Characterization of Araujia Mosaic Virus by In Vitro Translation Analyses

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ABSTRACT

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Araujia mosaic virus (AjMV), previously described as a possible new potyvirus group member, was partially purified and compared further with other potyviruses by in vitro translation analyses. Isolated AjMV RNA was translated in a rabbit reticulocyte lysate system, and the translation products were tested for their relatedness to other potyviral proteins by immunoprecipitation tests followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Two AjMV RNA translation products, with estimated molecular weights of 49,000 daltons (49kd) and 53kd, were similar in size and serological reaction to tobacco etch virus (TEV) nuclear inclusion proteins. Antiserum to TEV capsid protein reacted with the presumed AjMV capsid protein while antiserum to TEV cylindrical inclusion protein did not react with any of the AjMV translation products. Antiserum to dasheen mosaic virus cylindrical inclusion protein reacted with the presumed AjMV cylindrical inclusion protein produced in vitro. Antiserum to tobacco vein mottling virus helper component protein reacted with an 81kd AjMV translation product. A proposed gene order of translation for AjMV genome is as follows: 5′ end 81kd helper component-related protein—49kd protein—40kd protein—70kd cylindrical inclusion protein—53kd protein—32kd capsid protein—3′ end. The results presented here provide further evidence that AjMV is a distinct member of the potyvirus group.

Additional key words: potyvirus purification, gene order of translation map.

Araujia mosaic virus (AjMV) was tentatively described as a new member of the potyvirus group (4) on the basis of particle morphology, aphid vector transmission, and the induction of characteristic cylindrical inclusions in infected tissues. Extracts of AjMV-infected leaf tissue did not react in immunodiffusion tests with capsid antisera to 13 different potyviruses (4). The serological relationship of AjMV to other potyviruses remained unresolved due to the lack of a homologous antiserum. Information about this relationship is of interest in viral classification and is necessary in the evaluation of AjMV as a biocontrol agent for certain pestiferous members of the Asclepiadaceae (eg, milkweed vine).

Although we have been unable to produce a specific, high-titer antiserum to AjMV because of difficulties in the purification of the virus, we have isolated RNA suitable for in vitro translation studies from partially purified AjMV preparations. The AjMV RNA translation products were analyzed and compared by immunoprecipitations and sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) to a number of other potyviruses. Certain products of AjMV RNA translation were serologically related to three distinct gene products (one capsid protein and two nuclear inclusion proteins) of tobacco etch virus (TEV). In addition, certain AjMV products were also found to be related to dasheen mosaic virus (DMV) cylindrical inclusion protein and to tobacco vein mottling virus helper component protein (16). Products of AjMV translation did not react with complete homology with all of the antisera available from any one of ten different potyviruses.

MATERIALS AND METHODS

AjMV was cultured in *Morrenia odorata* and *Araujia sericofera* by mechanical transmission (4). The virus culture was maintained under quarantine in a greenhouse.

Virus purification. One hundred grams of infected *Morrenia* and *Araujia* (leaf tissue) harvested 8–10 wk after inoculation, were homogenized in 200 ml of cold 20 mM HEPES buffer (pH 7.5), 0.5 g of sodium sulfite, 0.04 g of phenyl methyl sulfonate (dissolved in 2.5 ml of dimethyl sulfoxide), 50 ml of chloroform, and 50 ml of carbon tetrachloride. The homogenate was centrifuged at 1,020 *g* for 5 min. Pellets were extracted with 100 ml of buffer (as above) and recentrifuged. The supernatants were combined, made up to 1% Triton X-100 and 5 mM $MgCl_2$, and stirred for 1 hr. The material was centrifuged at 10,400 *g* for 10 min. The supernatant was centrifuged at 90,000 *g* for 1 hr, with 1.5 ml of a 20% sucrose cushion at the bottom of each centrifuge tube. Pellets were resuspended, with the aid of a tissue grinder, in a small volume of buffer containing 5 mM $MgCl_2$ and 0.1% mercaptoethanol. The resuspended material (3–6 ml per tube) was layered on top of a Cs_2SO_4 (1.28 g/cm^3) solution (5 ml per tube, Beckman SW 41 rotor) and centrifuged at 180,000 *g* for 16 hr. The virus zone (15–18 mm from the bottom of the centrifuge tube) was collected and diluted with an equal volume of buffer before centrifugation at 12,100 *g* for 10 min. Virions were precipitated from the supernatant by the addition of 6–8% polyethylene glycol 6000 and centrifugation at 12,100 *g* for 10 min. The final pellet was resuspended in 0.5–1.0 ml of buffer or 20 mM tris, pH 8.2.

RNA isolation and in vitro translation. AjMV RNA was isolated from partially purified virus by the dissociation of the virions with 100 mM ammonium carbonate (pH 9.0) containing 1 mM EDTA and 1% SDS (2) followed by rate zonal density gradient centrifugation in linear log sucrose gradients (3). The 39 S RNA was collected and precipitated by the addition of sodium acetate (pH 5.0) at a final concentration of 100 mM, and two volumes of 100% ethanol. The RNA was resuspended in a small volume of H_2O and stored at −85 C. Translation in the mRNA-dependent reticulocyte lysate was done as previously described (5). Lysate was obtained from Green Hectares, Oregon, WI 53575.

Analysis of the translation products. Analyses of the translation products by SDS-PAGE were performed as previously described

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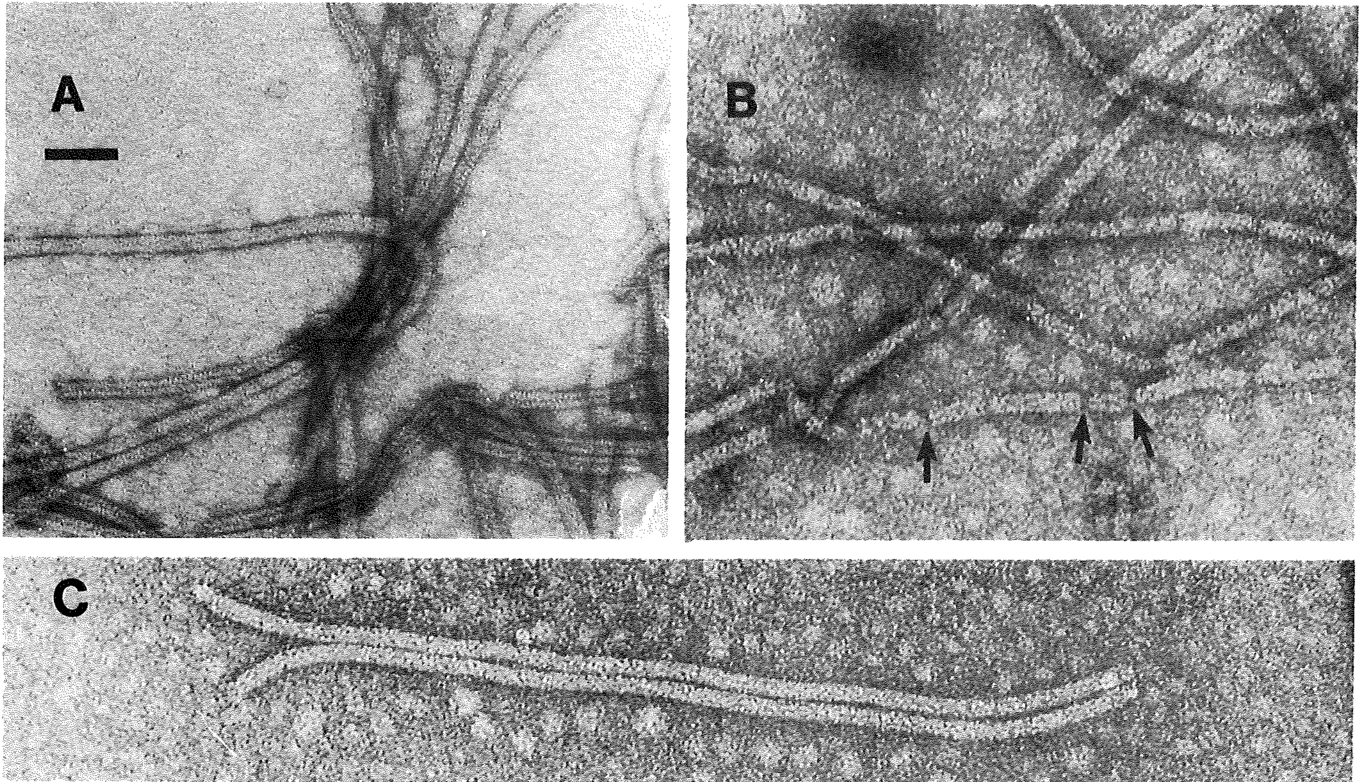

Fig. 1. Electron micrograph of Araujia mosaic virus (AjMV) particles: **A,** immediately after purification without $MgCl_2$ in the buffer; **B,** after storage for 3 days at 4 C; and **C,** after purification with $MgCl_2$ and storage at 4 C for 5 mo in 20 mM tris (pH 8.2). The particles were negatively stained with uranyl acetate. The arrows in **B** point to some apparent discontinuities in a virus particle. The protein subunit detail visible in **A** may be due to weak bonding between the AjMV capsid protein subunits. Bar represents 55.5 nm.

(5,6). The immunoprecipitation procedure has been described (6,9,10).

Antisera to tobacco vein mottling virus (TVMV), potato virus Y (PVY) helper component (HC) protein, and to extracts from noninfected tissue prepared by the procedure to purify HC protein were kindly provided by Thornbury and Pirone (16). Antisera to the TEV nuclear inclusion protein have been described by Dougherty and Hiebert (6). Antiserum to TVMV antiserum was obtained from G. V. Gooding, NC State University, Raleigh. Antiserum to DMV cylindrical inclusion protein was provided by F. W. Zettler, University of Florida, Gainesville. Antiserum to AjMV capsid protein was prepared by immunization of a rabbit with SDS-PAGE purified AjMV capsid protein according to procedures described elsewhere (10).

Electron microscopy. Samples from purified virus preparations were negatively stained with 2% uranyl acetate and viewed with a Hitachi H-600 electron microscope.

RESULTS

Virion purification. Stability of the virions appeared to be one of the problems in AjMV. Virions purified in the 20 mM HEPES buffer without $MgCl_2$ showed unusual protein subunit detail (compared to other potyviruses) when viewed by electron microscopy (Fig. 1A). After storage for 3 days at 4 C, the virions showed marked fragmentation and disintegration (Fig. 1B). Virions purified in the presence of 5 mM $MgCl_2$ are shown in Fig. 1C. In two trials, yields of virions were 2–3 times greater when $MgCl_2$ was present in the buffer compared to purification without $MgCl_2$. In addition, virions purified in the presence of $MgCl_2$ were more soluble after Cs_2SO_4 density-gradient centrifugation. Yields of up to 3.5 mg per 100 g tissue were obtained by using buffer containing $MgCl_2$.

Another problem encountered in AjMV purification was the loss of virions if polyethylene glycol 6000 precipitation was attempted immediately after tissue homogenization in buffer without $MgCl_2$ and clarification. Therefore, we had to concentrate the virions by

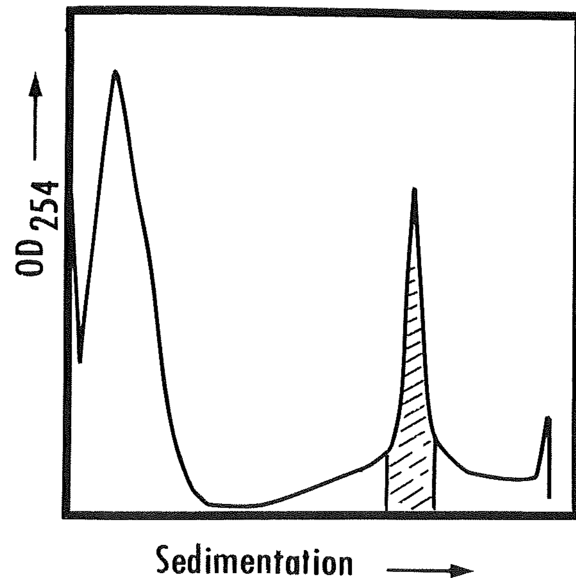

Fig. 2. Sedimentation profile of AjMV RNA after the dissociation of partially purified virus preparation in a SDS-ammonium carbonate-EDTA (pH 9.0) solution and centrifugation in a linear-log sucrose gradient at 260,000 *g* for 5 hr at 14 C. The figure illustrates the optical density measured at 254 nm versus depth in the gradient tube. The shaded area under the main peak represents the RNA collected for use in translation experiments.

high-speed centrifugation (90,000 *g* for 1 hr) at this stage. Interestingly, polyethylene glycol precipitation of the virions after Cs_2SO_4 density gradient centrifugation did not appear to be detrimental.

Contamination of the virus preparations was indicated by the number of protein bands detected when they were analyzed by SDS-PAGE (*unpublished*). There were several bands around

24,000 daltons (24kd) to 32kd and several at the 14–18kd range. An attempt to prepare antisera specific to AjMV capsid protein, presumed to be in the 28–32kd range, by preparatory electrophoresis (10) resulted in an antiserum which reacted indistinguishably with healthy and with infected tissue extracts in immunodiffusion tests (*unpublished*). However, the antiserum was suitable for in vitro translation product analysis (see below).

RNA isolation. The ammonium carbonate-SDS-EDTA dissociation procedure, followed by fractionation on linear-log sucrose gradients, was suitable for the isolation of AjMV RNA (Fig. 2). The large amount of ultraviolet absorption at the top of the gradient (Fig. 2) may represent nonvirion material in the virus preparations used for RNA isolation.

In vitro translation. The RNA isolated from virions purified in the presence of $MgCl_2$ was a more efficient messenger than that isolated from virions purified in the absence of $MgCl_2$. The AjMV RNA stimulated the synthesis of products in the rabbit reticulocyte lysate up to 22× the endogenous levels.

The analysis of the products of AjMV RNA translation by immunoprecipitation and SDS-PAGE is shown in Fig. 3. The primary product of AjMV RNA translation had an estimated size of 81kd (Fig. 3, lane b). This product reacted with the antiserum to TVMV-HC protein (Fig. 3, lane g), but not to PVY-HC protein (Fig. 3, lane h). Antiserum to AjMV capsid protein immunoprecipitated a product around 32kd (presumed to be the capsid protein), 28kd (presumed to be a premature termination of the capsid protein gene) and a number of products ranging from 50 to 100kd in size (Fig. 3, lane c). A major immunoprecipitation product at 85kd (Fig. 3, lane c) was also immunoprecipitated by antiserum to TEV 54kd nuclear inclusion protein antiserum (Fig. 3, lane d). The TEV 54kd nuclear inclusion protein antiserum immunoprecipitated a number of products ranging from 50 to 100kd in size (Fig. 3, lane d) and a product around 300kd. Antiserum to the TEV 49kd nuclear inclusion protein immunoprecipitated several products around 50 and 160kd (Fig. 3, lane e). Antiserum to DMV cylindrical inclusion protein immunoprecipitated a product around 70kd (presumed to be the AjMV cylindrical inclusion protein subunit) and major products at 100, 110, and 160kd (Fig. 3, lane f). The 160kd product reacted with both the 49kd nuclear inclusion protein and the DMV cylindrical inclusion protein antisera. Products reacting with more than one antiserum may be due to "readthroughs" of adjacent genes on the AjMV genome during in vitro translation. Antiserum to AjMV cylindrical inclusion protein has not been prepared.

The products of AjMV RNA translation were tested for serological relatedness with antisera to other potyviral-specified proteins. The results are given in Table 1. Among the serological combinations tested, there was no complete homology with all the available antisera to the viral-specified proteins of any one potyvirus. For example, AjMV products reacted efficiently with three of the four available antisera to TEV-specified proteins, and with the two available TVMV antisera. However, AjMV products did not react with pepper mottle virus cylindrical inclusion protein antiserum which does react efficiently with TVMV translation products (E. Hiebert, *unpublished*).

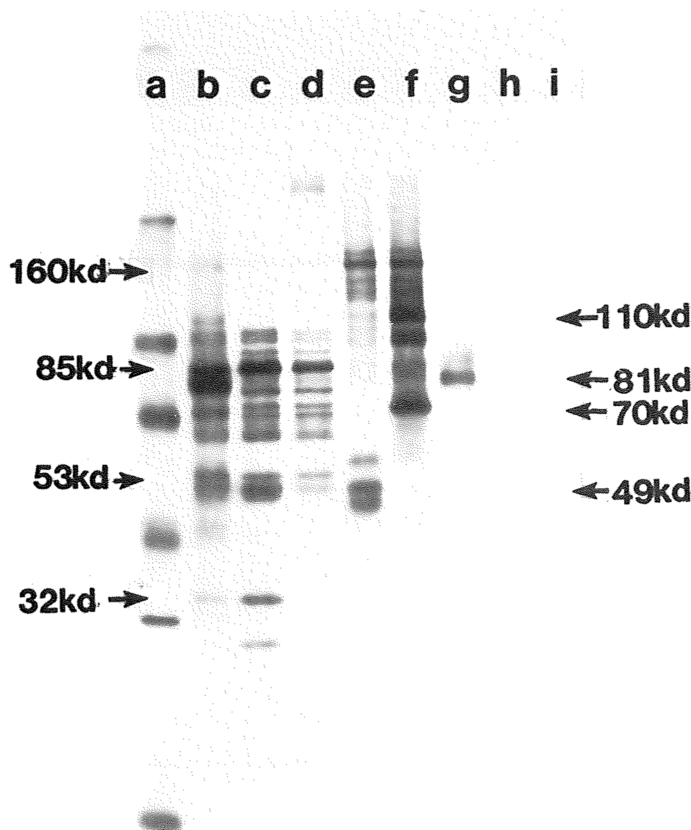

Fig. 3. Analysis of the in vitro translation products of AjMV RNA by immunoprecipitation and SDS-PAGE. The figure illustrates ^{35}S methionine-labeled products detected by fluorography. Lane a—^{14}C-labeled molecular weight markers from the top: myosin, 200,000 daltons (200kd); phosphorylase-b, 93kd; serum albumin, 67kd; ovalbumin, 43kd; carbonic anhydrase, 29kd; and lysozyme, 14.3kd. Lane b—AjMV total products. AjMV products immunoprecipitated by antibodies to AjMV capsid protein (lane c), to tobacco etch virus (TEV) 54kd nuclear inclusion protein (lane d), to TEV 49kd nuclear inclusion protein (lane e), to dasheen mosaic virus cylindrical inclusion protein (lane f), to tobacco vein mottling virus helper component protein (lane g), to potato virus Y helper component protein (lane h), and to extracts from uninfected tissue prepared by a procedure to purify helper component protein (lane i). Molecular weights of some of the major translation products are given on the sides of the figure.

DISCUSSION

The serological relationships of AjMV-specified proteins to the proteins of a number of different potyviruses have been evaluated.

TABLE 1. Serological reactivities of Araujia mosaic virus cell-free translational products with antisera to various potyviral-specified proteins

Antiserum to:	Reaction[a]
Bean yellow mosaic virus cylindrical inclusion protein (gladiolus isolate) (12)[b]	−
Dasheen mosaic virus capsid protein (1)	+
Dasheen mosaic virus cylindrical inclusion protein[c]	++
Pepper mottle virus cylindrical inclusion protein (15)	±
Potato Y virus helper component protein (16)	−
Soybean mosaic virus capsid protein[d]	++
Soybean mosaic cylindrical inclusion protein[d]	+
Tobacco etch virus capsid protein (5)	++
Tobacco etch virus 49kd nuclear inclusion protein (6)	++
Tobacco etch virus 54kd nuclear inclusion protein (6)	++
Tobacco etch virus cylindrical inclusion protein (6)	−
Turnip mosaic virus cylindrical inclusion protein (Florida isolate) (11)	−
Tobacco vein mottling virus capsid protein[e]	++
Tobacco vein mottling virus helper component protein (16)	++
Watermelon mosaic virus 1 capsid protein (13)	+
Watermelon mosaic virus 1 cylindrical inclusion protein (13)	±
Watermelon mosaic virus 2 capsid protein (13)	++
Watermelon mosaic virus 2 cylindrical inclusion protein (13)	±

[a]++ = strong reaction, + = weak reaction, ± = very weak reaction, and − = no reaction detected. Serological reactions were determined by immunoprecipitation of the in vitro translation products of Araujia mosaic virus RNA tested with various antisera. The serological reactions were evaluated by the intensity of the immunoprecipitated product(s) compared with the product(s) in the nonimmunoprecipitated product lane, in the fluorograms developed after SDS-PAGE.

Sources of the antisera are as follows:
[b]Numbers in parentheses are Literature Cited reference numbers.
[c]F. W. Zettler, University of Florida, Gainesville.
[d]E. Hiebert and D. E. Purcifull (*unpublished*).
[e]G. Gooding, North Carolina State University, Raleigh.

Three genetic products of AjMV RNA (representing ~38% of the potential genetic information) were serologically related to the coat protein and the two nuclear inclusion proteins of TEV. The cylindrical inclusion protein of AjMV (representing ~22% of the potential genetic information) was serologically related to DMV cylindrical inclusion protein, but not to TEV cylindrical inclusion protein. A distinct 81kd product (representing ~25% of the potential genetic information) of AjMV RNA translation was serologically related to TVMV-HC protein, but not to PVY-HC protein. Among the potyviral antisera tested in this report, the serological relationship of AjMV cylindrical inclusion protein to those of other potyviruses was not correlated with a corresponding relationship of the respective capsid protein. For example, TEV and soybean mosaic virus (SMV) capsid protein appeared to be closely related to AjMV capsid protein, but the TEV and SMV cylindrical inclusion proteins were not related and weakly related, respectively, to AjMV cylindrical inclusion protein. The serological comparisons listed in this report do not nearly cover all the possible combinations that could be tested among the large potyviral group. However, we believe the results provide further evidence that AjMV is a distinct member of the potyvirus group.

AjMV was difficult to purify and study serologically in relation to other potyviruses (4). Antiserum prepared to AjMV capsid protein reacted with healthy as well as AjMV-infected tissue in immunodiffusion tests (*unpublished*). We have been unable to purify the AjMV cylindrical inclusion protein using procedures (10) developed for other potyviral cylindrical inclusion proteins (*unpublished*). This report demonstrates that in vitro translation analyses of a viral RNA can be used in characterization and viral relationship studies in which conventional techniques have failed. In addition, the availability of serological probes for five different potyviral gene products (representing ~85% of the potential genetic information) made the in vitro translation product analyses very useful for studies of viral relationships among the large potyvirus group.

Serological reactions were not detected previously (4) in unilateral immunodiffusion tests with leaf extracts from AjMV infected *M. odorata* by using capsid protein antisera showing positive reactions in this report. The immunoprecipitation technique, used here, with labeled proteins is more sensitive than the SDS-immunodiffusion technique for detecting serological reactions (E. Hiebert, *unpublished*). The lack of a serological reaction in the unilateral serological tests (4) may also have been due to insufficient antigen in the tissue extracts. The components of tissue extracts of *M. odorata* give nonspecific precipitates in SDS-immunodiffusion tests and this may have obscured a positive reaction.

The addition of $MgCl_2$ to the buffer for AjMV purification stabilized the virions, increased the virus yield, improved virion solubility during purification, and improved the quality of isolated RNA. We have not evaluated the optimum concentration of $MgCl_2$ for AjMV purification. Divalent cations have been considered as agents in causing aggregation of potyviruses, thus as being detrimental in virion purification. For certain viruses (such as watermelon mosaic virus 2) a chelating agent such as ethylenediaminetetraacetate (EDTA) appears to be beneficial for purification and for the reduction in virion aggregation (E. Hiebert, *unpublished*). For watermelon mosaic virus 1, however, the use of EDTA is detrimental (E. Hiebert, *unpublished*). It is our opinion that $MgCl_2$ should be considered for potyviral purification where virion fragmentation is an obvious problem.

The improvement in RNA quality isolated from virions purified in the presence of $MgCl_2$ was evident in template activity and in the production of large products during in vitro translation analysis. RNA isolated from virions purified in the absence of $MgCl_2$ had low template activity (50% less) and produced insignificant amounts of capsid protein, cylindrical inclusion protein, and products with molecular sizes >100kd during in vitro translation (*unpublished*). This was consistent with our previous report (7) which showed that fragmented potyviral RNA has low template activity and produces only small products.

Based on the analysis of the in vitro translation products of AjMV RNA and the similarity of these results to other potyviral RNA translations (7,8), a gene order of translation map for the AjMV RNA is proposed (Fig. 4). Details for the basis of the gene order on the potyviral genome have been described elsewhere (7). Some of the AjMV translation products cross-reacting with more than one specific antiserum (Fig. 3), illustrated below the map (Fig. 4), were used to link genes on the map. For example, an 85kd product of AjMV reacted with both the capsid protein and TEV 54kd nuclear inclusion antisera and thus appeared to be similar to the 85kd TEV translation product. The latter has been shown by serology and peptide mapping to consist of nuclear inclusion proteins (7, and E. Hiebert, *unpublished*). No probe exists for the third gene on the map nor has this gene product been identified in vivo for any potyvirus. The genetic map accounts for most, if not all, of the genetic information in the AjMV genome.

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AjMV
5' 81kd 49kd 40kd 70kd 53kd 32kd 3'
160kd 85kd
110kd

TEV
5' Vpg 87kd 49kd 50kd 70kd 54kd 31kd poly A 3'
Helper Component
Nuclear Inclusion
Cylindrical Inclusion
Nuclear Inclusion
Capsid

Fig. 4. Proposed gene order of translation for AjMV RNA. The molecular weights of the gene products are presented above the map. Some of the products reacting with more than one antiserum are given below the map. These products were used in linking genes on the map. The proposed gene order of translation for TEV RNA is illustrated below the AjMV RNA map for comparison. The third gene from the left has not yet been associated with any virus-specified protein found in vivo. Details of the TEV genome have been described elsewhere (14).

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