Effect of Ethidium Bromide and Acridine Orange on the In Vitro Synthesis of Bromegrass Mosaic Virus RNA

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ABSTRACT

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The effect of ethidium bromide and acridine orange on the in vitro synthesis of bromegrass mosaic virus (BMV) RNA was investigated by measuring the incorporation of [^{3}H] uridine-5′-monophosphate into BMV-RNA replicative form by a crude RNA polymerase preparation from infected barley leaves. At the concentration of 100 μg/ml, both dyes strongly inhibited the synthesis of the "plus" strand of BMV-RNA. Using the same experimental system, formycin B at the concentration of 100 μg/ml had no effect, although it is a very active inhibitor of BMV synthesis in vivo. Ethidium bromide also inhibited the in vitro synthesis of cellular single-stranded RNA by a comparable fraction from healthy leaves.

In order to investigate the effect of potential inhibitors on the replication of bromegrass mosaic virus (BMV), we have developed a technique based on the study of a specific step of BMV synthesis; i.e., the in vitro transcription of viral RNA. In previous work (4, 9) we have shown that a crude extract from barley leaves infected with BMV contains a specific RNA polymerase activity which catalyzes the synthesis of the "plus" genomic strand of BMV-RNA on a "minus" strand template, the deproteinized product of the replication being mainly in the form of double-stranded viral RNA (replicative form).

In previous experiments using this system, we have shown that blasticidin-S which inhibits the in vivo synthesis of BMV when applied to inoculated leaves, did not interfere with the in vitro synthesis of BMV-RNA (5). In the present paper, we show that the dyes ethidium bromide and acridine orange which are known to inhibit the in vitro synthesis of DNA-dependent RNA, also inhibited the RNA-dependent synthesis of BMV-RNA when mixed with the crude RNA polymerase preparation from BMV-infected leaves.

MATERIALS AND METHODS

Barley seedlings were inoculated with BMV at the one-leaf stage (3). At 4 days after inoculation, the second leaf was harvested and used as a source of crude RNA polymerase preparation. The leaf extract that sedimented between 1,000 *g* for 5 min and 10,000 *g* for 10 min constituted the active fraction which was resuspended in incubation buffer pH 8.6 (4), mixed with [^{3}H] UTP (uridine-5′-triphosphate), actinomycin D (20 μg/ml) unless otherwise stated, and the necessary ingredients for RNA synthesis. Inhibitors were added to the mixture at the concentration of 100 μg/ml. The active fraction then was incubated for 10 min at 30 C, followed by deproteinization with phenol and SDS. In some experiments, ethidium bromide was added to the active fraction in incubation buffer, and the mixture was centrifuged at 10,000 *g* for 10 min; the supernatant liquid was discarded and the pellet was resuspended in incubation buffer (either with or without addition of 50 μg/ml of BMV-RNA) and was treated as above to promote the labeling of RNA.

In a first series of experiments, the deproteinized RNA was precipitated twice from the water phase by mixing with two volumes of ethanol, and the final product in 2× SSC (SSC = 0.15 M NaCl, 0.015 M Na acetate, pH 7.0) was either incubated or not with RNase (5 μg/ml for 30 min at 37 C). The material then was mixed with carrier protein, brought to 5% trichloracetic acid, and filtered through Millipore membranes HAWP 025. The acid-insoluble radioactivity was measured in a Nuclear Chicago spectrometer (8). Nonincubated zero-time aliquots were deproteinized immediately upon mixing the active fraction with the various ingredients for RNA synthesis; the mean zero-time values were substracted from 10 min values to obtain net counts per min.

In a second series of experiments, the water phase after deproteinization of the active fraction was passed through a G-50 Sephadex column (4), in order to better separate the RNA product from the nonincorporated

nucleotide precursors. The RNA material collected from the front of elution was precipitated with ethanol, resuspended in 2 × SSC, and either treated or not treated with RNase. It then was mixed with carrier protein and acid precipitated as described above. In some experiments, the RNA obtained after filtration through G-50 Sephadex was submitted to electrophoresis in polyacrylamide gels as described by Kummert (4).

RESULTS

A screening of potential inhibitors of the in vitro synthesis of RNA-dependent RNA was performed, using labeled RNA prepared by two successive ethanol precipitations (Table 1, exp. A_1 and A_2). Under these experimental conditions, although zero-time incorporations were rather high (250-300 cpm/min), it was apparent that ethidium bromide and acridine orange notably inhibited the incorporation of radioactivity into RNA, while formycin B had little effect.

In experiments using Sephadex G-50 filtration (Table 1, exp. B_1-B_4), zero-time values were lower 20-50 cpm), thus giving more accurate results. The inhibitory effect of both acridine orange and ethidium bromide on the labeling of RNA by the active fraction from BMV-infected barley leaves was confirmed, together with the lack of activity of formycin B. Experiments B_3 and B_4 were performed using the same leaf preparation and represent samples taken after column fractionation, either with (B_3) or without (B_4) subsequent ethanol precipitations. The RNA product labeled by the active fraction from BMV-infected leaves was essentially double-stranded, as indicated by its resistance to RNase. Ethidium bromide also strongly inhibited the labeling of cellular RNA by a comparable fraction prepared from healthy leaves; essentially this RNA was single-stranded, as indicated by its sensivity to RNase in 2 × SSC.

When the active fraction was preincubated with ethidium bromide at 100 μg/ml, centrifuged to eliminate most of the drug, and the washed pellet was resuspended and used as polymerase preparation, RNA labeling was less inhibited than with the standard incubation carried out in the presence of the dye (54% and 58% inhibition instead of 84% and 74%, respectively, for two independent experiments). Addition of BMV-RNA (50 μg/ml) to the incubation buffer of the washed pellet preincubated with ethidium bromide did not affect the

TABLE 1. Effect of potential inhibitors on the incorporation of [^{3}H]-UMP (tritiated uridine-5-monophosphate) into RNA by a cell-free extract from barley leaves either healthy or infected with bromegrass mosaic virus[a]

Experiment	Experimental conditions	Actinomycin D (20 μg/ml)	Inhibitor (100 μg/ml)	Radioactivity into RNA[b]	
				No RNase	RNase[c]
A_1	healthy	+	none	35	35
	infected	+	none	348	270
	infected	+	ethidium bromide	64	24
	infected	+	acridine orange	37	14
	infected	+	formycin B	349	192
A_2	infected	+	none	293	321
	infected	+	ethidium bromide	33	33
B_1	healthy	−	none	1,458	25
	healthy	+	none	90	71
	healthy	−	ethidium bromide	89	19
	healthy	+	ethidium bromide	53	17
B_2	infected	+	none	251	178
	infected	+	ethidium bromide	19	13
	infected	+	acridine orange	11	8
	infected	+	formycin B	202	144
B_3	infected	+	none	955	897
	infected	+	ethidium bromide	206	108
B_4	infected	+	none	476	297
	infected	+	ethidium bromide	69	13

[a]Incorporation of [^{3}H]-UMP into RNA was performed by incubating the crude RNA polymerase fraction for 10 min as described previously (3).

A: the deproteinized RNA was precipitated twice successively with ethanol.
A_1 and A_2: mean values of two replicates
B: the deproteinized RNA was passed through a G-50 Sephadex column and then precipitated with ethanol.
B_1 and B_2: mean values of three replicates
B_3 and B_4: values of individual samples

[b]Results are expressed as acid-insoluble net counts/min/assay.

[c]Product incubated for 30 min at 37 C with 5 μg/ml of RNase in 2 × SSC.

[d]Abbreviations: UMP = uridine-5′-monophosphate; SSC = standard saline citrate buffer containing 0.15 M NaCl and 0.015 sodium citrate, pH 7.0.

level of inhibition (56%, instead of 54% without added RNA).

The analysis of product RNA of experiments B_3 by electrophoresis in polyacrylamide gels (Fig. 1) showed that the labeling of virus-specific RNA, known from previous experiments (4, 8, 9) to correspond to BMV replicative forms, was almost entirely inhibited by ethidium bromide.

DISCUSSION

The lack of effect of formycin B in our experiments was expected because the inhibitory effect of this drug depends on its conversion in vivo into nucleotides which then interfere with RNA synthesis (1).

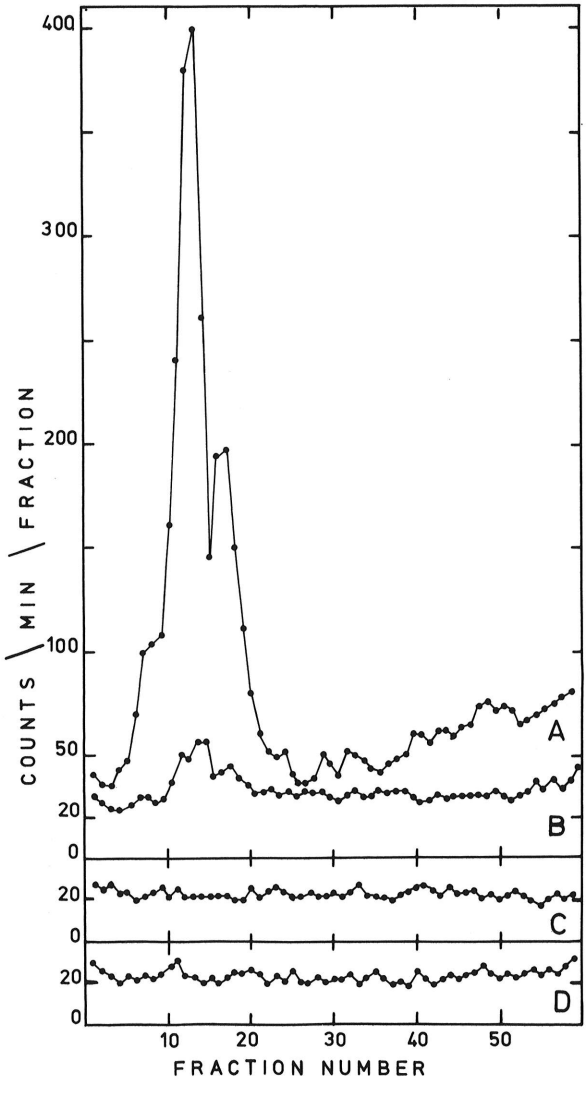

Fig. 1. Effect of ethidium bromide on the in vitro labeling of bromegrass mosaic virus (BMV)-RNA. The crude RNA polymerase preparation from barley leaves was incubated with [^{3}H]-UTP (= tritiated uridine-5′-triphosphate), actinomycin D, and the necessary ingredients for RNA synthesis. The deproteinized RNA was submitted to electrophoresis for 3 hr at 5 mA/tube in 2.4% acrylamide gels (4).

Acridine orange and ethidium bromide strongly inhibited the actinomycin-resistant labeling of RNA in our in vitro system, known from previous experiments to label "plus" strand of BMV-RNA by virtue of a viral RNA polymerase associated with an endogenous "minus" strand template (9). The deproteinized radioactive product thus obtained was mainly integrated into complete double-stranded BMV-RNAs (replicative forms) (4). Under such conditions, labeling is probably the result of the elongation of existing RNA molecules rather than the initiation of new RNA molecules (6). Because of the almost complete inhibition of RNA labeling obtained with ethidium bromide and acridine orange, it appears that both dyes induce an immediate blockage of the enzymic synthesis of BMV-RNA; furthermore, ethidium bromide also was effective in blocking the DNA-dependent RNA polymerase activity of the active fraction prepared from healthy leaves, as assayed without actinomycin D.

Acridine orange and ethidium bromide are known to bind to DNA and RNA. Although binding to DNA has been generally considered to result from the intercalation of dye molecules, association of acridine orange to cellular RNA was thought to result from the binding of aggregated dye (7).

Inhibition of the in vitro activity of phage R 17 replicase by ethidium bromide has been previously described (2). This drug also was shown to inhibit the replication of encephalomyocarditis in L cells, probably by complexing with double-stranded viral RNA (10). Little is known about how the dyes used in this study associate with viral replicative RNAs from plants.

Partial reversibility of the ethidium bromide-induced inhibition of BMV-RNA polymerase activity by removal of the inhibitor, indicates that at least part of its effect does not result from denaturation. A major part of the inhibitory effect was maintained after removal of the drug and washing of the RNA synthesizing fraction; this may be due either to stable binding of ethidium bromide to the replicative complex responsible for the labeling of BMV-RNA, or to irreversible denaturation of this complex. Further analysis of the problem was hampered by the great lability of the RNA-synthesizing complex in extract of BMV-infected leaves.

Because of the efficient inhibition of an already characterized in vitro BMV-RNA synthesis system, ethidium bromide and acridine orange will be useful in studies dealing with the replication of this and other RNA viruses of plants.

The specificity of action of ethidium bromide differs from that of actinomycin D; the latter inhibits specifically the synthesis of RNA on a DNA template, whereas ethidium bromide inhibits both DNA-dependent and RNA-dependent synthesis of RNA. Further work is in progress to find inhibitors of RNA synthesis on an RNA template, that do not inhibit RNA synthesis on a dsDNA template (11).

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