Double-Stranded RNAs in Mycelial Cultures of *Agaricus bisporus* Affected by La France Disease

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ABSTRACT

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Diseased mycelial cultures of *Agaricus bisporus* derived from basidiocarps affected by La France disease contained nine major double-stranded RNA (dsRNA) segments ranging from 2.50 to 0.53×10^6 molecular weight (MW). The highest concentration of the dsRNAs (about 60 ng/g fresh weight of mycelium) occurred in cultures grown at 21 and 24 C compared with those grown at 16 or 30 C. Incorporation of [5, 6] ^{3}H-uridine into the dsRNAs was first detected in diseased mycelium after 14 days of labeling and continued up to 28 days. Hybridization analysis revealed that the disease-specific dsRNAs labeled in vivo shared sequence homology with the dsRNAs isolated from symptomatic basidiocarps. Healthy mycelial cultures did not show these dsRNAs either by staining with ethidium bromide or radiolabeling. Our findings that the dsRNAs previously detected in diseased basidiocarps are also associated with and actively replicating in diseased mycelial cultures, further support their etiologic role in La France disease.

Additional keywords: common cultivated mushroom, mycovirus, X-disease.

In 1950, Sinden and Hauser (14) coined the name La France disease to describe a serious infectious disorder of the common cultivated mushroom, *Agaricus bisporus*. Today this name is used to describe numerous symptoms, all of which are associated with the presence of viruslike particles (VLPs) (13,17). The most diagnostic symptoms attributed to La France disease are a delay in the appearance of basidiocarps, development of basidiocarps with small misshapened pilei and elongated stipes (drumstick syndrome), and a reduced yield that manifests as barren areas in the production beds.

The causative role of viruses in the La France disease was first proposed after the discovery of several types of VLPs in affected basidiocarps (6). Since this initial report, random mixtures of 25 nm and 34–35 nm spherical particles and a 19-$\times$ 50-nm bacilliform particle have been associated with the disease (9,17). Additional supportive evidence for a viral etiology is the detection of discrete, high molecular weight, double-stranded RNA (dsRNA) molecules (5,7,8,11,18) in diseased tissues. Variation in the number and size of dsRNAs among diseased basidiocarp isolates would suggest that several viruses occur in *A. bisporus*. However, a specific virus(es) has been implicated as the causal agent based on the close association of a unique set of nine dsRNAs ranging in size from 2.50 to 0.53×10^6 MW (18). In this study, we show that the dsRNAs previously described from symptomatic basidiocarps are also present in diseased mycelial cultures. In addition, we provide evidence that they are actively replicating in diseased tissue.

MATERIALS AND METHODS

Source and maintenance of cultures. A diseased culture (V700) of a commercial white strain was derived from pileus tissue of symptomatic basidiocarps containing the nine dsRNAs associated with La France disease. Cultures of healthy, commercial white strains (PSU310 and PSU342) lacking the dsRNAs were obtained from The Pennsylvania State University Mushroom Spawn Laboratory. These cultures were derived from healthy basidiocarp tissue and had been maintained in the collection for more than 12 yr. Cultures were grown on either potato-dextrose agar or in YPPS broth (4 g of yeast extract, 1 g of KH_2PO_4, 15 g of soluble starch, and 0.5 g of $MgSO_4/L$) and routinely transferred to fresh medium at 3- to 6-wk intervals.

Stationary liquid culture. Mycelium was raised in stationary liquid culture as follows: 75 ml of YPPS broth contained in a 250-ml flask was seeded with a 4-mm-diameter mycelial plug and maintained at 21 C without agitation. In temperature studies, at least 12 flasks per strain were incubated for 10 wk at each of four temperatures, 16, 21, 24, and 30 C ± 1 C. Cultures to be maintained at 30 C were first grown at 24 C for 1 wk and then transferred to 30 C for the remaining 9 wk. Mycelium was harvested by vacuum-filtration and stored at −20 C.

Radiolabeling in vivo. Under aseptic conditions, mycelium grown in stationary liquid culture was shaken briefly with 3-mm-diameter glass beads in distilled water and blotted onto filter paper. Seven grams of washed mycelium was transferred to a 125-ml flask containing 25 ml of YPPS broth amended with 100 μCi/ml of [5, 6] ^{3}H-uridine (Spec. Act., 42 Ci/mmole, ICN Biomedicals, Inc., Costa Mesa, CA). After 0, 7, 14, 21, and 28 days of growth at 21 C, 0.5 g of mycelium was harvested and stored at −20 C. At each sampling date, a drop of incubation medium was plated onto YPPS agar to monitor for microbial contamination.

Isolation of dsRNA. Unlabeled dsRNA was isolated from 10 g of frozen mycelium by phenol extraction and cellulose column chromatography (18). For the isolation of ^{3}H-labeled dsRNA, the standard protocol was modified such that 0.5 g of mycelium was homogenized first with a mortar and pestle and then with a Dounce homogenizer in the presence of the standard extraction solutions and 0.8 g of pulverized glass. The final dsRNA pellet was resuspended in 300 μl of sterile distilled water.

Isolation of total ^{3}H-labeled RNA. Two grams of ^{3}H-uridine-labeled mycelium were homogenized first with a mortar and pestle and then with a Dounce homogenizer in the presence of 0.67 ml of 10% SDS, 6 ml of TNE (100 mM Tris, 100 mM NaCl, and 10 mM EDTA, pH 8.5), 8 ml of TNE-saturated phenol,

and 0.8 g of pulverized glass. The emulsion was separated by centrifugation at 10,000 g for 10 min and the nucleic acids were precipitated from the upper aqueous phase by the addition of 2.5 volume of cold 95% ethanol at −20 C. The nucleic acid precipitate was recovered by centrifugation at 12,000 g for 20 min, washed twice with cold 70% ethanol containing 3 M sodium acetate, pH 4.5, dried with a stream of nitrogen gas, and resuspended in 1 ml of distilled water.

Gel electrophoresis. DsRNAs were electrophoresed in 3% polyacrylamide gels as described elsewhere (18). After electrophoresis, dsRNAs were detected by staining with ethidium bromide (18) or fluorography (1). Stained dsRNAs were visualized with ultraviolet light (302 nm) and photographed with type 55 Polaroid film. Molecular weights of the dsRNAs were estimated from their electrophoretic mobilities relative to the $BstE$ II restriction endonuclease fragments of lambda DNA (New England BioLabs, Beverly, MA).

Confirmation of dsRNA. The dsRNA nature of nucleic acids was confirmed by an ionic strength-dependent sensitivity to hydrolysis by RNase A (Sigma Chemical Co., St. Louis, MO) as described by Wach et al (18). To determine the ribonuclease sensitivity of the total ^{3}H-labeled RNA, the reaction mixture also included 5 μg of RNase T1 per milliliter (Sigma).

Quantification of dsRNA. Quantification of the unlabeled dsRNAs was done by laser densitometry (Model 2202 Ultrascan, LKB Instruments, Inc.) with the photographic negatives of the polyacrylamide gels. The dsRNAs of reovirus-3, kindly provided by Dr. Wolfgang Joklik, Duke University Medical Center, Durham, NC, were used as a standard.

Determination of acid-insoluble radioactivity. The extent of RNA synthesis was assayed by measuring the incorporation of ^{3}H-uridine into cold trichloroacetate (TCA)-precipitable material (4).

Molecular hybridization. RNA hybridizations on Biodyne nylon membranes were performed according to the manufacturer (ICN). DsRNA samples were heat-denatured at 100 C for 5 min, quick-cooled, and spotted onto the membranes. The membranes were baked under vacuum at 80 C for 1 hr. Prehybridization was conducted at 42 C for 1 hr in 0.4 ml of prehybridization solution per 100 cm^2 of membrane. After prehybridization, ^{3}H-labeled dsRNAs were heat-denatured as before, added to the hybridization solution, and allowed to hybridize at 42 C overnight. After hybridization, the membranes were washed four times with 2× SSC (1× = 0.15 M NaCl and 15 mM sodium citrate, pH 7) containing 0.1% SDS for 5 min at room temperature, twice with 0.1× SSC with 0.1% SDS for 15 min at 50 C, air dried, treated with EN^{3}Hance (Du Pont Co., Boston, MA), and exposed to Kodak X-Omat AR film at −20 C.

Data analysis. Statistical analysis of the data was done by Fisher's protected least significant difference test at $P = 0.05$ (15).

RESULTS

Diseased mycelial cultures. We routinely obtained diseased mycelial cultures of light cream, off-white, white, and hybrid white varieties of *A. bisporus* by subculturing the pileus tissue of symptomatic basidiocarps that contained the nine dsRNAs associated with La France disease in their stipe tissue. Cultures derived from severely diseased basidiocarps (drumstick syndrome) typically grew slowly, had appressed and brownish-colored mycelia, and caused a more pronounced discoloration of the growth medium than healthy cultures. These cultures invariably contained a specific set of dsRNAs which upon electrophoresis in 3% polyacrylamide gels was resolved as eight segments of 2.50, 2.05, 1.90, 1.70, 1.10, 0.89, 0.58, and 0.53 × 10^6 MW (Fig. 1). The 1.90 × 10^6 MW dsRNA segment detected by electrophoresis in 3% polyacrylamide gels is actually a doublet that migrates in 0.9% agarose gel as 1.95 and 1.85 × 10^6 MW dsRNAs (data not shown).

The diseased culture used in this study, V700, was representative of more than 15 diseased cultures we had examined with respect to vegetative characteristics and dsRNA profile. In a replicated experiment, we found that V700 grown for up to 20 wk with
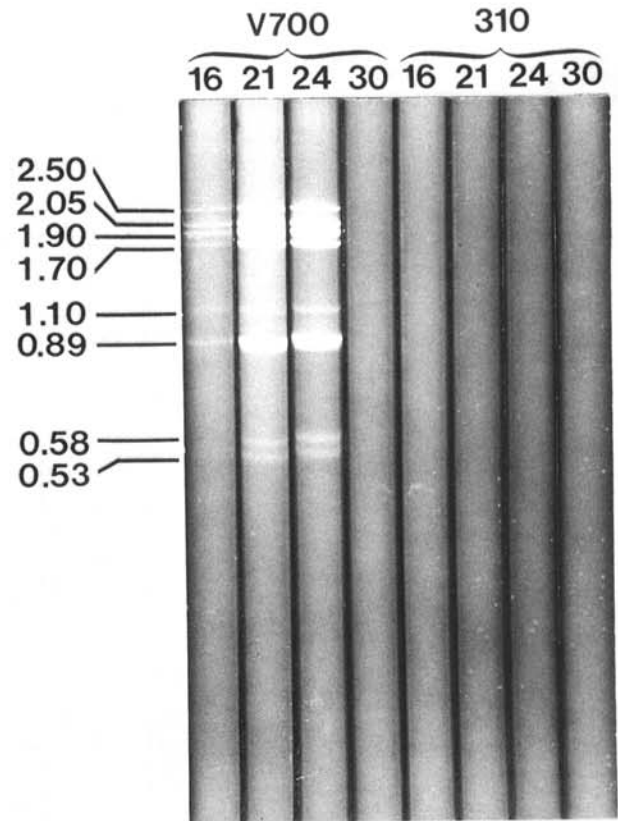
Fig. 1. DsRNA analysis of diseased and healthy mycelial cultures grown at different temperatures. DsRNA were extracted from mycelium of diseased (V700) and healthy (PSU310) white strains that had been grown in stationary liquid culture for 10 wk at 16, 21, 24, and 30 C. Electrophoresis was through 3% polyacrylamide gels at 5 mA/gel for 3.5 hr. Stained with ethidium bromide. Molecular weights (× 10^6) are indicated.

TABLE 1. Comparative growth and dsRNA content of healthy (PSU342) and diseased (V700) mycelial cultures[a]

Culture	Source of mycelial plug	Time (wks)	Area of growth (cm^2)[b]	Yield of dsRNA (ng/g fresh wt. of mycelium)[c]
Healthy (PSU342)	Center	0	···	0
	Center	10	43.5 b	0
	Center	20	43.9 b	0
	Periphery	0	···	0
	Periphery	10	52.3 a	0
	Periphery	20	55.7 a	0
Diseased (V700)	Center	0	···	147
	Center	10	6.6 cd	108
	Center	20	4.0 d	98
	Periphery	0	···	63
	Periphery	10	11.7 c	73
	Periphery	20	9.6 cd	74

[a] Healthy (PSU342) and diseased (V700) cultures were grown at 21 C on YPPS agar. The cultures were transferred at 5-wk intervals to fresh YPPS agar using 4-mm-diameter mycelial plugs consistently removed from either within 1 cm of the center (Center) or 1 cm of the margin (Periphery) of the culture. At 0, 10, and 20 wk, the areas of growth of the 5-wk-old cultures were determined and mycelial plugs were taken to initiate stationary liquid cultures for dsRNA analysis.

[b] Mean area of growth of the culture at the time of transfer which was calculated by averaging the values for the maximum culture diameter and the diameter at right angles to the maximum. Means followed by the same letter are not significantly different at $P = 0.05$ using Fisher's protected least significant difference test. Means are based on three observations in each of two experiments.

[c] DsRNA was isolated from 10 g fresh weight of mycelium that had been grown in stationary liquid cultures in YPPS broth at 21 C for 10 wk. Mean yields of dsRNA from V700 were not significantly different at $P = 0.05$ using Fisher's protected least significant difference test. Means are based on one observation in each of two experiments.

routine subculturing at 5-wk intervals was a reliable source of inoculum for dsRNA-containing stationary liquid cultures (Table 1). Typical of severely diseased cultures, the vegetative growth rate of V700 perpetuated from either the older mycelium at the center of the culture or the younger mycelium at the periphery was significantly lower than that of healthy culture PSU342. Yields of dsRNA from V700 stationary cultures ranged from 63 to 147 ng/g of mycelium and were not influenced by the age of the mycelial inoculum. No dsRNA was detected in healthy culture PSU342.

Effect of temperature on the concentration of dsRNA. When V700 was grown at 21 and 24 C, five to 10 times more dsRNA accumulated than at either 16 or 30 C. In two experiments with one observation each, the mean yield of dsRNA for cultures maintained at 16, 21, 24, and 30 C was 5, 60, 57, and 11 ng of mycelium, respectively. However, only the yield at 21 C was significantly higher from that obtained at 16 and 30 C. DsRNA was not detected at any temperature in healthy culture PSU310. In one experiment, V700 maintained at 30 C appeared to be heat-cured of the nine dsRNAs (Fig. 1). In a replicate experiment, however, the dsRNAs were still detected after 9 wk of growth at 30 C. No qualitative differences in the dsRNA profile existed among the diseased cultures of V700 grown at the different temperatures.

In vivo radiolabeling of dsRNA. RNA synthesis was detected in healthy and diseased mycelium labeled in vivo with ^{3}H-uridine for 14 and 40 days (Table 2). Initially, we measured the sensitivity of the total ^{3}H-labeled RNA to hydrolysis by RNase to compare the level of dsRNA synthesis in healthy and diseased tissues. We found a similar proportion (10–14%) of the total ^{3}H-labeled RNA in healthy and diseased cultures to behave like dsRNA based on a resistance to RNase at high ionic strength. However, the fact that about 7% of the ^{3}H-labeled RNA also resisted RNase treatment at low ionic strength indicated that only a fraction of the RNase-resistant RNA was authentic heteroduplex RNA. Similarly, we found no evidence for a disparity in dsRNA synthesis between healthy and diseased cultures based on the elution profiles of the ^{3}H-labeled RNA upon cellulose chromatography (data not shown). However, gel electrophoresis and fluorographic analysis revealed that the ^{3}H-labeled RNA from diseased mycelium eluting in the dsRNA fraction from cellulose consisted of eight dsRNA segments corresponding in size to those detected in diseased cultures by ethidium bromide staining (Fig. 2). Signal in these dsRNAs was first detected 14 days after labeling began and increased for at least 28 days. In contrast, healthy mycelium lacked these dsRNAs, but instead contained heterodispersed low molecular weight RNA which migrated near the dye front (not visible in Fig. 2) as well as high molecular weight material which was also present in diseased tissue.

Hybridization analysis showed that the dsRNAs labeled in vivo in diseased mycelium shared sequence homology with the dsRNAs isolated from diseased, but not healthy, basidiocarps (Fig. 3).

On the other hand, the heterodispersed RNA labeled in healthy cultures failed to hybridize with the dsRNAs from either healthy or diseased basidiocarps.

DISCUSSION

The most compelling evidence to date for a viral etiology of La France disease is the coincidence in basidiocarps of a set of nine dsRNAs and characteristic symptoms (7,8,11,18). We now provide evidence from electrophoretic, radiolabeling, and hybridization studies that the identical dsRNAs also prevail in diseased mycelial cultures. In all likelihood, the dsRNAs reported here are analogous to those of unspecified size observed in diseased cultures of *A. bisporus* (2,5). Moreover, the results of our radiolabeling studies suggest the disease-related dsRNAs are synthesized in vivo and are the principal dsRNAs undergoing replication in diseased mycelium. Assuming replication is a requisite for pathogenesis, our data further support a causal role for viruses in the disease.

We were unable to detect an increased accumulation of dsRNA in diseased mycelium based on the RNase resistance and behavior upon cellulose chromatography of the total cellular ^{3}H-labeled
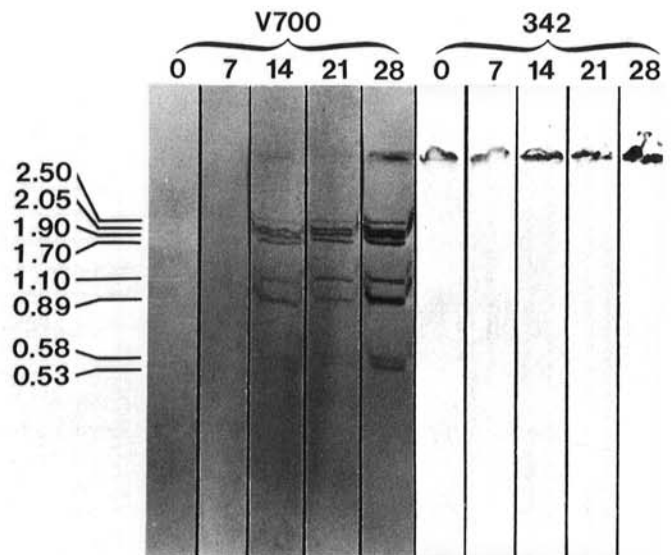

Fig. 2. Fluorogram of the dsRNAs labeled in vivo with ^{3}H-uridine in diseased and healthy mycelium. Diseased (V700) and healthy (PSU342) cultures were grown at 21 C in YPPS broth amended with 100 μCi [5, 6] ^{3}H-uridine per milliliter. At 0, 7, 14, 21, and 28 days of growth, 0.5 g of mycelium was sampled for dsRNA analysis. ^{3}H-labeled dsRNAs were electrophoresed through 3% polyacrylamide slab gels at 100 V for 3.5 hr and detected by fluorography. Molecular weights ($\times$ 10^6) are indicated.

TABLE 2. Comparison of the ribonuclease sensitivity of the total RNA radiolabeled in vivo with ^{3}H-uridine in healthy and diseased mycelium[a]

| Culture | Exp. # | Net Radioactivity (CPM)[a] | | | | | |
| | | 0.3 M NaCl | | | 0.03 M NaCl | | |
		−RNase (A)	+RNase (B)	% RNase Resistance (B/A)	−RNase (A)	+RNase (B)	% RNase Resistance (B/A)
Healthy (PSU310)	1	157,149 d	22,535 f	14	164,375 cd	10,852 fg	7
Healthy (PSU342)	2	47,525 e	6,271 g	13	56,678 e	4,836 g	9
Diseased (V700)	1	174,893 bc	17,421 fg	10	184,042 b	10,906 fg	6
Diseased (V700)	2	253,688 a	24,600 f	10	258,707 a	17,915 fg	7

[a]Healthy (PSU310 and PSU342) and diseased (V700) cultures were grown in YPPS broth amended with 100 μCi/ml of [5, 6] ^{3}H-uridine at 21 C for 14 days (Exp. 1) and 40 days (Exp. 2). In each experiment, total ^{3}H-RNA was extracted from 2 g fresh weight of mycelium as described in the Materials and Methods, adjusted to either 0.3 M NaCl or 0.03 M NaCl, and treated with either sterile water (−RNase) or 20 μg of RNase A and 5 μg of RNase T1 per milliliter (+RNase). After incubation at 33 C for 30 min, TCA-insoluble radioactivity was determined. Means followed by the same letter are not significantly different at $P = 0.05$ using Fisher's protected least significant difference test. Means are based on two observations in each experiment.

RNA. This was somewhat unexpected because we anticipated that the presence of the disease-related dsRNAs, assuming they were undergoing replication in vivo, would be reflected in an increased level of radiolabeled dsRNA. Instead, due to a preponderance of low molecular weight dsRNA-like nucleic acid in healthy tissue, the disease-related ^{3}H-labeled dsRNAs could only be resolved after gel electrophoresis and fluorographic analysis. The precise origin of this RNA in healthy mycelium is unknown, but it is frequently detected by ethidium bromide staining in dsRNA preparations of healthy basidiocarp isolates (Romaine, *unpublished results*). One possibility is it represents regions of intramolecular hydrogen bonding in cellular single-stranded RNA. It is worth mentioning that some healthy strains of *A. bisporus* contain a major 1.6×10^6 MW dsRNA and associated minor larger dsRNAs (10), although they were not detected in healthy strains used for this study. Also we are mindful that the healthy cultures used here were not of the same origin as the diseased culture (i.e., identical strain and crop), which might limit the interpretation of our results. At the same time, it is important to note that they represent popular commercial strains and have vegetative and reproductive traits deemed typical of white mushroom types. Moreover, it is well recognized that differences among commercial strains of *A. bisporus* are negligible because of the low genetic diversity within the species (12) and the rampant and unregulated exchange of strains among spawn manufacturers.

We observed that the temperature of growth had a pronounced effect on the accumulation of dsRNAs in diseased mycelium. The dsRNAs accumulated to their highest levels in cultures maintained at 21 and 24 C. In one of two experiments, they were undetectable in cultures grown at 30 C. Our findings agree with earlier reports of sporadic success in using heat therapy to cure diseased cultures of VLPs (17). The presence of a relatively low level of dsRNA

in cultures reared at 16 C was somewhat surprising because this is the temperature at which basidiocarps of *A. bisporus* normally develop and at which La France disease ordinarily occurs. This observation implies a less than optimal rate of replication of the dsRNAs in diseased basidiocarps under standard cultivation conditions. However, Detroy et al (3) have shown that synthesis of viral dsRNA closely paralleled mycelial growth in *Penicillium stoloniferum*. If this is generally true, then we would expect dsRNA synthesis to proceed maximally near 24 C in mycelium and near 16 C in basidiocarps, since this is within the optimal temperature range for vegetative and reproductive growth of *A. bisporus*, respectively (19).

The nine dsRNAs that we have associated with diseased basidiocarps and mycelial cultures appears to comprise the minimal pathogenic unit. Their presence correlates positively with the disease, they are acquired and lost in concert by the host, and they are the most actively synthesized dsRNAs in diseased tissues. Whether all of the dsRNAs are essential for pathogenesis or whether some might be terminally conserved remnant RNAs (16) or satellite RNAs (5) should be considered.

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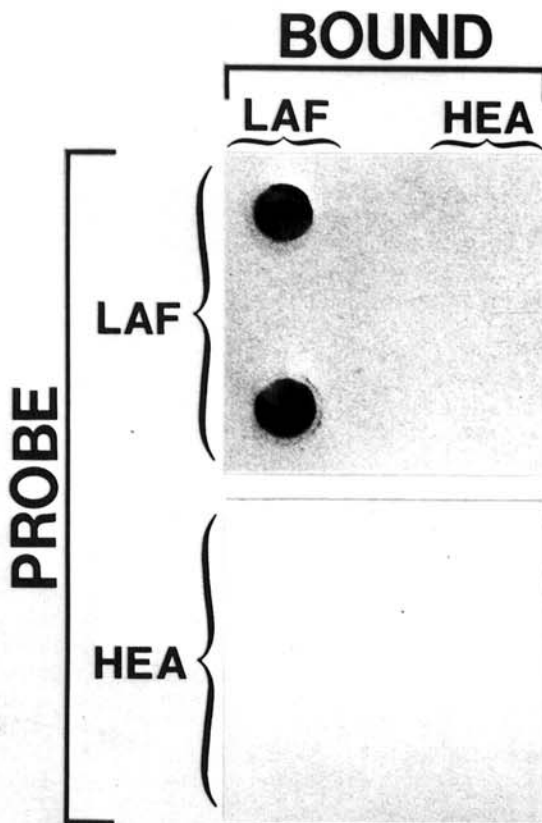
Fig. 3. Hybridization analysis of the in vivo ^{3}H-labeled dsRNAs from diseased mycelium to the disease-specific dsRNAs isolated from basidiocarps. Aliquots (20 μl) of the dsRNA fraction of healthy (HEA) and diseased (LAF) basidiocarps were bound (BOUND) in quadruplet to Biodyne nylon membranes and probed (PROBE) in replicate with the ^{3}H-dsRNA labeled in vivo after isolation from healthy (HEA) or diseased (LAF) mycelium.