Use of a ^{32}P-Labeled Transcribed RNA Probe for Dot Hybridization Detection of Plum Pox Virus

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ABSTRACT

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Subcloning of an 800-bp *Taq*I fragment derived from the 3′ region of cDNA clone of plum pox virus (strain D) RNA in the *Acc*I site of the transcription vector plasmid Bluescribe (pBS$^+$) generated plasmid pBPPV1. This plasmid served as a template for phage T_7 RNA polymerase to synthesize ^{32}P-labeled complementary RNA (cRNA) copies of the viral RNA, which were used as probes in dot blot hybridization assays. A sensitivity of 4 pg of purified virus (PPV-D) per spot was attained with this type of probe. When the above sensitivity was compared with that of a similar nick-translated DNA probe (100 pg virus/spot) or of enzyme-linked immunosorbent assay (ELISA) (1 ng virus/assay), the RNA probe was, respectively, 25 and 250 times more sensitive. We have compared dot-hybridization with an RNA probe to ELISA in routine indexing of infected apricot trees. Almost 100% of positive samples in ELISA were also positive in hybridization, while 76% of samples negative in ELISA indexed positive by hybridization.

Additional keywords: immunoenzymatic assay, molecular hybridization.

A reliable and sensitive diagnosis of plum pox virus, a potyvirus inducing the plum pox (Sharka) disease of stone fruit trees (10), is of prime importance in countries where a disease control program by eradication and sanitary selection is practiced and in countries where the disease has not been introduced yet, but where severe quarantine measures are taken to avoid introduction. Problems encountered by enzyme-linked immunosorbent assay (ELISA) (1) currently used for virus detection are the uneven distribution of the virus and its low concentration in the infected trees (16).

We recently developed a dot blot hybridization assay using a cloned cDNA representing the 3′ end of the viral RNA (18). This technique proved to be of interest when actual amounts of virus per test were considered. It could detect as low as 100 pg of virus per assay and was therefore more sensitive than ELISA (1 ng/assay). In terms of concentration of the virus in the plant the above sensitivity represents 20 ng/ml as compared to 4 ng/ml for ELISA, because of the respective volumes tested in the two methods (18). Analogous results were also observed for other viruses in detection assays with DNA probes (2,13). To increase the hybridization sensitivity one could concentrate the nucleic acids in the plant extracts or use larger samples, but this often increases the level of nonspecific reactions. It has been reported that RNA probes, synthesized by using transcription vectors (5,11,14) carrying phage RNA polymerase promoters, present several advantages over DNA probes (15,17). As those RNA probes consist of single-stranded RNA copies of one of the strands of the cDNA integrated in the plasmid, there is no loss of activity by self reassociation of the probe during hybridization procedures. The RNA-RNA hybrids are more stable than the DNA-RNA ones and permit post-hybridization washings under more stringent conditions, thus increasing the sensitivity and eliminating the possible nonspecific reactions. In addition, an optional RNAse treatment makes it possible to digest any nonspecifically bound RNA sequences.

In the case of plant pathogens, RNA probes are used currently with good results only for the detection of potato spindle tuber viroid (9). We developed this system for the diagnosis of PPV by subcloning in the transcription vector Bluescribe (pBS$^+$) an 800-bp fragment from the 3′ terminal region of the viral RNA previously cloned in pUC9 (18). The performance of the radioactive-labeled transcript RNA probe was tested with purified virus, infected plant samples from the glasshouse, and with field samples in routine indexing conditions. As low as 4 pg of virus (e.g. 0.2 pg of viral RNA) was detected per assay, which is 25 times more sensitive than with the cloned cDNA probe pPPV9A and 250 times more sensitive than with ELISA.

MATERIALS AND METHODS

PPV strains, plasmids, and bacteria. PPV strain D, an apricot isolate from southeastern France was used for virus cloning. Probes were also tested against strain PPV-M, a peach isolate from northern Greece, belonging to the second virus serological group (8). These virus strains were propagated in *Pisum sativum* 'Express Genereux.' For PPV cloning, plasmid pUC9 and *Escherichia coli* JM103 cells were used (7,18). Subcloning was carried out in the vector plasmid Bluescribe (Stratagene), carrying phage T_3 and T_7 RNA polymerase promoters placed at each side of the multiple cloning site thus allowing synthesis of both plus and minus RNA copies. The plasmids were propagated in *E. coli* strain JM83.

PPV cDNA analysis and subcloning. Recombinant plasmid pPPV30 was purified and the inserted fragment was isolated from it, as already described (18). The insert was mapped by digestion with various restriction enzymes (Boehringer)(12). The enzyme *Taq*I was used to provide a specific fragment that could be directly subcloned in pBS$^+$ by ligation in the *Acc*I site. After *Acc*I digestion, the plasmid was dephosphorylated by using calf intestinal alkaline phosphatase (CIP) to prevent its recircularization during ligation (12). The ligation mixture was used to transform *E. coli* strain JM83 cells (6). Recombinant clones were selected as being β-galactosidase negative and ampicillin resistant. Plasmid pBPPV1 was isolated (3) and characterized by restriction enzyme digestion for the presence and orientation of the insert inside the plasmid.

Probe synthesis and hybridization conditions. Plasmid pBPPV1 was linearized by *Hin*dIII, cutting at a site 18 bases downstream of the insert. After proteinase K treatment to remove all contaminating RNAses, the plasmid preparation could be used for RNA probe synthesis. The Promega Biotec kit (Riboprobe Gemini

System) was used with the phage T_7 RNA polymerase and (^{32}-P) CTP (800 Ci/mmole), as the radioactive precursor. Further treatment with RNAse-free DNAseI degraded the plasmid DNA. The probe was recovered after phenol extraction and ethanol precipitation.

Hybridization conditions (15) were as follows: prehybridization was carried out at 50 C for 3 hr in 50% formamide, 50 mM phosphate buffer, pH 6.5, 5× SSC (1× SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7), 0.1% sodium dodecyl sulfate (SDS), 1 mM ethylenediaminetetraacetate (EDTA), 0.05% bovine serum albumin (BSA), 0.05% ficoll, 0.05% polyvinyl pyrrolidone (PVP), and 200 μg/ml of denatured salmon sperm DNA (1 ml of buffer per 10 cm^2 of membrane). Hybridization was at 50 C for 15 hr with fresh hybridization solution after addition of denatured RNA probe (1× 10^6cpm/ml of hybridization buffer). The membranes were then washed four times, for 20 min, at 65 C in 0.1× SSC, 0.1% SDS. Autoradiography of the membranes was at −80 C for 48 hr using X-ray film (Kodak-XAR) and intensifying screens.

Field indexing trials. They were performed on samples from a small, isolated, naturally infected orchard of southern France.

Experiment 1. Leaves from 10 twigs from an infected apricot tree were individually assayed, giving a total of 241 samples. Symptom-bearing leaves were noted as symptom positive (+).

Experiment 2. One hundred twenty twigs were assayed for each of two infected trees, one with generalized symptoms (No. 2-1), the other with only localized symptoms (No. 2-2). Three leaves per twig constitute one sample. Samples from symptom-bearing twigs were noted symptom positive (+) regardless of the actual presence of symptoms on the tested leaves themselves.

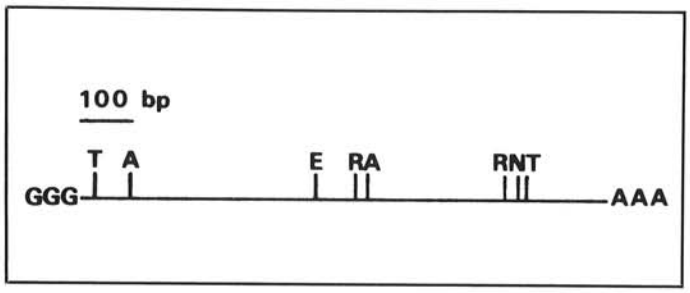

Fig. 1. Restriction endonuclease map of pPPV30 clone. Abbreviations correspond to: A, *Alu*I; E, *Eco*RI; N, *Nde*I; R, *Rsa*I; T, *Taq*I.

Experiment 3. Forty infected trees were assayed by using our standard sampling procedure: Each tree was divided into five areas, three twigs were collected within each area and usually pooled to be tested as one sample, as in experiment 2. Samples coming from symptom-bearing areas of the tree were noted symptom positive (+) regardless of the actual presence of symptoms in the sample. A total of 253 samples were obtained and assayed in this way.

Sample preparation for dot-blotting. Purified virus solutions in 50 mM citrate buffer, pH 8.3, were diluted in 12 × SSC, 6% formaldehyde, and spotted on a 20× SSC saturated nitrocellulose membrane. One-gram pea or peach samples were ground with 4 ml of 50 mM citrate buffer, pH 8.3, and were diluted fivefold in 12× SSC with 6% formaldehyde. From these preparations, 10-μl aliquots were spotted. In the case of apricot samples the extraction buffer was supplemented with 2% PVP, so that the same extracts could also be used in ELISA tests. Membranes were then air-dried and baked under vacuum at 80 C for 2 hr.

Enzyme-linked immunosorbent assay. Two PPV-D antisera prepared in our laboratory were used in double antibody sandwich ELISA. In the tests performed with purified virus, pea, and peach extracts, rabbit immunoglobulins were utilized at 1 μg/ml for coating and the alkaline phosphatase conjugate at a dilution of 1/2,000. One-gram samples were ground in 4 ml of 50 mM citrate buffer and 250 μl were used per well. The SANOFI commercial PPV detection kit based on a goat antiserum to PPV-D was also used (sensitivity of the same order as that of the rabbit reagents), when an apricot orchard of southern France (Montpellier) was indexed. In this case, the grinding buffer also contained 2% PVP. The threshold of detection was established as previously described (8).

RESULTS

Recombinant clone analysis. The cloning of PPV-D cDNA has already been described (18). Clone pPPV30 contains an insert of about 1,000 bases overlapping with clone pPPV9 but extending further to the 5′ region of the viral RNA. Its mapping revealed a *Taq*I site near the 5′ end (Fig. 1).

Subcloning of the *Taq*I fragment of the PPV-D insert. Digestion of the PPV30 insert by enzyme *Taq*I resulted in a fragment of approximately 800 bp lacking the 3′ Poly-A region. The latter may result in nonspecific cross reactions with polyadenylated normal plant RNAs or viral RNAs (18). This 800-bp fragment was then directly subcloned in pBS^+ as described in Materials and Methods, generating pBPPV1. Restriction analysis showed that pBPPV1 contains an insert of the expected size (Fig. 2A), oriented as shown in Figure 2B.

RNA probe synthesis. RNA transcripts complementary to the viral RNA were synthesized by T_7 RNA polymerase, after

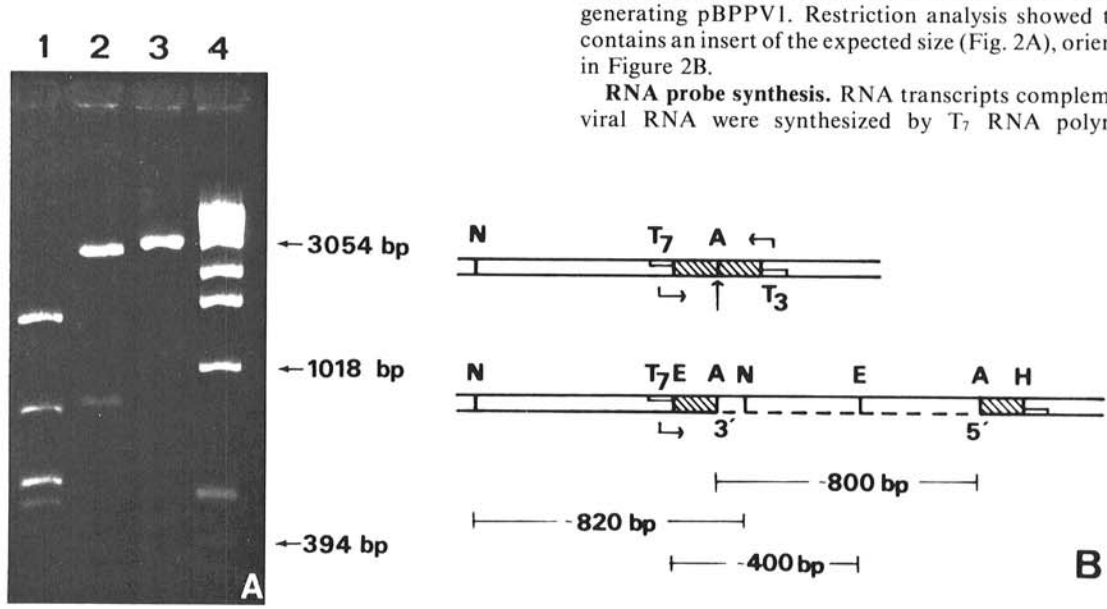

Fig. 2. Insert excision and determination of orientation in pBPPV1. **A,** Digestion with restriction enzymes was followed by electrophoretic separation of the fragments through an 1% agarose gel; pBPPV1 was digested by: lane 1: *Taq*I; lane 2: *Nde*I; lane 3: *Eco*RI; lane 4: 1-kb ladder (BRL). **B,** Orientation of the PPV insert in pBPPV1. ═ pBS^+, ▒▒ polylinker of pBS^+, ═ T_3 or T_7 promoters, ┄┄ insert, ↳ transcription direction, ↑ insertion site. Abbreviations correspond to: A, *Acc*I; E, *Eco*RI; H, *Hind* III; N, *Nde*I; T, *Taq*I.

linearization of the plasmid by *Hind*III downstream of the region corresponding to the 5′ region of the viral RNA. A probe with specific radioactivity of about 4×10^8 cpm/μg was usually obtained, and we calculated that approximately four molecules of transcripts were synthesized from one molecule of plasmid template.

Sensitivity of detection by dot blot hybridization and comparison with that of ELISA. Purified virus of strains D and M, representing the two viral serotypes, was used in dot-blot hybridization to evaluate the sensitivity of the method. In the autoradiograph shown in Figure 3, the last visible spots correspond to 4 pg of PPV-D and 20 pg of PPV-M. The virus being 5% RNA, the above sensitivity corresponds to a detection of 0.2 pg and 1 pg of viral RNA per spot. In terms of concentration, the above values correspond to 0.4 ng/ml (4 pg in a 10-μl spot) of the PPV-D strain and 2 ng/ml of the PPV-M strain. Concentrations detectable by ELISA (sandwich method) are 4 ng/ml (i.e., 1 ng per assay) for the D strain and 16 ng/ml for the M strain, respectively).

Plant extracts were assayed with both methods. The RNA probe detected PPV-D and M in pea at dilution end points of 1:78,125 and 1:15,625, respectively, in peach infected with PPV-D and M, detection was to a dilution of 1:3,125 in both cases (Fig. 4). There was a total absence of nonspecific reactions with the healthy sap extracts. The same samples were assayed in parallel by the ELISA test. Table 1 shows the minimal quantity of plant tissue necessary for detection by ELISA and hybridization with the RNA probe. In all cases, the RNA probe gave a 375 times more sensitive detection. Because the volumes of the samples were different (250 μl for ELISA as compared to 10 μl for molecular hybridization) this still gave a 15-fold greater sensitivity for molecular hybridization, based on virus concentration in sample.

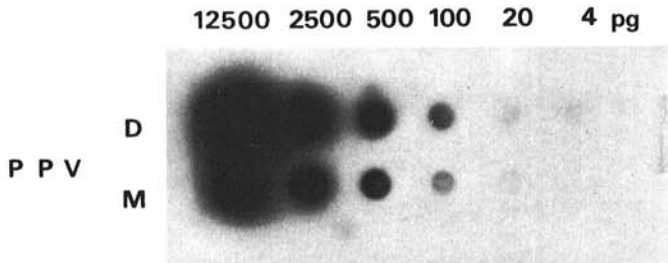

Fig. 3. Detection of PPV-D and -M purified virions by hybridization with the RNA probe synthesized from pBPPV 1. Samples were diluted in 12× SSC, 6% formaldehyde; B, Buffer.

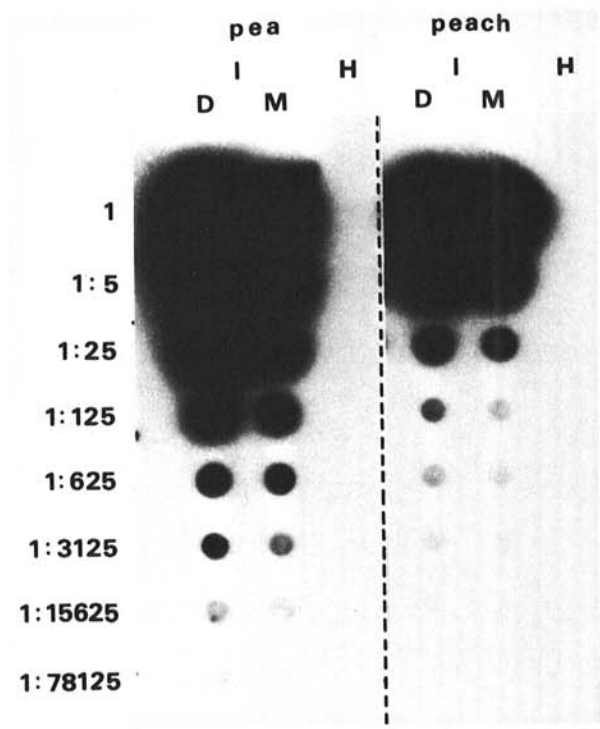

Fig. 4. RNA probe hybridization to crude sap extracts from pea and peach, healthy (H) or infected (I) (D or M strains). Samples were extracted in citrate buffer and diluted in 12× SSC, 6% formaldehyde.

Large-scale application of ELISA and molecular hybridization. A field indexing was done to confirm the superiority of molecular hybridization with the RNA probe in laboratory experiments and to determine its potential for routine application. We used a grinding buffer that allowed the same extracts to be tested by the two techniques. Citrate buffer (50 mM) with 2% PVP performed as well as the traditional PBS-Tween-PVP-Nicotine buffer in ELISA or the citrate buffer used in hybridization (data not shown). Three field indexing experiments were performed as described in Materials and Methods on samples collected in a small, isolated, naturally infected orchard of southern France.

The results of these three experiments are given in Table 2. There is a very strong correlation between the presence of symptoms (as defined in Materials and Methods) and the detection of the virus by ELISA (86.5% detection in symptom positive samples vs. 15% in symptom negative ones). By comparison, molecular hybridization (MH positive) seems less affected (98 vs. 77.5%). There is also an almost perfect correlation between MH and ELISA in ELISA positive samples (only three MH negatives out of 337 ELISA positives, i.e., less than 1%). On the other hand, MH was able to detect the virus in ELISA negative samples (301 out 397 samples, i.e., 76%). This effect is especially strong on symptom negative samples in which ELISA does not detect the virus efficiently (15%), whereas MH detects the virus in 77.5% of the same samples. The superiority of MH is confirmed by the overall results of field

TABLE 1. Comparison of detection sensitivities of plum pox virus by enzyme-linked immunosorbent assay (ELISA) (antibodies to D strain) and molecular hybridization (RNA probe synthesized from pBPPV1)

Origin of the infected material	Minimum quantity of infected material (μg) necessary for detection of PPV	
	ELISA[a]	Hybridization[b]
PPV-D pea	12	0.03
PPV-M pea	60	0.16
PPV-D peach	300	0.8
PPV-M peach	300	0.8

[a] ELISA: 1-g sample was ground in 4 ml of buffer, serially fivefold diluted and applied as 250 μl.
[b] Molecular hybridization: 1-g sample was ground in 4 ml of buffer, serially fivefold diluted and applied as 10 μl spots.

TABLE 2. Comparison of the detection of PPV in apricot samples[a] by enzyme-linked immunosorbent assay (ELISA) and molecular hybridization (MH) (RNA probe synthesized from pBPPV 1) in three field indexing trials

Symptoms[b]	Experiment No.[b]	Nb samples	ELISA positives		ELISA negatives	
			MH (+)	MH (−)	MH (+)	MH (−)
+	1	100	91	0	8	1
	2-1	68	60	0	7	1
	2-2	6	5	0	1	0
	3	144	119	0	21	4
Subtotal		318	275(86.5%)	0(0%)	37(11.5%)	6(2%)
−	1	141	18	2	88	33
	2-1	52	15	0	32	5
	2-2	114	10	1	81	22
	3	109	16	0	63	30
Subtotal		416	59(14%)	3(1%)	264(63.5%)	90(21.5%)
Total		734	334(45.5%)	3(0.5%)	301(41%)	96(13%)

[a] One-gram samples were extracted with 4 ml of buffer, 10-fold diluted and applied as 250 μl in ELISA and 10 μl in MH.
[b] Experiments and definition of symptom positive (+) and symptom negative (−) samples as in Materials and Methods.

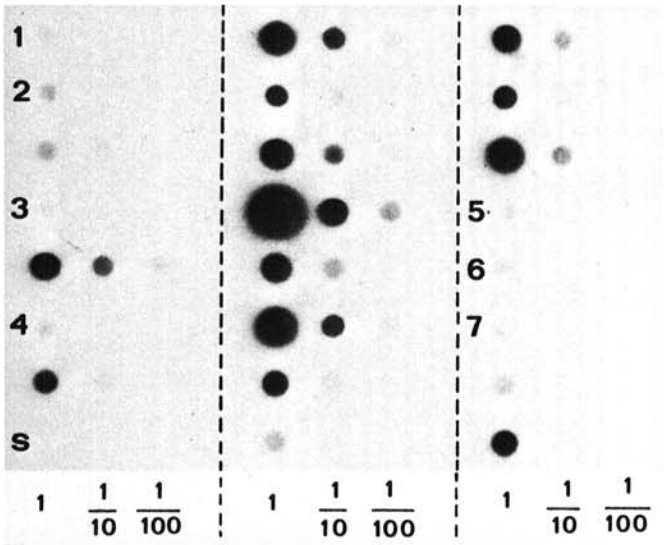

Fig. 5. Dot blot hybridization using the RNA probe and crude extracts from PPV infected apricot samples. One-gram samples were ground in 4 ml of 50 mM citrate buffer, 2% PVP and then serially (10-fold) diluted in 12× SSC, 6% formaldehyde. S, healthy apricot; 1, 2, 3, 4, 5, 6, 7: samples negative in the ELISA test.

indexing experiment 3 in which 40 infected trees were assayed with the standard sampling protocol. By analyzing five samples composed of three twigs, ELISA showed 36 of these trees to be infected, whereas all were found to be infected using MH.

An autoradiograph of hybridization with some field samples is given in Figure 5. Samples positive in hybridization but negative in ELISA correspond to the faint spots (1 to 7), which probably indicates a low concentration of viral RNA. These samples are considered as positive because we never observed any hybridization with healthy plant material.

DISCUSSION

Virus detection problems encountered with immunological methods and the promising results obtained with molecular hybridization with DNA probes (18) prompted us to try the hybridization assay with an RNA probe and to test it under field conditions.

The limits of detection with the RNA probe synthesized from pBPPV1 were 4 pg of PPV-D, the strain used to produce the clone, and 20 pg of PPV-M, representing the second viral serogroup. In terms of concentration, these values correspond to 0.4 and 2 ng/ml. With these performances the RNA probe is 25 times more sensitive than the DNA probe pPPV9A (18) and 10–15 times more sensitive than the ELISA test, depending on the nature of the sample (purified virus or sap extract, respectively).

The high sensitivity of the RNA probe is accompanied with high specificity, in terms of an absence of a nonspecific response with healthy plant extracts or of general background noise. This facilitates interpretation of results.

In routine testing of apricot samples, molecular hybridization detected virus in 76% of the samples negative by ELISA while only 1% of the ELISA positive samples indexed negative by molecular hybridization. These tests were performed with the same extracts for both diagnostic test procedures, an important factor when comparing results, because the virus is well known to be unevenly distributed in infected plants. It is not possible to conclude if the advantage of hybridization as compared to ELISA comes only from an increase in sensitivity or if nonencapsidated viral RNA was present in the samples negative in ELISA. Nevertheless, it is clear from the intensity of spots that in such samples we are close to the detection limits of molecular hybridization, very likely in the range of 0.2–2 pg of RNA per spot. If this RNA was encapsidated, it would correspond to 0.4–4 ng of virus per milliliter, which is usually less than that detectable by ELISA.

Thus, the RNA probe, apart from the general advantages of the dot-hybridization assay (4), provides a very sensitive method for PPV detection. Additional incentive comes from the fact that very promising results have been obtained with bark from infected plants. The possibility of using bark samples must be confirmed with dormant material. This would be of major interest for the testing of introduced trees and for an application to quarantine measures.

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