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Homology Between Double-Stranded RNA and Nuclear DNA of Yeast

MICHAEL VODKIN

Department of Biology, University of South Carolina, Columbia, South Carolina 29208

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The relationship between mycoviral double-stranded (ds) RNA and host cell DNA was investigated. Radiolabeled ds RNA was denatured and reannealed in the presence and absence of denatured DNA. RNA from killer strains of the yeast *Saccharomyces cerevisiae* and from nonkiller derivatives was utilized. The above-mentioned strains, as well as one that lacks all ds RNA, were sources for extracted DNA. Net hybridization of ds RNA to DNA occurred regardless of the strains from which the respective nucleic acids were prepared.

Several laboratories (1, 22) established that double-stranded (ds) RNA with a molecular weight of 2.4×10^6 occurs in most strains of bakers' yeast (*Saccharomyces cerevisiae*). In the strains that harbor the killer factor, an additional ds RNA (1.4×10^6 daltons) is present which codes directly for or regulates the production of a toxin (21). The latter species of ds RNA is correlated with the presence of the killer factor but is missing from strains that have been cured of killing either by cycloheximide or by heat treatment (5, 22). Each species of ds RNA is encapsulated in separate virus-like particles (9).

The occurrence of ds RNA in yeast is not an isolated phenomenon of fungal biology. Viruses and virus-like particles are common in other fungi, and almost all have genomes consisting of ds RNA (12). The origin of fungal ds RNA is presently unknown. Gillespie and Gallo (7) recently postulated a theory on the origin of RNA genomes of tumor viruses. According to their theory, such tumor virus RNA evolved recently from host cell DNA by "paraprocessing," the escape of an RNA transcript from the usual nucleolytic processing that precedes translation. Paraprocessing may also have been important for the formation of mycoviral RNA. If the ds RNA genomes in yeast were created in this manner, there should be homology between ds RNA and host cell nuclear DNA. Experiments described in this paper indicate that such homology does indeed exist.

MATERIALS AND METHODS

Yeast strains. All strains of *S. cerevisiae* of the A8209B series were described previously (20). A8209B is a killer strain; A8209NK1 and A8209BNK5 are nonkillers derived spontaneously and with cycloheximide, respectively. Petite (*rho*$^-$) strains were derived from all three with ethidium bromide under conditions which led to the loss of all detectable mitochondrial DNA (8). JM15 is a nonkiller derived by mutagenesis with ethyl methane sulfonate from FM11. FM11 is a killer strain whose genotype was described previously (14).

Labeling, extraction, and purification of ds RNA. Cells were grown with 50 μCi of carrier-free ^{32}P per ml in complete medium from which the inorganic phosphate had been removed (16). The ds RNA was extracted and partially purified according to Vodkin et al. (21). It was further purified by chromatography on a CF11 cellulose column (15), and the peak fractions of radioactivity eluting in 0% ethanol were pooled. All preparations were routinely tested for purity by their expected sensitivity to nucleases (20) and their homogeneity on gel electrophoresis.

DNA extractions and purification. For all strains except JM15, DNA was extracted from *rho*$^-$ derivatives. Cells were grown in complete medium, harvested by centrifugation at $4,000 \times g$ for 10 min at 4°C, and then suspended in buffered saline solution. They were disrupted by blending for 1 min in a Braun homogenizer with glass beads (0.45 to 0.50 mm). DNA was solubilized in the homogenate by making the solution 5% with sodium dodecyl sulfate and then incubating it at 60°C for 10 min. The DNA was purified by the basic protocol of Marmur (12) with the following modification. Prior to treatment with pancreatic RNase, the DNA preparation was subjected to a digestion with RNase III. The final purification step was centrifugation in a CsCl density gradient. Some preparations were also passed through a cellulose CF11 column, and the DNA was pooled from fractions eluting in 35% ethanol.

RNA-DNA hybridization. DNA was denatured by the addition of 0.1 volume of 1 N NaOH, and after 15 min its pH was adjusted to 7.0 with 1 M KH_2PO_4. RNA was denatured in 40% formamide-1× SSC (1× SSC is 0.15 M NaCl-0.015 M sodium citrate, pH 7.0) by heating for 5 min at 90°C and then quick-cooling (6). Subsequent hybridization was performed either by diluting with the same buffer and incubating at

37°C, or by diluting with 2× SSC and incubating at 65°C for up to 3 h. Both methods yielded the same results. Hybridization was monitored by diluting portions in 2× SSC and incubating them at room temperature for 30 min with 20 μg of pancreatic RNase per ml. Tubes containing no DNA, native yeast DNA, or denatured calf thymus DNA served as controls.

Cesium sulfate-density gradient centrifugation. After hybridization, the reaction mixture was diluted with 2× SSC and digested with 20 μg of pancreatic RNase per ml. The RNase was removed with phenol, and the nucleic acids in the aqueous layer were precipitated with carrier rRNA and ethanol. The precipitate was suspended in a small volume of 0.05 M Tris (pH 7.4), and Cs_2SO_4 solution in the same buffer was added to make a final density of 1.54 g/cm³. The solution was centrifuged at 80,000 × *g* for 66 h at 20°C in a Beckman L5-65 ultracentrifuge with a type 65 rotor. Fractions of 0.2 ml were collected in chilled tubes, and the refractive indexes were determined immediately with a Bausch and Lomb refractometer. Each fraction was precipitated with trichloroacetic acid and assayed for radioactivity, as described previously (20).

RESULTS

Purity of DNA and ds RNA. It is crucial in the present hybridization experiments that the purified DNA and ds RNA should not be cross-contaminated. In the purified DNA preparation, any contaminating ds RNA will not be hydrolyzed by the usual purification with pancreatic RNase in 1× SSC; nor will it be removed entirely by isopycnic CsCl centrifugation. The reality of this problem was emphasized by the fact that purified, labeled ds RNA added to a DNA preparation prior to CsCl density gradient centrifugation comigrated with DNA in the gradient. Two steps were taken to free the DNA from contaminating ds RNA. The DNA from these strains was treated with RNase III and then centrifuged to equilibrium in CsCl. This treatment was sufficient to remove the purified, labeled ds RNA that had been added to a DNA preparation. DNA was extracted from nonkiller mutants, which lack all detectable ds RNA (Vodkin, unpublished data). These strains were also examined by an independent laboratory (G. Fink, personal communication), and the analysis was confirmed.

Our purified ds RNA is judged to be pure on the basis of two independent criteria. The nuclease resistance pattern of labeled ds RNA from the different strains is shown in Table 1. The ds RNA was resistant to DNase and to pancreatic RNase in high salt. However, the latter resistance was lost after denaturation. The ds RNA was hydrolyzed by pancreatic RNase in low salt and by RNase III. The above pattern was expected for ds RNA and demonstrated the absence of any significant contaminating DNA.

TABLE 1. *Effect of nucleases on ds RNA from various strains*[a]

Treatment	cpm		
	A8209 B[b]	A8209 BNK1	A8209 BNK5
None	1,194	449	514
Pancreatic RNase (low salt)	15	29	12
Pancreatic RNase (high salt)	1,195	476	506
RNase III	20	18	15
DNase[c]	1,170	453	502
Denatured and treated with pancreatic RNase (high salt)	80	51	33

[a] Double-stranded RNA, labeled with ^{32}P, from the indicated strains was subjected to various nuclease treatments. The numbers in the table represent cold acid-insoluble counts per minute that remained after treatment. Pancreatic RNase at a final concentration of 20 μg/ml was incubated with nucleic acids for 30 min at room temperature in either 0.1× SSC (low salt) or 2× SSC (high salt). Incubations were terminated by the addition of 100 μg of bovine albumin in an excess of chilled 5% trichloroacetic acid. The precipitate was collected by filtration on 25-mm type A Gelman glass-fiber filters. The filters were dried and monitored for radioactivity with a liquid scintillation system.

[b] Source of ds RNA.

[c] As a control, tritium-labeled DNA was added to all assays involving DNase. In such experiments 90% of the input was rendered acid soluble after the digestion.

Electrophoresis in 5% polyacrylamide gel of ds RNA extracted from the various strains reveals only the expected bands after staining with methylene blue (not shown). After the gels had been sliced and monitored for radioactivity, only the peaks with the mobility expected for ds RNA were apparent (Fig. 1). The ds RNA of A8209B shows a second peak of radioactivity; the ds RNA of the other two strains shows only a single peak. Approximately 95 to 99% of the applied radioactivity of each sample was accounted for in these peaks. The residual activity barely entered the gel and may represent an aggregation of ds RNA.

Hybridization. The hybridization of denatured ds RNA to DNA from each strain is depicted in Fig. 2, which is representative of experiments that have been repeated many times. In actuality, two competing reactions occurred simultaneously: the hybridization of RNA to DNA as well as the hybridization of RNA to RNA. The nuclease assay used to moni-

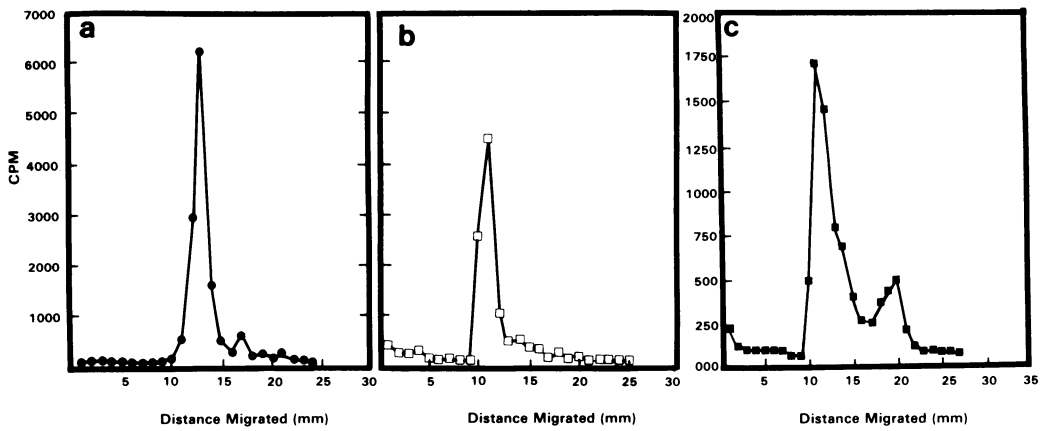

FIG. 1. *Gel electropherograms of radiolabeled ds RNA isolated from different strains. Cells were grown in the presence of ^{32}P, and ds RNA was extracted and purified as described in the text. The sample was subjected to electrophoresis in 5% polyacrylamide gel. After being fixed and stained, the gels were sliced longitudinally into 1-mm slices and analyzed for radioactive content by Cerenkov counting. (a) ds RNA of A8209B; (b) ds RNA of A8209NK1; and (c) ds RNA of A8209NK4.*

tor hybridization did not distinguish between these two structures. The control in each graph is a measure of the RNA-RNA hybridization. The amount of self-annealing of RNA shown in Fig. 2a is significantly lower than that observed in Fig 2b. In some cases, the specific activity of the ds RNA was greater than 10^6 cpm/μg with the labeling protocol used. In hybridization experiments involving such RNA, it was possible to start with a lower R_0t and thereby minimize the self-annealing of single-stranded RNA. The reaction was concentration dependent and appeared to show complex kinetics. These kinetics probably result from the fact that the ds RNA itself contains some nucleotide sequences that are reiterated (G. Fink, personal communication). The data presented in Fig. 3 demonstrate that hybridization in 40% formamide at 37°C does not appear to alter the results.

All the various DNA preparations, including one that had no detectable ds RNA, had sequences similar or identical to the ds RNA. Regardless of the strain from which the DNA had been extracted, there appeared to be no significant difference in the net amount of hybridization (RNA-DNA). These hybridizations were performed with DNA in excess (assuming that there is one copy of the ds RNA/haploid genome) to detect nucleotide sequences of the dsRNA that were complementary to those in the DNA. However, it is difficult to measure accurately the proportion of DNA complementary to ds RNA under conditions of excess denatured RNA because of its rapid self-annealing. It is quite possible, therefore, that the DNA from the killer strain and its nonkiller mutant derivatives differ in the concentration of sequences that can hybridize to ds RNA. It is also difficult to detect whether the smaller ds RNA component of A8209B hybridizes to DNA. During the extreme conditions required for denaturation there is some breakage of the ds RNA, and thus electrophoretic analysis of the products of hybridization is not conclusive.

Although DNA used during these studies was not extracted from nuclear preparations, the hybridization probably represents a reaction with nuclear DNA. The major contaminant of extranuclear DNA, namely mitochondrial DNA, has been eliminated from all strains except JM15. However, circular DNA with the same buoyant density as nuclear DNA was reported in yeast (4), and hybridization of the ds RNA to this and other contaminating DNA cannot be excluded. It is also possible that the ds RNA could be homologous to mitochondrial DNA, as has been reported for the single-stranded RNA of a virus in *Neurospora* (10).

Cs_2SO_4 density gradient centrifugation. To resolve RNA-DNA from the RNA-RNA hybrid, a portion of the hybridization mixture was analyzed on a Cs_2SO_4 density gradient. As controls, hybridization mixtures with native DNA and denatured RNA or with denatured DNA and native RNA were analyzed in parallel experiments. Two discrete peaks of radioactivity were resolved at buoyant densities of 1.62 and 1.57 g/cm^3 (Fig. 4a). The former peak also appeared in controls: a hybridization mixture with native DNA (Fig. 4b) and a hybridization mixture with native ds RNA (Fig. 4c). The first peak is ds RNA since its buoyant density value agrees

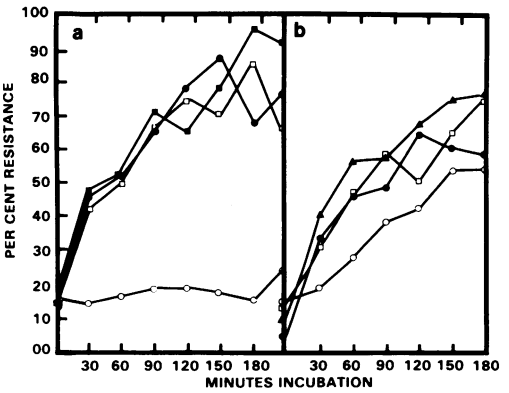

FIG. 2. *Hybridization of ds RNA in aqueous system. Radiolabeled ds RNA (specific activity, 0.4 × 10^6 to 2 × 10^6 cpm/μg) in 0.1 ml of 40% formamide-1 × SSC was denatured by heating to 90°C followed by quick-cooling. The denatured RNA was mixed with alkaline-denatured DNA and diluted to 1 ml with 2 × SSC. (The control either lacked DNA or contained either native yeast DNA or denatured calf thymus DNA.) The mixture was incubated at 65°C for up to 3 h. At time intervals, 20- to 50-μl portions, representing 1 to 5 ng of RNA, were removed and mixed with 1 ml of 2 × SSC containing 20 μg of pancreatic RNase. After incubation at room temperature for 30 min, the reaction was stopped by addition of carrier bovine serum albumin and cold 5% trichloroacetic acid. The solution was filtered, and the filters were monitored for radioactivity. (a) Six nanograms of ds RNA from A8209NK1 hybridized to 50 μg of DNA; (b) 100 ng of dsRNA from A8209-NK5 hybridized to 125 μg of DNA. Symbols: ○, control; □, A8209B DNA; ■, A8209NK1 DNA; ●, A8209NK5 DNA; ▲, JM15 DNA.*

with those of ds RNA cited in the literature (11). The second peak, present only under the actual conditions of hybridization, represents the RNA-DNA hybrid with its expected lower buoyant density.

DISCUSSION

The results of these studies show that ds RNA, after denaturation, reanneals to host cell DNA. Even the strain that lacks detectable ds RNA has sequences in the DNA that are identical to or closely resemble those of the ds RNA. These hybridizations directly involve the ds RNA in question and contrast the indirect methods utilized by Shalitin and Fisher (17) in a recent work. They showed that some of the ds RNA in yeast contains a polyadenylic acid [poly(A)] sequence. They also isolated single-stranded, poly(A)-containing RNA with a relatively long half-life. This RNA is at least twice the genome size of the large species of ds RNA, and it hybridizes to 2.5% of the cellular DNA. DNA isolated from a nonkiller strain that lacked one of the ds RNA components showed a proportional decrease in sequences that could hybridize to the poly(A) RNA. They suggest that the poly(A) RNA is a precursor of ds RNA either in transcription or replication. They concluded further that the nonkiller strain used in their study was missing the DNA complementary to one of the ds RNAs. However, they did not directly hybridize ds RNA of the killer nor did they hybridize the single-stranded poly(A) to ds RNA to show that these sequences are related.

Three hypotheses on the origin of ds RNA in yeast can be discussed in connection with the present hybridization results: (i) DNA codes for ds RNA; (ii) ds RNA codes for the complementary DNA sequences; and (iii) ds RNA replicates independently of the DNA sequences but is derived from them initially by a paraprocessing event. There are two alternatives for the first possibility: the ds RNA can result from either the symmetric transcription of complementary DNA strands or the asymmetric transcription of a palindrome of one DNA strand.

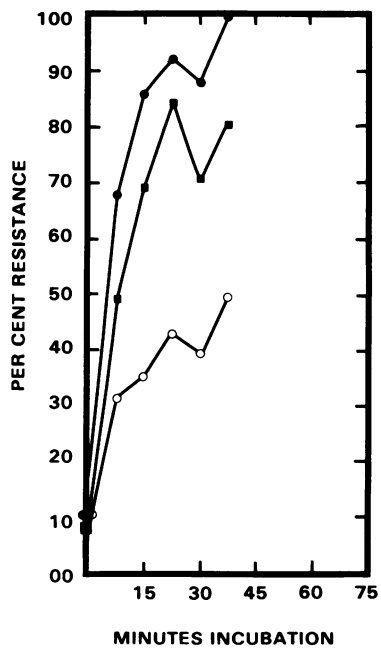

FIG. 3. *Hybridization of ds RNA to DNA in a formamide system. Conditions of reaction were the same as described in the legend to Fig. 2, except that the hybridization solution consisted of 40% formamide-1 × SSC, and incubation was at 37°C. Twenty nanograms of ds RNA from A8209B hybridized to 80 μg of DNA. Symbols: ○, control; ■, A8209B DNA; ●, A8209NK5 DNA.*

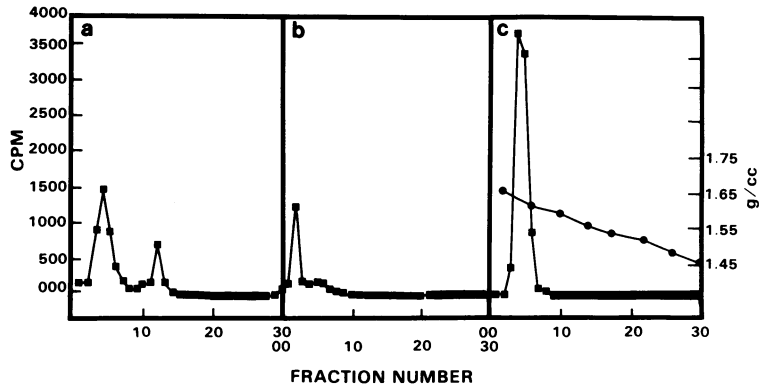

FIG. 4. *Analysis of hybridization mixture on Cs_2SO_4 isopycnic density gradients. A hybridization mixture consisting of ds RNA and DNA from strain A8209B was incubated at 65°C for 3 h. The mixture was digested with pancreatic RNase at a final concentration of 20 μg/ml. The RNase was removed with phenol, and the nucleic acids were precipitated with ethanol. The precipitate was suspended in 0.05 M Tris (pH 7.4) and adjusted to a final density of 1.54 g/cm³ with solid Cs_2SO_4. The mixture was centrifuged at 80,000 × g for 66 h at 20°C. The centrifuge tube was pierced and fractions were collected. Radioactivity was measured as described in the legend to Fig. 2. (a) Hybridization mixture with denatured RNA and DNA; (b) hybridization mixture with denatured RNA and native DNA; (c) hybridization mixture with native RNA and denatured DNA.*

The latter RNA with an inverse repeat would show a concentration-independent rate of self-annealing and twice the expected molecular weight on formamide gel electrophoresis (under denaturing conditions). There is no evidence for either observation (cf. Fig. 2a with b; unpublished data), although ds RNA with hairpin loops can be synthesized from DNA with RNA polymerase in vitro (13).

It is also conceivable that ds RNA could arise from transcription of complementary strands of DNA. Double-stranded RNA was reported in other eukaryotic cells, presumably uninfected by exogenous virus (3). It is not known whether or not this RNA is encapsulated. In at least one case, the ds RNA can hybridize to its cellular DNA (6). It appears unlikely that the ds RNA specific for the killer factor replicates in the above manner because that RNA can be irreversibly cured at high frequency with cycloheximide or heat treatment.

If the information flow in yeast proceeds from ds RNA to DNA, at least one prediction can be made. Virus-like particles are expected to contain reverse transcriptase activity. Such activity has not been reported in the literature, and the hypothesis cannot be eliminated at this time.

Finally, there is the possibility that ds RNA and its complementary DNA replicate independently of each other. Under this hypothesis, one would expect the virus-like particles to contain an RNA-dependent RNA polymerase. There is evidence of the existence of such an enzyme in another ds RNA mycoviral system and of the functioning in vivo of this enzyme (2). Thus, the hypothesis of independent replication of ds RNA and its complementary DNA is presently favored.

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