Isolation of Replicative Intermediates during Brome Mosaic Virus (BMV) Development.

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Summary: BMV development was studied in well defined physiological conditions, avoiding the use of antibiotics or excision of the roots. For an adequate labelling, to distinguish between host and viral RNAs, different periods of exposure to ^{32}P were required, depending upon the post-inoculation time of infected plants and corresponding age of healthy control. Within the period of normal outlook of the plants, the rate of ^{32}P incorporation into BMV RNA was much higher than into barley ribosomal RNA in infected tissue or in comparable healthy tissue.

Analysis of the replicative structures isolated from infected plants revealed that only the three largest RNAs (A,A',B) had their own replicative intermediates (R I). This material was shown to contain a certain amount of intact viral RNA species, which were stable when treated with heat and formamide, excluding the possibility of hidden breaks.

Introduction

BMV is one of the smallest known viruses with a multipartite genome[1] whose genetic information is distributed among five types of RNA components.[2] We designate the RNA components as A,A',B,C and D in order of decreasing size. The infectivity properties only reside among the three largest RNA components which are present in three types of structurally similar virions. An overall picutre of BMV multiplication and replication *in vivo* is still rather confusing. Hiruki[3] and Semal and Kummert[4] found only one replicative form for BMV, Lane and Kaesberg[2] observed five replicative forms corresponding to the five single stranded RNAs. Kummert and Semal[5] *in vitro* and Philipps et al[6] *in vivo* found two well defined peaks of double stranded RNAs, corresponding to the three largest RNAs. Plant virology is beset with a number of inevitable problems. We investigated the BMV – development in natural conditions. We avoided the use of actinomycine D, which effects variably from inhibition to stimulation in the multiplication of certain viruses.[7,8,9] The roots were not excised as uptake of radioactivity through cut leaves may lead to artefacts.[10,11,12].

Materials and Methods

Seeds of barley (Hordeum vulgare) var. Herta were germinated.[13] An overlapping set of experiments was carried out in chronological order, in principle before the outbreak of visible symptoms of virosis. Manually wounded healthy barley and BMV-inoculated barley were compared in most cases,[13] since an enhanced ^{32}P incorporation into host cellular RNA by wounding the tissue was observed.[12] The nucleic acids of the infected (or healthy) plants were labelled by uptake of carrier – free ^{32}P through the roots.

RNA was isolated by an optimized method, main features have already been described.[14]

High molecular weight nucleic acid was fractionated by 2.4% polyacrylamide gel electrophoresis[15] and/or formamide gel electrophoresis. To prepare 5% formamide slab gels: 100 ml of formamide was stirred in 4 grams of mixed bed ion-exchange resin of coarse mesh (Amberlite) for 2 hours (h) for deionization. The formamide was then filtered twice through filter paper. 0.57 g of Na_2HPO_4 $12H_2O$, 0.053 g of Na $H_2PO_4H_2O$ (formamide – buffer) was added to 100 ml of deionized formamide. To 75 ml of this solution 2.55 g of acrylamide monomer, 0.45 g of N,N-methylene – bisacrylamide, 0.75 ml of ammonium per-sulphate (0.1g/ml), 150 μl of N,N,N',N'-tetramethylethylene diamine were added. The ethanol-precipitated pellets were dissolved in deionized formamide, and after addition of sucrose (final concentration 6%) and 5 μl of 0.022% bromophenol blue, layered on slab gels in a non-aqueous formamide buffer. 0.02M phophate buffer pH. 7.5 was used as electrophoresis buffer.

After electrophoresis, polyacrylamide gels were sliced and radioactivity was measured[13]. The formamide slab gels were run for specified time at 100 V and then autoradiographed.

Detection of Labelled RNA by Trichloroacetic acid (TCA) precipitation

The RNA was precipitated with 15 volumes of 10% TCA for half an hour at $0^{o}C$, and filtered off on a Whatman GF/C glass filter. This was successively washed twice with TCA and twice with ethanol. The filter paper was dried under Infra-Red light and then the radioactivity on each filter was determined.

Results and Discussion

In order to follow the role of intermediates *in vivo*, the material investigated ranged from the same day-infected leaves labelled for a certain period, to the leaves harvested late in infection.

Different rates of the ^{32}P incorporation were observed in healthy and infected plants, depending upon the time and duration of labelling. The rate of ^{32}P incorporation in case of healthy tissue was most significant, when plants were labelled during a period when host-rRNAs are actively being synthesized, e.g. in younger leaves.[12] In later stages of leaf development, the rate of ^{32}P-incorporation into host-rRNAs decreased, with concomitantly increased rate of viral RNA synthesis (Fig. 1). This can be further seen in Table 1, where the acid-insoluble material, before first ethanol precipitation, and normalized to 5mCi of ^{32}P in each batch of plantlets, is shown as a function of labelling time and period Cerenkov radiation before TCA-precipitation represents the total uptake of the label in each case. After TCA-precipitation, Cerenkov or liquid scintillation counts refer to incorporation of label in acid precipitable nucleic acids. Our results indicate that the uptake of ^{32}P becomes much less efficient in sick plants, i.e. late in infection, as compared to healthy control (Table 1). When the material extracted from such plants, is subjected to acid precipitation, a larger proportion of precipitable radioactive label is observed in infected plant extracts, reflecting a higher incorporation of ^{32}P into viral RNA species. This is in agreement with the finding of Philipps et al[16], in case of BMV-barley and Pring[17] in case of Barley Stripe Mosaic Virus-Barley system. This interpretation is further supported by the fact, that incorporation of ^{32}P in host rRNAs is higher after a long labelling period, in case of healthy barley than in the virus infected one (Fig. 2). This severe decrease of the rate of ^{32}P incorporation into host RNA in the inoculated leaf is indicative of the irreversible changes occurring in the tissue[17]. These changes may

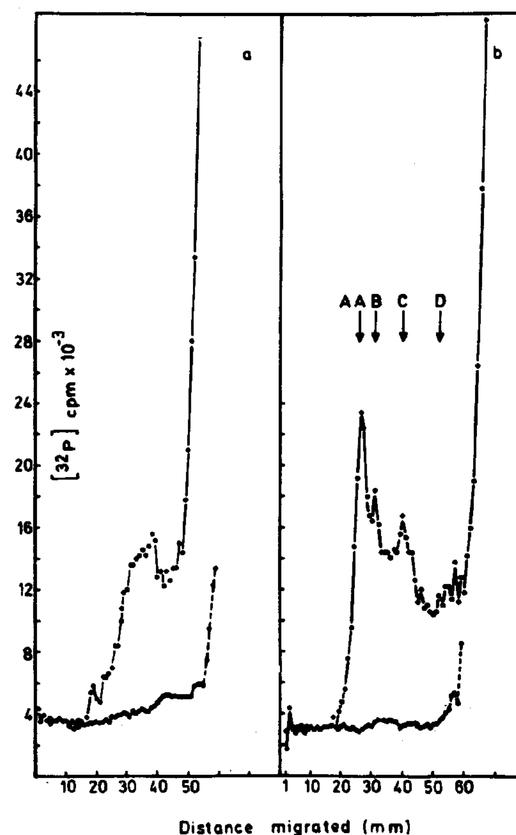

Fig. 1 Electrophoretic analysis of differential labelling in case of wounded control plantlets.

The deproteinized material was extracted from 2.985 g of freshly harvested barley. The RNAs were labelled with 5 mCi of ^{32}P for 26 h after 4 days of inoculation, either with (b) or without (a) virus-containing suspension. A sample in 50 μl (one-sixth of total extract) was used for loading. Electrophoresis in 2.4 % polyacrylamide gel was performed at 5 mA per gel for 135 min. The material was from

(a) Manually wounded barley.

(b) Manually BMV-inoculated barley.

O- - -O Untreated extract

O- - - -O Extract in STME buffer (0.1M NaCl, 0.005 Tris, 0.001 M $NaCl_2$, 0.0001 M EDTA, pH 7.0) treated with 3 U of T_1-RNase (15 min at $28^{o}C$) and with 1 μg ofDNase (30 min) at $0^{o}C$.

Table 1. Trichloroacetic acid precipitation scheme. cpm [^{32}P] per mg of leaf.

Infection time before labelling (days)	Duration of of labelling with 5 mCi ^{32}P (h)	Uninfected tissue			Infected tissue		
		Before TCA precipitation	After TCA– precipitation		Before TCA precipitation	After TCA precipitation	
		Cerenkov counting	Cerenkov counting	+LS counting	Cerenkov counting	Cerenkov counting	LS counting
Zero	24	17233	157	1657	57893	543	6550
Zero	24	59553	590	8440	56263	1323	18117
Zero	48	62717	570	7603	116050	1620	18520
Zero	48	42020	1290	18210	74890	980	15517
2	24	–	–	–	54633	673	8000
2	48	–	–	–	82550	1017	12467
2	48	39493	920	11930	83437	2303	33453
2	72	–	–	–	85637	1177	14537
2	120	116243	1880	24517	–	–	–
4	26	156593	397	1783	55297	717	3030
5	40*	17820	120	1230	25890	300	3240
6	36	65417	297	1567	19690	447	2677
6	36	70300	207	1163	20707	327	1940
7	24	63600	210	2617	14563	73	870
7	40*	5717	107	323	2133	80	263
8	48	8057	53	663	12727	63	830
·6	96	67200	297	1667	47357	483	2337
9.5	36*	11613	37	130	4607	33	167

Asterisks: cases where low specific activity of [^{32}P] was used.
+LS: Liquid Scintillation.

reflect a selective degradation of the host rRNAs, as Hirai and Wildman[18] observed a reduction in ^{32}P labelling of 16 S and 23 S chloroplast (Ch) RNA in tobacco, infected with TMV-vulgare, while the 18 and 25 S cytoplasmic (Cy) RNA species readily incorporated ^{32}P. Fraser[19] found a similar effect with TMV-vulgare, while a severe strain (TMV-flavum) inhibited ^{32}P incorporation into rRNAs of both cytoplasmic and chloroplastic nature.

Such detrimental changes might occur right after the onset of infection, since we observed even in freshly infected tissue[13] a profile between Ch_1 and Cy_2 which did not fit into our tabulated R_M-values of either host rRNA or viral RNAs. Such a peak with R_M 1.2 – 1.3, relative to the heavy cytoplasmic (Cy_1) ribosmal RNA peak taken as 1, was also observed by Philipps et al[16], without further emphasis. There is no obvious reason for ^{32}P incorporation in a new discrete species of viral or host cellular RNA. A degradation product with a rather resistant core of reproducible size would equally fulfill the conditions for well-resolved migration position.

We have produced the evidence for two classes of double stranded RNA molecules (RF) and a true precursor role was attributed to a heterodisperse material, the replicative intermediate (RI) in BMB-barley system.[13] We clearly observed this heterogeneous RNA, refer to it as replicative structures (RS), during the replication cycle at an early time[13], when the BMV-induced RNA polymerase activity is at its maximum rate.[20] This was further supported by experiments with long-term radioactive labelling (Fig. 2), where ^{32}P-incorporation into both strands would be expected even if there

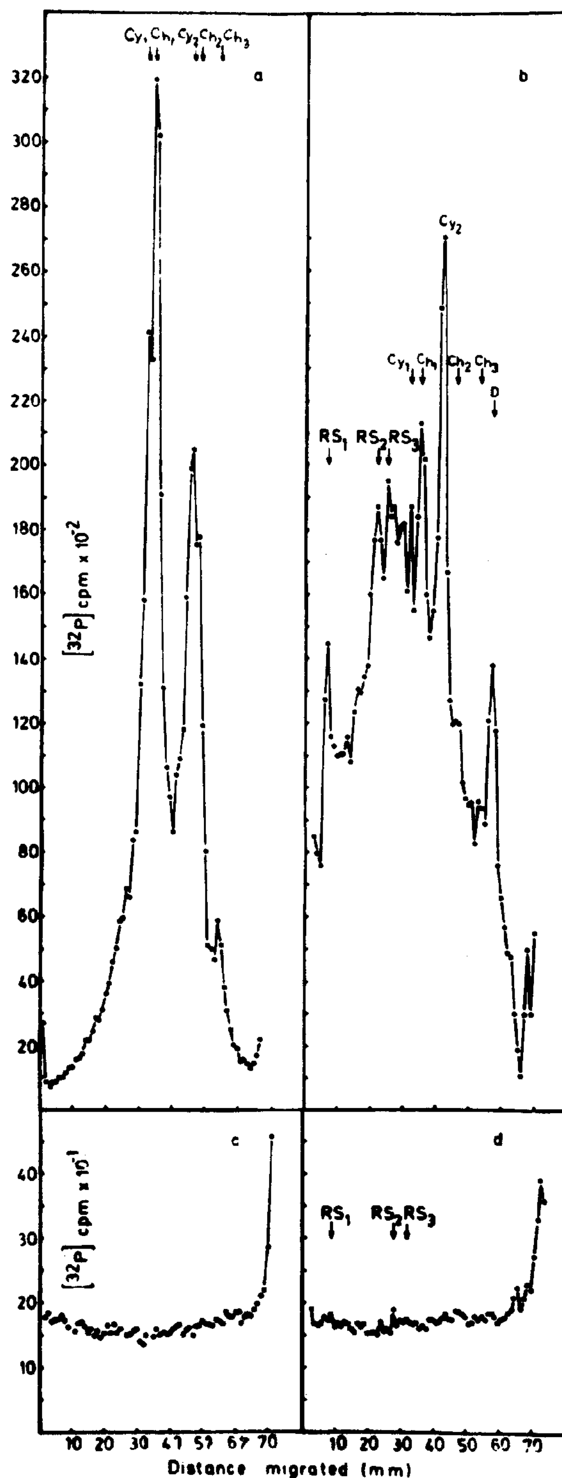

Fig. 2. Results of long time labelling.

Electrophoretic pattern of nucleic acids extracted from 4.54 g of freshly harvested barley leaves.

Labelling with 2.5 mCi of ^{32}P was started 6 days after inoculation and lasted for 4 days.

Samples of 50 μl in 2 SSC (0.15 M NaCl, 0.015M Sodium citrate, pH: 7.0). containing 0.3% SDS (one-sixth of total extract) were used. Electrophoresis was carried out in 2.4% polyacrylamide gel at 5 mA per gel for 3.5 h in (a) and (b); for 4.5 h in (c) and (d).

(a) and (c) Mechanically wounded barley.

(b) and (d) Mechanically BMV-inoculated barley.

(a) and (b) Treated with 1 μg of DNase for 30 min at 0.°C.

(c) and (d) Treated with 3 U of T_1-RNase for 30 min at 30°C and subsequently with 1 μg of DNase for 30 min at 0°C.

Only the positions of the host rRNA have been marked in (b) to justify the standard Cy_1 for the determination of R_M-Values for replicative structures, although viral SS RNAs coexist dominantly in nearly the same position. The positions of RS_1, RS_2 and RS_3 in (d) correspond with those in (b), with respect to their R_M-values.

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was largely asymmetric synthesis during replication[21]. We consistently found three distinguished replicative structures with Relative mobility (R_M) values 0.22, 0.69 and 0.78 respectively. This material was not present in the corresponding healthy control. It gave a similar pattern after DNase treatment. When the material was digested with RNase (Fig. 2d), three very faint peaks were found with a relatively high underlying background. If present at all, the fully double stranded profiles corresponding to RS_1, RS_2 and RS_3 certainly have lower activity than their "RS" counterparts. As the radioactively labelled heterodisperse RNA species show up more clearly after a longer labelling time than after a short one, it reflects the slow synthesis and release of viral RNA strands.

Since the background radioactivity makes it improbable to assign variation in activity to the presence of replicative structures, the material of the zone of "RS" was electrophoretically eluted after a run of 8 hours at 5 mA/gel. The eluted material was then coprecipitated with yeast RNA and was analyzed electrophoretically on 5% formamide gels supported on 10% polyacrylamide gels.

The autoradiographs of such material shows three distinct bands corresponding to A, A, and B components as envisaged by R_M-values of standard BMV-RNA, which are the only infectious components[2]. These retained their identity even after incubation at 100°C for 3 minutes (Fig. 3) excluding the possibliity of hidden breaks. An estimation of the molecular weight of replica-

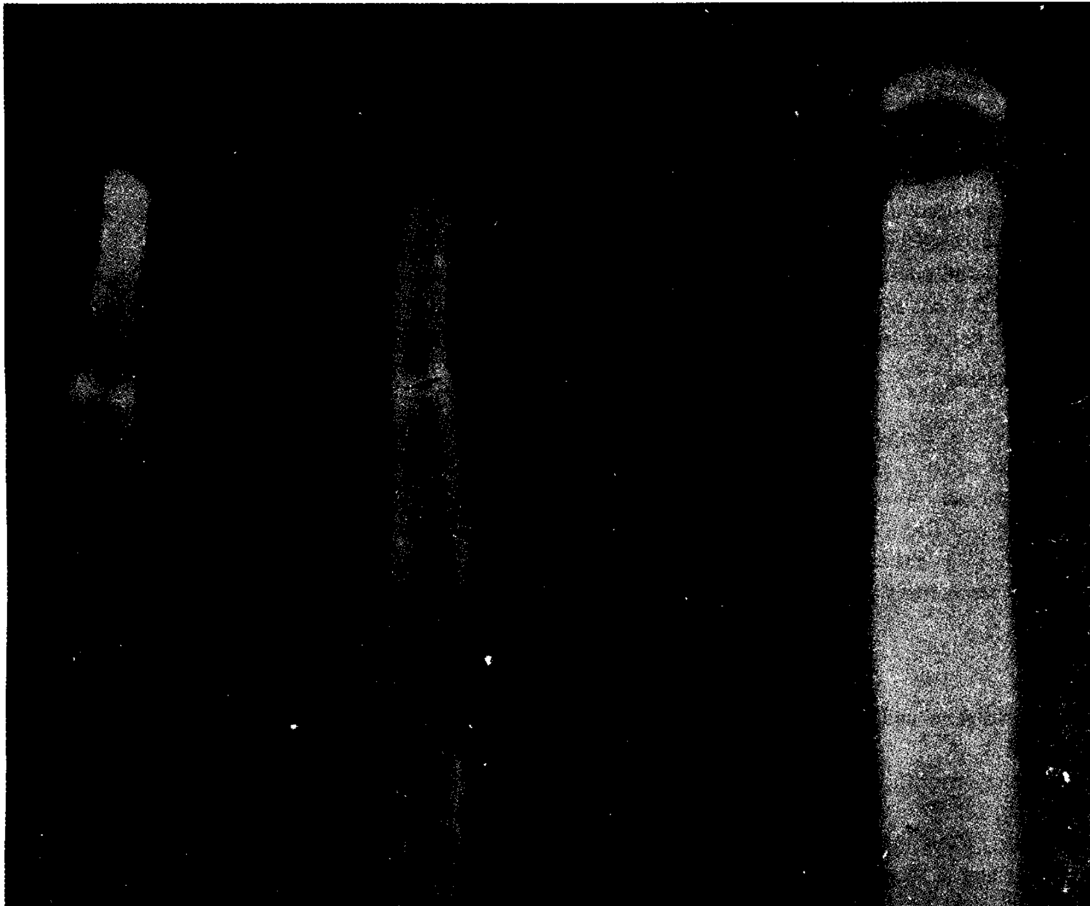

Fig. 3. Stability of intact viral RNA species in formamide gel.

The deproteinized extract from 4 grams of freshly harvested barley, which were labelled for 48 h after 4 days of inoculation. The nucleic acids (¼th of total) were first fractionated on 2.4% polyacrylamide gels. The material was eluted from the zone of Replicative Structures, coprecipitated with 84 μg of yeast RNA and then dissolved in 100 μl of deionized formamide. The material was fractioned on 5% formamide slab gels. Electrophoresis was performed at 100 volts for 8 h.

(a) and (c) Manually BMV-inoculated barley.

(b) and (d) Manually wounded barley.

(a) and (b) Material (½ of the eluted) treated with formamide for 3 minutes at room temperature.

(c) and (d) Material (¼ of the eluted) incubated with formamide for 3 minutes at 100°C.

(e) ^{32}P-labelled MS2 RNA in 50 μl of formamide as external marker.

tive structures was not performed, since single stranded (SS) markers could not be used for this purpose. The use of DNA or double stranded RNA as marker is inconvenient, since the structure of double stranded material is highly dependent on experimental conditions.

Attempts to anneal the freshly synthesized material, either to its endogenous complement or to cold viral RNA mostly remained unsuccessful. We could not find the replicative intermediate (s) for C and D components. Therefore, it can be inferred that C and D are generated from large components by specific cuts by some cellular or viral induced endonucleases.

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