Proc. Nat. Acad. Sci. USA
Vol. 70, No. 4, pp. 1069–1072, April 1973

A Nucleic Acid Associated with a Killer Strain of Yeast

(double-stranded RNA/cesium sulfate)

MICHAEL H. VODKIN AND GERALD R. FINK

Section of Genetics, Development, and Physiology, Cornell University, Ithaca, New York 14850

Communicated by Adrian M. Srb, January 26, 1973

ABSTRACT **A crude membrane fraction was isolated from both killer *M*(k) and isogenic nonkiller *M*(o) strains of yeast labeled with [^{3}H]adenine. Nucleic acids were extracted from this fraction and centrifuged to equilibrium in an ethidium bromide–Cs_2SO_4 solution. A peak of radioactivity coincident with a single fluorescent band was present in the membrane fraction isolated from cells of the killer strain but was absent from that isolated from nonkiller cells. This material has been characterized as double-stranded RNA by treatment with various nucleases and by chromatography on a cellulose CF-11 column. Sedimentation in a sucrose gradient indicates an S value of 8–10.**

Killer strains of yeast possess the following properties: (*a*) killing, (*b*) immunity, and (*c*) non-Mendelian inheritance. Killer cells, *M*(k), release a toxin that kills sensitive cells. The killer determinant (k) is an extrachromosomal element, that is dependent on a nuclear gene *M* for its maintenance. (1, 2).

Under conditions reported to delete mitochondrial DNA in yeast (3), killer cells still retain the extrachromosomal determinant (k). This result suggests that the killer determinant does not reside in mitochondrial DNA and does not require mitochondrial function for its expression. In a previous paper (2), it was shown that nonkiller *M*(o) strains can be derived from killer *M*(k) strains. A comparison of the nucleic acid profiles of these otherwise isogenic strains could provide information concerning the molecular structure of the killer determinant.

MATERIALS AND METHODS

Strains. The original killer strain A8209B ρ^+ *M*(k) has been described (1). Three other strains have been derived from it (Table 1). A8209B ρ^+ *M*(o) is a spontaneous nonkiller derivative. A ρ^- killer and a ρ^+ nonkiller were induced in the ρ^+ *M*(k) and ρ^+ *M*(o) strains, respectively, with prolonged ethidium bromide (EtBr) treatment (3).

Media. Yeast was grown in medium containing by weight 2% glucose, 0.5% yeast extract (Difco), and 1% bacto peptone (Difco). Radioactive labeling was done in a medium that contained 2% glucose and 0.7% yeast nitrogen base without amino acids (Difco), supplemented with 125 μM histidine.

To label nucleic acids, 50 ml of a late log-phase culture of the appropriate strain was inoculated into 1 liter of medium. 1 mCi of [8-^{3}H]adenine was added, and the culture was incubated at 30° for 16–18 hr (24–27 hr for ρ^- strain) in a gyrotory shaker.

Nucleic Acid Extraction and EtBr–Cs_2SO_4 Centrifugation. By the procedure of Clark-Walker (4), a crude membrane fraction was obtained from 25–40 g of yeast broken in a Braun homogenizer. EtBr at a concentration of 500 μg/ml was present in all buffers used during the isolation. The pellet was lysed in 2.5 ml of 1% Brij 35. To the lysate was added 0.5 ml 0.1 M EDTA (pH 7.0) + 2 ml of 1.75 mg/ml EtBr in 0.1 M sodium phosphate (pH 7.0). The solution was centrifuged at 22,000 × *g* for 20 min. The supernatant solution was decanted and 1.5 g of Cs_2SO_4 was added together with enough distilled H_2O to bring the final volume to 8 ml. The resulting solution was adjusted to an initial refractive index of 1.375 by addition of H_2O or Cs_2SO_4. The solution was again centrifuged as before. A pellicle floating on top was discarded, and the remaining solution was used for the density-gradient centrifugation.

EtBr–Cs_2SO_4 Density Gradient Centrifugation. The solution was placed in a polyallomer tube, overlayed with liquid paraffin, and centrifuged at 10° in a type-65 Spinco rotor at 40,000 rpm for 48 hr. After centrifugation the tubes were photographed with a UV-light source and then punctured. Fractions were collected, and aliquots thereof were monitored for radioactivity.

Radioactivity Assay of Fractions. 100 μg of bovine-serum albumin was added to each aliquot from the gradient. Nucleic acids were precipitated by the addition of excess cold 5% trichloroacetic acid and collected by filtration on 25-mm type-A Gelman glass-fiber filters. After drying, the radioactivity on the filters was determined on a Mark 1 Nuclear-Chicago liquid scintillation system. The scintillation cocktail consisted of toluene containing 0.4% 2,5-diphenyloxazole (PPO) and 0.05% 1,4-bis-2-(4-methyl-5-phenoxazolyl)-benzene (POPOP).

RNase Treatments. Appropriate fractions from an EtBr–Cs_2SO_4 centrifugation were pooled, diluted 5-fold with distilled H_2O containing 50 μg/ml of carrier tRNA, and precipitated with 2 volumes of cold 95% ethanol. The pellet was collected by centrifugation at 10,000 × *g* for 5 min and resuspended in distilled H_2O. The nucleic acid was treated with 20 μg/ml of pancreatic RNase in final salt concentrations of either 0.1 × SSC (SSC, 0.15 M NaCl–0.015 M sodium citrate) or 2 × SSC for 30 min at 20°. Treatment with RNase III

Abbreviations: EtBr, ethidium bromide; SSC, 0.15 M NaCl–0.015 M sodium citrate.

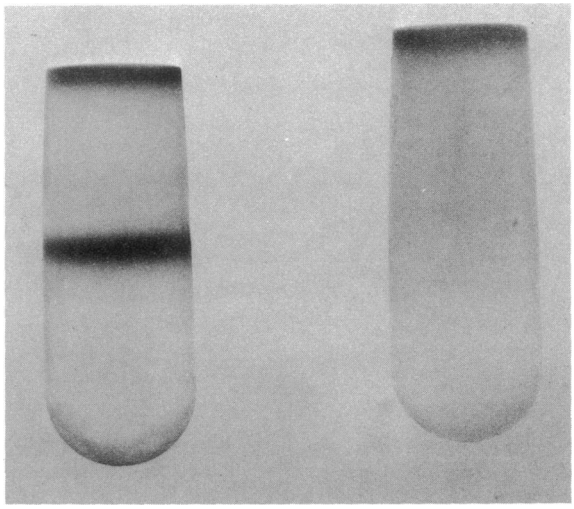

FIG. 1. Ultraviolet photograph of EtBr–Cs_2SO_4 gradients from killer (*left*) and nonkiller (*right*) strains. A membrane preparation from 25–40 g of either killer or nonkiller strains was isolated (4). This fraction was lysed in 1% Brij 35, and the lysate was centrifuged in an EtBr–Cs_2SO_4 solution until equilibrium was reached. Then the gradients were photographed with UV illumination.

was performed according to Robertson, Webster, and Zinder (5). Treatment with alkali was at a final concentration of 0.3 N NaOH for 30 min at 37°. As standards [^{14}C]tRNA (single-stranded) and ^{3}H-labeled polio virus RNA (double-stranded, replicative form) were treated with nucleases.

Cellulose CF-11 Chromatography was performed according to Robertson *et al.* (5), except that distilled H_2O was used in the final wash.

Chemicals. [*8*-^{3}H]Adenine was obtained from New England Nuclear Corp., Boston, Mass. EtBr and pancreatic RNase were obtained from Calbiochem, Los Angeles, Calif. Brij 35 was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. Cs_2SO_4 was obtained from Harshaw Chemical Co., Solon, Ohio. The double-stranded, ^{3}H-labeled replicative

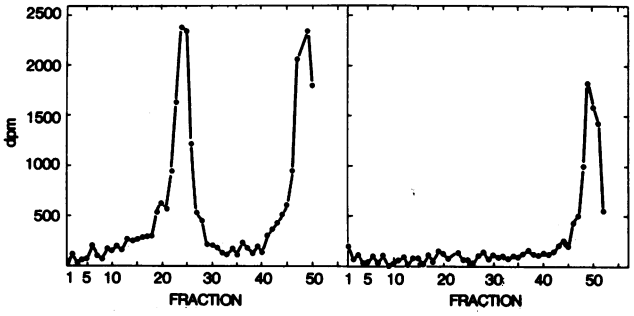

FIG. 2. Radioactive profiles of EtBr–Cs_2SO_4 gradients from killer (*left*) and nonkiller (*right*) strains. About 5 g of cells grown in 1 mCi of [*8*-^{3}H]adenine was mixed with 20–35 g of carrier cells. The procedure used in Fig. 1 for isolating membranes was followed. After centrifugation, tubes were punctured and fractions were collected. Nucleic acids of each fraction were precipitated by excess cold 5% trichloroacetic acid, collected on glass-fiber filters, and monitored for radioactivity. Centrifugation of an extract from a mixture of labeled killer with unlabeled nonkiller cells gave a radioactive fluorescent band; centrifugation of an extract from a mixture of unlabeled killer with labeled nonkiller cells gave a nonradioactive fluorescent band.

TABLE 1. *Origin of strains used in this study*

Killers		Nonkillers
A8209B $\rho^+ M$(k)	spontaneous →	A8209B $\rho^+ M$(o)
↓EtBr		↓EtBr
A8209B $\rho^- M$(k)		A8209B $\rho^- M$(o)

form of polio virus RNA was prepared in the laboratory of David Baltimore with the aid of Charles Cole. RNase III was purified up to the 0.2 M supernatant step in the procedure of Robertson *et al.* (5) from *Escherichia coli* MRE 600, kindly provided by these authors.

RESULTS

The EtBr–Cs_2SO_4 gradients run on crude membrane lysates of killer or nonkiller strains were clearly different in that the killer strain gradient contained a fluorescent band that was missing in the nonkiller strain gradient (Fig. 1). This difference is seen whether or not the respective killer and nonkiller strains are ρ^+ or ρ^-. The radioactive profiles of the above gradients also reveal a distinct difference between killer and nonkiller strains (Fig. 2). Gradients from the killer strains contain a peak coincident with the fluorescent band that cannot be detected in the nonkiller strains. When aliquots of the radioactive fractions are rerun in an EtBr–Cs_2SO_4 gradient the radioactive peak again appears in the middle of the gradient. In addition, all gradients display variable amounts of a radioactive peak at the top.

The properties of the radioactive nucleic acid unique to killer strains were analyzed. The nucleic acid was degraded both by pancreatic RNase in low-salt concentration and by alkaline hydrolysis (Table 2). Therefore, we conclude that the nucleic acid in question is RNA. Its resistance to pancreatic RNase in high salt and sensitivity to RNase III indicate that the RNA is double-stranded. In the above experiments tRNA and polio virus RNA served as single- and double-stranded RNA controls, respectively. As an additional criterion for double-stranded RNA, the nucleic acid was characterized by cellulose column chromatography. To calibrate the column, a mixture of [^{14}C]tRNA, ^{3}H-labeled polio virus RNA, and unlabeled rRNA was chromatographed. Transfer RNA eluted with 35% ethanol, rRNA with 15% ethanol, and polio RNA only with distilled water. The recovery of polio virus RNA in this fraction was 80% of the input. Labeled nucleic acid from the killer strains, fractionated on an EtBr–Cs_2SO_4 gradient, was applied to an identical column of cellulose. As shown in Fig. 3, radioactivity was eluted only by the aqueous solvent, in the double-stranded region of the chromatogram.

The sedimentation velocity of the nucleic acid and its homogeneity with respect to molecular weight were determined by centrifugation in a 5–25% sucrose gradient. A single radioactive peak appears in the gradient with an apparent sedimentation coefficient of 8–10 S (Fig. 4). From the above data we conclude that the double-stranded RNA has a unique sedimentation velocity in sucrose.

By the procedures used in this study, a species of double-stranded RNA can be isolated reproducibly (8 times) from the membrane fraction of killer strains. However, both killer and

TABLE 2. *Effect of nucleases on nucleic acids*

Treatment	Nucleic acid		
	[^{14}C]tRNA	^{3}H-Labeled polio RNA	^{3}H-Labeled killer strain
None	2470	6400	7000
Pancreatic RNase (low salt)	135	0	345
Pancreatic RNase (high salt)	115	6100	6400
RNAase III	2100	105	350
Alkali	120	70	500

[^{14}C]tRNA, polio virus[^{3}H] RNA, or [^{3}H]nucleic acid from killer strain were subjected to various treatments. The numbers in the table represent cold acid-insoluble dpm after treatment of each nucleic acid. Pancreatic RNase at a final concentration of 20 μg/ml was incubated with nucleic acids for 30 min at 20° in salt concentrations of either 0.1 × SSC or 2 × SSC. Treatment with RNase III was performed according to (5). Alkaline treatment was at a final concentration of 0.3 N NaOH for 30 min at 37°. Incubations were terminated by addition of 100 μg of bovine-serum albumin and excess of cold 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. The average counting efficiency was 85% for ^{14}C and 40% for ^{3}H. Treatment with DNase solubilized labeled bacterial DNA but did not affect labeled killer RNA.

nonkiller strains contain other species of double-stranded RNA. When either total RNA (phenol extracted) or the supernatant from the crude membrane fraction is examined in an EtBr–Cs_2SO_4 gradient, many fluorescent bands appear, and no reproducible difference between killer and nonkiller strains is observed. As an alternate isolation procedure, total RNA was chromatographed on a cellulose column. Elution profiles from killer and nonkiller strains were very similar. Nucleic acids appeared in the double-stranded RNA portion of the chromatograph. These nucleic acids acted like double-stranded RNA in their nuclease sensitivity but displayed considerable heterogeneity, with sedimentation values higher than that of the RNA unique to killer strains.

DISCUSSION

The fluorescent, radioactive band in the EtBr–Cs_2SO_4 gradients is associated specifically with the killer trait. The band has been extracted only from killer strains and not from spontaneously derived nonkiller strains. Curing a strain of mitochondrial DNA with EtBr alters neither the killing trait nor the fluorescent band. Thus, although the band is extracted from a membrane fraction (enriched for mitochondria in ρ^+ strains and promitochondria in ρ^- strains), its presence does not depend on mitochondrial DNA or functional mitochondria.

The band has been characterized as double-stranded RNA by three independent procedures. The exact relationship of this RNA to the killing trait is still uncertain. It could play a role in the replication and/or transcription of the information for the toxin produced by killer strains. Alternatively, double-stranded RNA could be present in both killer and nonkiller strains, but amplified or associated with this membrane fraction only in killer strains. Indeed, analysis of total RNA reveals the presence of double-stranded RNA in both killer and

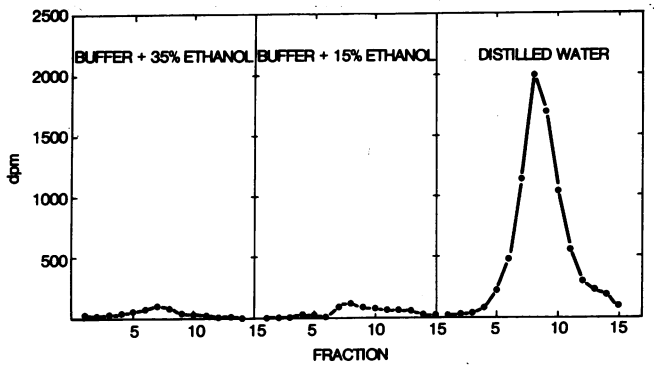

FIG. 3. Cellulose chromatography of labeled RNA from killer strain. Labeled RNA from killer strain, isolated from an EtBr–Cs_2SO_4 gradient as in Fig. 2, was suspended in 1 ml of 35% ethanol–65% 0.05 M Tris·HCl (pH 6.85)–0.1 M NaCl–0.001 M EDTA. The RNA was passed through a 10-ml column packed with cellulose CF-11. Fifteen 1-ml fractions were eluted with the same buffer; then 15-ml fractions were eluted with 15% ethanol–85% buffer; finally 15 1-ml fractions were eluted with distilled H_2O. All fractions were monitored for radioactivity.

nonkiller strains. However, the double-stranded RNAs common to both killer and nonkiller strains can be distinguished from that unique to killer strains in two respects: cellular localization and sedimentation coefficient.

Double-stranded RNAs have been described in yeast and other fungi (6–9). Berry and Bevan (10) studied independently isolated killer strains of yeast and found in the total nucleic acid extract a species of double-stranded RNA with an electrophoretic mobility on acrylamide gels similar to that of DNA and slower than that of the larger rRNA subunit. The difference between our RNA and that described by Berry and Bevan may be real or result from different isolation procedures and methods of characterization. In fact, the disparity between migration of double-stranded RNA on gels as compared with sucrose gradients has also been observed for reovirus RNA (11, 12).

All fungal viruses that have thus far been examined exhibit multiple double-stranded RNAs with different molecular weights. This situation may be analogous to the case for

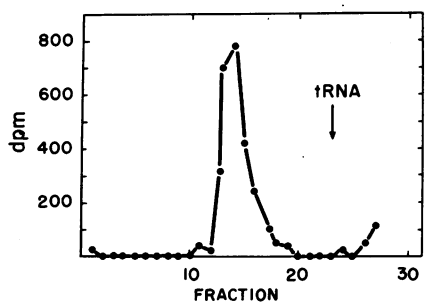

FIG. 4. Sedimentation in a sucrose gradient of labeled RNA from a killer strain. Labeled RNA from a killer strain was isolated from an EtBr–Cs_2SO_4 gradient. The RNA was precipitated with carrier tRNA and addition of 2 volumes of cold ethanol. The RNA was gently layered on 4.6 ml of a linear 5–25% sucrose gradient buffered with 0.01 M magnesium acetate (pH 5.2). Centrifugation was at 5° in an SW 65 Spinco rotor for 8 hr at 40,000 rpm. After centrifugation, the tube was punctured and fractions were collected. Each fraction was diluted with distilled H_2O and monitored for A_{260} and radioactivity.

reovirus, the genome of which is apparently composed of 10 separate pieces of double-stranded RNA. The RNA characterized by our study may represent only a portion of a segmented genome, that was detected by the procedures in this study. Alternatively, although our double-stranded RNA appears homogeneous with respect to buoyant density and sedimentation coefficient, it may be heterogeneous with respect to information content. A combined genetic and biochemical approach should distinguish between these hypotheses.

We thank Dr. Leon Heppel and Dr. David Wilson for critical comments on the manuscript. This research has been supported by Grant GB36644 NSF and Grant GM-1035-10 NIH.

1. Bevan, E. A. & Makower, M. (1963) *Proc. Int. Congr. Genet. 11th* 1, 202.
2. Fink, G. R. & Styles, C. A. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 2846–2849.
3. Goldring, E. S., Grossman, L. I., Krupnick, D., Cryer, D. R. & Marmur, J. (1970) *J. Mol. Biol.* **52**, 323–335.
4. Clark-Walker, G. D. (1972) *Proc. Nat. Acad. Sci. USA* **29**, 388–392.
5. Robertson, H. D., Webster, R. E. & Zinder, N. D. (1968) *J. Biol. Chem.* **243**, 82–91.
6. Banks, G. T., Buck, K. W., Chain, E. B., Himmelweit, F., Marks, J. E., Tyler, J. M., Hollings, F., Last, F. T. & Stone, O. M. (1968) *Nature* **218**, 542–545.
7. Banks, G. T., Buck, K. W., Chain, E. B., Darbyshire, J. E. & Himmelweit, F. (1969) *Nature* **223**, 155–158.
8. Lhoas, P. (1972) *Nature New Biol.* **236**, 86–87.
9. Border, D. J. (1972) *Nature New Biol.* **236**, 87–88.
10. Berry, E. A. & Bevan, E. A. (1972) *Nature New Biol.* **239**, 279–280.
11. Bellamy, A. R., Shapiro, L., August, J. T. & Joklik, W. K. (1967) *J. Mol. Biol.* **29**, 1–17.
12. Bellamy, A. R. & Joklik, W. K. (1967) *J. Mol. Biol.* **29**, 19–26.