0.2 ml of a solution containing 2 % sodium dodecyl sulphate, 2 % mercaptoethanol, 15 % glycerol, 0.002 % bromophenol blue in 0.05 M-tris-HCl, pH 6.8 (Laemmli, 1970; Paterson & Knight, 1975). This solution was used without further treatment for polyacrylamide gel electrophoresis. When quantitative counting of gel slices was used, uninfected protoplasts were labelled with \(^{14}\)C-leucine and infected ones with \(^3\)H-leucine; the protoplasts were mixed after sampling, the proteins extracted and then subjected to electrophoresis on cylindrical polyacrylamide gels (7 to 9 %). The gels were cut transversely into 1 mm-thick slices which were digested in hydrogen peroxide and ammonia (Goodman & Matzura, 1971) and then counted in a liquid scintillation counter (Sakai et al. 1977). Autoradiographs were prepared as below after slicing the gels longitudinally into 1.5 mm-thick sections (Watts et al. 1977).

The amounts of the high mol.wt. proteins were at the lower limit of detection for direct counting of gel slices. Gel slabs were therefore run using the method of Laemmli (1970); a 2 cm stacking gel (4 % acrylamide) and 10 cm running gel (12.5 % acrylamide) proved satisfactory.

Autoradiographs were prepared by drying the gels on to sheets of 3MM Whatman filter paper. Fluorography was used to detect \(^3\)H; the gel was infiltrated with diphenyloxazole in dimethylsulphoxide before drying down on filter paper (Laskey & Mills, 1975). The dried gels were applied to Kodak RP Royal X-Omat film and exposed at \(-20\) °C for periods up to 14 days before development (Bonner & Laskey, 1974).

The relative amounts of radioactivity in the different proteins were determined from the absorbances of the bands on the autogelogram, measured with a double-beam recording microdensitometer (Joyce-Loebl Co., Ltd, Mark IIIIB). When wide ranges of density were measured, two periods of exposure (7 and 14 days) were used to avoid saturating the film.

RESULTS

The distribution of radioactivity in the proteins was first measured quantitatively using cylindrical gels that were sliced transversely and counted by liquid scintillation. Fig. 1 shows a result obtained with proteins from protoplasts 24 h after infection with BMV. Comparison of the radioactivity in proteins from infected and uninfected material showed that there were three peaks of radioactivity found only in the infected protoplasts which corresponded closely with the three proteins detected in protoplasts infected with CCMV (Sakai et al. 1977). The peak at slice 52 was not reproducible. The mol.wt., determined by comparison with lysozyme oligomers and authentic coat protein were 2, 3, 6 and \(10 \times 10^4\). The amount of radioactivity in the peak with the highest mol.wt. was just within the limit of detectability of the method.

When the distribution of label was examined by autoradiography of cylindrical gels labelled with \(^{14}\)C-leucine, it was found that there was an additional faint band running at a position corresponding to a protein with mol.wt. of about \(1.07 \times 10^5\). A fluorograph of a time-course experiment is shown in Fig. 2. The four bands of radioactivity uniquely present
BMV proteins in protoplasts

Fig. 1. Synthesis of proteins in infected and uninfected, irradiated protoplasts. Irradiated, infected or uninfected protoplasts were grown for 24 h in medium containing $^3$H- and $^{14}$C-leucine, respectively. The protoplasts were then mixed and proteins were prepared, separated on 7.5% cylindrical gels and the relative amounts of $^3$H and $^{14}$C determined on 1 mm slices. Migration is from left to right. The protoplasts were 69% infected 20 h after inoculation.

Fig. 2. Fluorograph of proteins from infected and uninfected protoplasts separated on a slab gel. Irradiated, infected or irradiated, uninfected protoplasts were grown in medium containing $^3$H-leucine. Samples were taken at intervals, the proteins were prepared, and separated on a 12.5% slab gel. A fluorograph was then prepared. Pairs of samples (infected sample on the right) are marked with the time (h) from inoculation. The protoplasts were 51% infected 24 h after inoculation.
Fig. 3. Time-course of synthesis of virus-specific proteins in irradiated, infected protoplasts. The experiment is that illustrated in Fig. 2. The density of the bands P1, P2, P3 and P4 was measured and plotted in arbitrary units. ●, P1; ○, P2; △, P3; ▲, P4. Left-hand logarithmic scale refers to P1, right-hand linear scale to P2, 3 and 4.

in the infected samples can be readily observed in the later samples (16 h or more after infection). Fig. 3 is derived from microdensitometry traces of fluorographs of the gel used to produce Fig. 2. It shows that the smallest protein, P1 (coat protein), was synthesized over many hours at rates approaching logarithmic, but the 35000 mol.wt. protein (P2) reached a maximum about 18 h after infection and thereafter remained constant or fell slowly. In contrast, the two proteins with mol.wt. around 10⁶ were apparently synthesized in an approximately linear fashion closely parallel to each other with about twice as much label in the smaller molecule.

When the proteins were fractionated in a tris-phosphate buffer, the coat proteins of CCMV and BMV ran according to mol.wt. and could be resolved from each other, with CCMV coat protein migrating faster than that of BMV, but proteins P2 and P3 of the two viruses could not be resolved. If, however, tris-glycine was used as the electrode buffer and tris-HCl as the gel buffer (Laemmli, 1970), coat proteins and P2 proteins of the two viruses ran in different positions; the BMV proteins now ran ahead of the CCMV proteins, showing that the two viruses code for different proteins.

**DISCUSSION**

These results are in excellent agreement with those obtained using virus RNA as messenger in a cell-free extract of wheat germ (Shih & Kaesberg, 1973, 1976; Davies & Kaesberg, 1974) although the BMV was not the strain V5 used in this work. Shih & Kaesberg (1973) showed that *in vitro* BMV RNA 3 codes for a protein of mol.wt. 35000 and RNA 4 codes for coat protein. Later work with fractionated BMV RNA (Shih & Kaesberg, 1976) showed
that RNA 1 and 2 code for proteins of mol.wt. 1.2 and $1.1 \times 10^5$ respectively; these are rather larger than our estimates but well within the limits of uncertainty of the techniques used.

It is reasonable to assume, therefore, that the three proteins found in protoplasts infected with CCMV (Sakai et al. 1977) represent the gene products of RNA 3 and RNA 2. The fourth protein with mol.wt. $1.1 \times 10^5$ found in protoplasts infected with BMV corresponds to the gene product of RNA 1 which was not detected in CCMV infection. It would require virtually the whole of RNA 1 and 2 to code for the two largest proteins, while the two smallest proteins (P1 and P2) account for most of RNA 3; at most there would remain a section of RNA 3 equivalent to a protein of mol.wt. $2 \times 10^4$ not accounted for. RNA 4 appears to be the messenger for coat protein (Shih & Kaesberg, 1973; Davies & Kaesberg, 1974) but the gene for coat protein is on RNA 3 and RNA 4 is not required for infection (Lane & Kaesberg, 1971; Bancroft & Flack, 1972; Bancroft & Lane, 1973). The four proteins therefore account for over 90% of the genome of BMV.

Caution is needed when interpreting the kinetics of incorporation of radioactivity because of uncertainties about the specific radioactivities of pools; however, some comments seem in order. The functions of three of the proteins (P2, P3, P4) are not known. It has been proposed, with little supporting evidence, that the $3.5 \times 10^4$ mol.wt. protein is a sub-unit of the virus replicase (RNA-dependent RNA polymerase; Hariharasubramanian et al. 1973). The kinetics of synthesis of P2 certainly fit such a role, with a peak about 20 h after infection, suggesting close regulation of synthesis of a protein with intracellular function. The functions of P3 and P4 are quite unknown; even genetic studies have not satisfactorily attributed a role to RNA 1, although RNA 2 mutants have been associated with changes in the character of local lesions on assay plants both with BMV and CCMV (Bancroft & Lane, 1973). The kinetics of synthesis of P3 and P4 are similar to each other but very different from those of coat protein and P2; they cannot strictly be termed late proteins because they are in evidence almost as early as coat protein. They are synthesized more or less linearly with time and do not reach a maximum until very late in infection (more than 50 h). The amounts synthesized are very small compared with coat protein. If we assume the same proportion of leucine in all four proteins, then the numbers of molecules of each at 30 h after infection are in the ratios P1:P2:P3:P4 = 180:11:2:1. Since RNA 2 is implicated in lesion character, it is possible that the $1 \times 10^5$ mol.wt. protein has a role in facilitating the transmission of infection from cell to cell in the plant. The absence of P4 from CCMV infections may indicate that there is a qualitative difference in function compared with BMV, at least in tobacco and wheat, since the in vitro system also fails to give clear-cut evidence of a specific product of the RNA 1 of CCMV; it is however possible that it was not resolved or was below the level detectable by these methods.

REFERENCES


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