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No Homology Between Double-Stranded RNA and Nuclear DNA of Yeast

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We investigated the possibility of sequence homology between yeast DNA and one of the double-stranded RNAs present in many strains of *Saccharomyces cerevisiae*. These double-stranded RNAs are encapsidated in virus-like particles, which appear to be similar to the viruses of higher fungi. Contrary to a recent report (M. Vodkin, J. Virol. **21**:516-521, 1976), we find no such homology.

Killer factor is a cytoplasmically inherited genetic element in the yeast *Saccharomyces cerevisiae* (3, 12). It confers upon its host cells (killers) the ability to secrete several toxic proteins that kill sensitive cells (6, 24). The inheritance of the killer phenotype is independent of that of mitochondria (1, 24). The killer phenotype is associated with a double-stranded (ds) RNA (M) of 1.1×10^6 to 1.7×10^6 daltons (2, 4, 19, 20, 22). Another dsRNA (L), 2.5×10^6 to 3×10^6 daltons, is also present in all known strains that harbor M (2, 4, 19, 20, 22). These two dsRNA's are separately encapsidated in virus-like particles (VLPs), which are separable either by sucrose gradient centrifugation or by cesium chloride equilibrium gradient centrifugation (8; D. F. Pietras and J. A. Bruenn, unpublished data). Strains lacking M still synthesize L and L-containing VLPs. One of the strands of L encodes the major polypeptide of L-containing VLPs, as shown by in vitro protein synthesis (10). The VLPs have a viral transcriptase (9). Numerous nuclear mutations are known that affect the inheritance of M but have no effect on the inheritance of L (22). In many respects, then, these dsRNA-containing particles in yeast are similar to the dsRNA viruses of higher fungi (11). Killer factor has recently been reviewed in detail (14, 21).

Naturally, one of the questions that arises about replication of these dsRNA's in yeast is the presence or absence of nuclear DNA homologs. Recently, Vodkin (18) reported the presence of DNA homologous to the crude dsRNA of strains harboring only L. A rough calculation from his data indicates the presence of a minimum of 10 copies of L (our calculations) per nuclear genome. We have been unable to duplicate these experiments; on the contrary, we find, in carefully controlled experiments, at most 0.075 copies of L per nuclear genome and possible fewer than 0.025 copies per genome, using DNA from one of the strains reported to have DNA homologous to L. In similar experiments, other laboratories have also been unable to find any yeast DNA homologous to L (23; J. Hopper, personal communication).

The in vivo ^{32}P-labeled dsRNA used for these experiments was purified on 1.4% agarose gels, as previously described (5). An autoradiograph of such a gel is shown in Fig. 1. The yeast strain used, T158D SK (G. Fink), has both L and M, which are easily separated in this gel system. The extent of rRNA contamination, estimated from the amounts of 2′-*O*-methyl-dinucleoside disphosphates present in a total RNase T2 digest, was less than 0.1% (5). The T1 fingerprint of L purified in this manner revealed the presence of no major contaminating species (5), and pppGp end group determination implied a molecular weight of 2.9×10^6 (4). We have found no differences in the T1 fingerprints of L isolated from several strains. L isolated in this manner appears to be entirely ds by a number of criteria, including base composition, electron microscopy, resistance to pancreatic RNase or S1 nuclease at high salt, and the molecular weight of its denatured single strands (1.5×10^6) (4; J. A. Bruenn, unpublished data). The specific activity of preparations used for hybridizations was 0.5×10^6 dpm/μg.

DNA for these experiments was isolated from strain A8209B (one of the strains reported to have DNA homologous to L). Cells were grown under conditions of glucose repression to minimize the amount of mitochondrial DNA present. Cells were broken by one passage through a French press, and DNA was purified by repeated chloroform-isoamyl alcohol extractions, Pronase and pancreatic RNase digestions, and ethanol

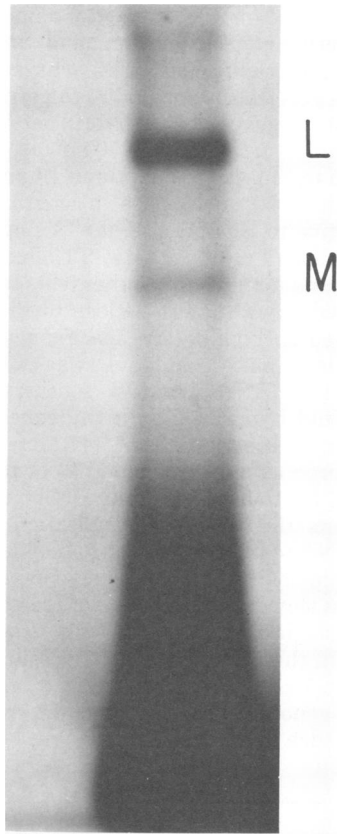

FIG. 1. *Purification of yeast dsRNA. Autoradiograph of a 1.4% agarose gel of crude yeast RNA isolated by phenol extraction of whole cells. The dsRNA's are indicated. Most of the remaining RNA is tRNA, 5S, and 5.8S RNA, although smaller quantities of 25S and 18S are present.*

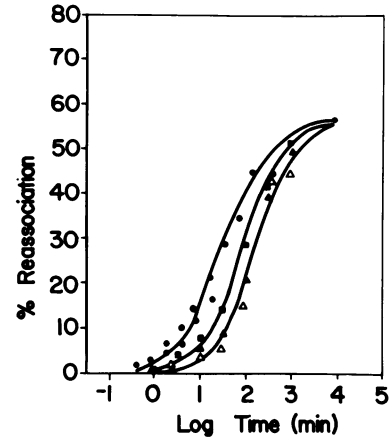

FIG. 2. *Renaturation kinetics of yeast DNA and L dsRNA. Reactions were carried out in a volume of 0.2 or 0.4 ml in sterile polypropylene tubes. Samples were denatured by boiling in water for 5 min; immediately after this, zero time points were taken. Samples were equilibrated without salt at 70°C for 10 min; the reaction was then started by adjusting the mix to 2× SSC (0.3 M sodium chloride, 0.03 M sodium citrate, pH 7). At the times indicated, points were taken into cold distilled water and subsequently assayed for S1 nuclease or RNase sensitivity. For analysis of single-copy DNA reassociation, samples were adjusted to final concentrations of 0.2 mM EDTA, 50 mM sodium chloride, 30 mM sodium acetate (pH 4.5), 0.6 mM zinc sulfate, and 0.052 N acetic acid and were then split into aliquots, one of which was treated with 10 U of S1 nuclease (Sigma) for 30 min at 37°C. For measurement of L renaturation, samples were adjusted to 2× SSC and split into aliquots, one of which was treated with 20 μg of pancreatic RNase per ml. Yeast DNA (1 mg) renatured with 5 μg of ^{3}H-labeled yeast single-copy DNA in 0.4 ml (●). L RNA (22 ng) renatured in a volume of 0.2 ml in the presence of 500 μg of yeast DNA (■). L RNA (22 ng) renatured in a volume of 0.4 ml in the presence (▲) and absence (△) of 1 mg of yeast DNA. Zero time values have been subtracted in all cases.*

and isopropanol precipitations (15). Before hybridization experiments, DNA was denatured by boiling in 0.2 N NaOH for 15 min and was then neutralized; this treatment provided DNA with an average size of about 1,000 nucleotides.

Figure 2 (●) shows the renaturation of ^{3}H-labeled, single-copy yeast DNA (prepared by nick translation; a gift from M. Rosbash) to a vast excess of unlabeled yeast DNA. The abscissa is expressed in units of time so that a direct comparison can be made with the rate of reassociation of L dsRNA in the same tube. S1 nuclease was used to determine the amount of labeled DNA remaining single stranded during the course of the reaction. It has been shown that when S1 nuclease is used to measure renaturation of randomly sheared DNA, the reaction does not follow ideal second-order kinetics but follows the form: $S/C_o = [1/(1 + kC_ot)]^{0.45}$ where S is the concentration of totally single-stranded DNA, C_o is the concentration of single-stranded DNA at the start of the reaction, t is time and k is the second-order rate constant (J. Morrow, Ph.D. thesis, Stanford University, Stanford, Calif., 1974). The continuous line which is fitted to the data (circles) in Fig. 2 presents an ideal fit by using the above relationship and taking the k value to be 0.17 M^{-1} s^{-1} and the end point of the reaction to be 60% reassociation. This is very close to the value of 0.23 M^{-1} s^{-1} predicted as the rate constant for yeast single-copy DNA under these conditions (13). Also shown in Fig. 2 is the renaturation of ^{32}P-labeled L dsRNA in the presence of yeast DNA that is renaturing at the rate shown by the circles. In one experiment (■) the concentration of the RNA was two times

higher than in a second experiment (▲). The observed rate of renaturation of the RNA was three times slower in the first case and six times slower in the second case than the reassociation rate of the single-copy DNA. L also renatured at the same rate in the presence (▲) or absence (△) of the renaturing yeast DNA. If we assume that RNA-DNA hybridization occurs at the same rate as DNA-DNA reassociation, we can conclude that there is at most one copy of the L sequence per six yeast genomes. If we assume that, as suggested, the rate of RNA-DNA hybridization lags behind DNA-DNA reassociation by a factor of 3 (7, 13), we can conclude that there is at most one copy of the L sequence per two copies of the yeast genome. It is difficult to fit the data of Vodkin to theoretical second-order kinetics. However, had we obtained the same result as Vodkin, we would have observed a 50- to 100-fold increase in the rate of reassociation of the L RNA in the presence of yeast DNA. The continuous lines that are fitted to the renaturation data obtained for the L RNA represent ideal second-order fits, taking the rate constant to be 560 M^{-1} s^{-1} and the final extent of the reaction to be 55%. These fits also allow for the fact that there is twice as much RNA in one case (■) as the other (▲). We can predict, by comparison with the renaturation rate of *Escherichia coli* DNA, that the second-order rate constant of renaturation for a dsDNA of the same complexity as L under these conditions would be about 640 M^{-1} s^{-1}.

We have repeated this experiment with α-[^{32}P]ATP-labeled single-stranded L to obtain greater sensitivity in the determination of L sequences in yeast DNA. Single-stranded RNA complementary to one of the strands of L was synthesized in vitro by using the transcriptase activity of purified L-containing VLPs (9). Reaction mixtures contained 0.5 mM UTP, CTP, and GTP, 3.86 μM α-[^{32}P]ATP at a specific activity of 259 Ci/mmol (Amersham), 50 mM Tris-hydrochloride (pH 7.4) 10 mM beta-mercaptoethanol, 5 mM $MgCl_2$, 0.1 mM EDTA, 20 mM NaCl, and a sucrose gradient fraction containing 0.8 absorbancy units at 260 nm of L VLPs in a total volume of 1 ml. Incubations were for 2 h at 37°C. L VLPs were extracted from strain S7 (C. McLaughlin) and grown to stationary phase by French pressing, low- and high-speed centrifugation, precipitation with polyethylene glycol, phase separation with dextran sulfate, removal of dextran sulfate with KCl, and sucrose gradient (5 to 20%) centrifugation, (procedure of M. Leibowitz, personal communication). Fractions with the highest transcriptase activity were used for the preparation of α-[^{32}P]ATP-labeled single-stranded L. The product of the reaction was isolated by phenol extraction, ethanol precipitation, and Sephadex G25 chromatography, and it was characterized by agarose gel electrophoresis, T1 fingerprinting, S1 nuclease digestion, and hybridization. The product ranged from 200 to 4,700 nucleotides in length (up to the size of denatured L) and averaged about 2,500 nucleotides in length; it was 97% sensitive to S1 nuclease; its T1 fingerprint included about 1/3 of the large T1 oligonucleotides of L; and it hybridized either to its template or to added L with a $R_0t_{1/2}$ close to that of in vivo labeled L (Fig. 3). The specific activity of the product single-stranded L was 1.3×10^8 dpm/μg. This RNA was 97% sensitive to S1 nuclease and hybridized with the expected kinetics to its template dsRNA (extracted along with the product single-stranded L) or to added L (Fig. 3). Neither calf thymus DNA nor yeast DNA alters the rate of hybridization of this labeled RNA to its template (Fig. 4). The rate of hybridization of the labeled probe to its template is 40 times slower than the rate of reassociation of yeast single-copy DNA in this experiment (Fig. 4). On the basis of the above assumptions, fewer than 1 copy of L sequence in 13 copies of the yeast genome and possibly as few as 1 copy of L sequence in 40 copies of the yeast genome are implied.

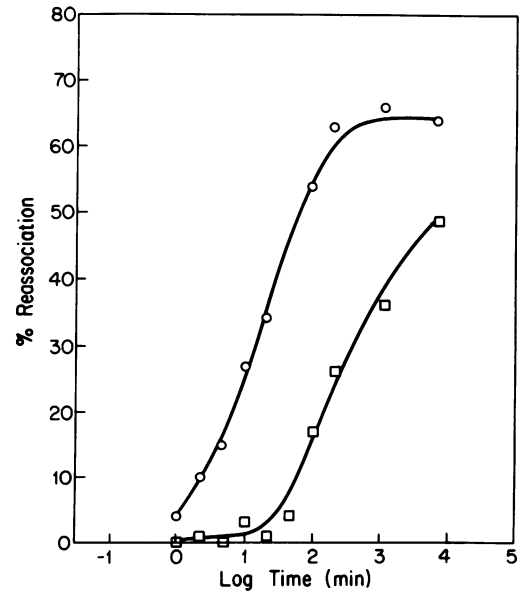

FIG. 3. *Approximately 2 ng of ^{32}P-labeled single-stranded L RNA was renatured in the presence* (○) *or absence* (□) *of 242 ng of added unlabeled L in a volume of 0.1 ml. In both cases, about 5 ng of unlabeled template L is also present. Conditions for the denaturing, renaturing, and assaying extent of duplex formation are essentially the same as described for Fig. 2.*

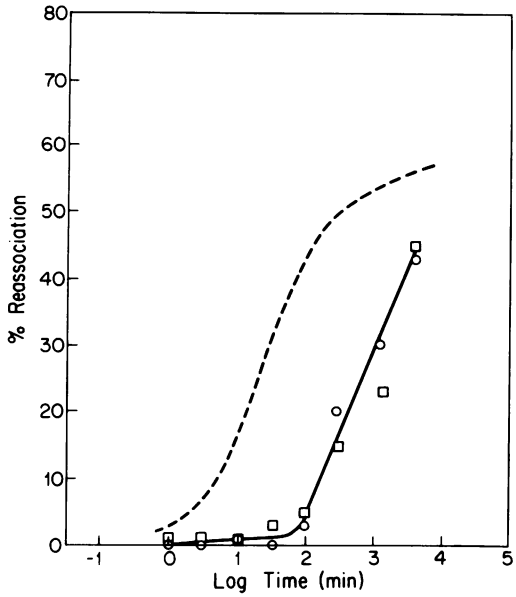

FIG. 4. *Approximately 1 ng of ^{32}P-labeled single-stranded L RNA was renatured in the presence of 900 μg of yeast DNA (○) or 900 μg of calf thymus DNA (□) in a volume of 0.360 ml. Conditions for the denaturing, renaturing and assaying extent of duplex formation were essentially the same as described for Fig. 2. The discontinuous line shows the rate of renaturation of single-copy yeast DNA at the concentration used in this experiment (taken from Fig. 2).*

We are unable to reconcile our results with those of Vodkin (18). The dsRNA and and the DNA used for our experiments renatured with kinetics close to those predicted. We have sufficient yeast DNA to allow a 20-fold (with dsRNA probe) or 100-fold (with single-stranded RNA probe) sequence excess over the L RNA if one copy of the L were present per copy of the yeast genome. We conclude that there is at most 1 copy of L per 13 yeast genome equivalents and possibly fewer than 1 copy of L per 40 yeast genome equivalents. A similar failure to detect yeast DNA species homologous to L has been reported by Wickner (23) and Hopper (personal communication).

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