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Two New Double-Stranded RNA Molecules Showing Non-Mendelian Inheritance and Heat Inducibility in *Saccharomyces cerevisiae*

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Certain strains of *Saccharomyces cerevisiae* were found to have a complex nuclear defect (designated clo$^-$) that makes cells unable to maintain some L-B and some L-C double-stranded RNAs at 25°C. The clo$^-$ strains were not defective in maintenance of L-A, M_1, or M_2 double-stranded RNAs. Most clo$^-$ strains lacking L and M carry small amounts of two double-stranded RNA species intermediate in size between L and M and denoted T (2.7 kilobase pairs) and W (2.25 kilobase pairs). Some strains carry both T and W, some carry neither, and some carry only W; no strains carrying only T have been found. Both T and W show 4+:0 segregation in meiosis and efficient transmission by cytoplasmic mixing (cytoduction), indicating that they are non-Mendelian genetic elements. T and W do not cross-hybridize with each other or with L-A, L-B, L-C, M_1, M_2, or chromosomal DNA. T and W are apparently distinct from other known non-Mendelian genetic elements (2μ DNA, [*rho*], [*psi*], 20S RNA, [URE3]). In most strains the copy number of both T and W is increased about 10-fold by the growth of cells at 37°C. This heat inducibility of T and W is under control of a cytoplasmic gene. T and W double-stranded RNAs are not found in a purified L-containing virus-like particle preparation from a strain containing L-B, T, and W double-stranded RNAs. The role, if any, of T or W in the killer systems is not known.

The killer system of yeast is unique among eucaryotic virus and plasmid systems in the detail in which interactions among viral components and host components have been explored and the number of host components known to be involved. This reflects the ease with which these aspects can be investigated in *Saccharomyces cerevisiae* more than any unusually close integration of virus and host. Although most double-stranded (ds) RNA viruses have 10 or more segments, almost all of which are dependent on all the others for their propagation, the situation in *S. cerevisiae* was thought to be much simpler, with only two ds RNA species (L and M) and the former independent of the latter for its replication. However, the following nine distinct ds RNAs have now been described in *S. cerevisiae* (reviewed in references 5, 32, and 33). (i) M_1, M_2, and M_3 determine the secretion of and immunity to the K_1, K_2, and K_3 killer toxins, respectively (2, 3, 16, 27, 37). (ii) L-A-HN, L-A-HE, L-A-E, and L-A-X are closely related to each other in sequence, encode the major virus-like particle proteins, and carry various combinations of the cytoplasmic genes [HOK], [NEK], and [EXL] (4, 12, 19, 20, 31, 34, 36). (iii) L-B and L-C are the same size as the various L-A variants, but are unrelated to L-A in sequence. L-B and L-C show considerable sequence homology to each other (19, 20, 36).

We find that clo$^-$, a complex chromosomal defect, results in the loss of several forms of L-B and L-C. Minor ds RNA bands migrating between M and L ds RNA were previously observed (35), but were not characterized, and attempts to do so were complicated by the presence of large amounts of L ds RNA. Using clo$^-$ strains, we show that among these minor ds RNA species, two (called T and W) show non-Mendelian inheritance and are not dependent on any of the L or M ds RNAs for their maintenance or replication. T and W do not show detectable hybridization with each other, with any of the L or M ds RNAs, or with yeast DNA.

A single strain may carry five different ds RNA molecules as follows: at least one form of L-A, either L-B or L-C, T, W, and at least one of the M ds RNAs.

MATERIALS AND METHODS

***S. cerevisiae* strains and media.** The strains used are listed in Table 1. SD medium contained 0.67% yeast nitrogen base without amino acids (Difco Laboratories) and 2% dextrose, supplemented as needed with additional nutrients. YPAD contained 1% yeast extract, 2% peptone, 2% dextrose, and 0.04% adenine sulfate. YPG was the same as YPAD with dextrose replaced by 3% (vol/vol) glycerol. Agar plates contained 2% agar.

Genetic methods. Standard methods of meiotic analysis were used (17).

Cytoduction. A cytoplasmic genome can be transferred from one haploid strain to another without diploidization or other change of nuclear genotype by heterokaryon formation, using the *kar1* mutant, which is defective in nuclear fusion (6). Recipient cells were usually [*rho*^{0}] (mitochondrial DNA eliminated by growth on ethidium bromide), and donor cells were [*rho*$^+$]. After mating recipient and donor cells on YPAD plates for 6 h, cells with donor nuclei were counterselected by plating the mating mixture for single colonies on appropriate media. Diploids were screened out by replica plating to minimal medium, and [*rho*^{0}] clones were eliminated by replica plating to YPG plates. Respiratory competent clones having the recipient nuclear genotype were the cytoductants.

Scoring plasmid phenotypes. [URE3-1] was tested (1, 13) by transferring colonies to a freshly spread lawn of the *ura2*$^-$

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TABLE 1. Strains of *S. cerevisiae*

Designation	Genotype[a]	L species[b]	T, W species	Reference
1815	α *trp1*(Am) *pet18-1 met8-1*(Am) *SUP4-3* clo$^-$	L-o	NT	This work
S140	**a** *mkt1-1 lys11* [HOK-o(39°C)] [NEX-o(39°C)] [KIL-o]	L-B	T, W	(20)
1773	**a** *arg9* [HOK-o] [NEX-o] [KIL-o]	L-o	T, W	(20)
S161	**a** *lys11 mkt1* [HOK-o(39°C)] [EXL-o(39°C)] [KIL-o]	L-C	NT	(20)
AN33	α *arg1 thr1* [EXL] [HOK-o] [NEX-o] [KIL-o]	L-A-E + L-C	T-o, W-o	(31)
1686	α *arg1 thr1* [EXL-o(37°C)] [HOK-o] [NEX-o] [KIL-o]	L-C	T-o, W-o	(20)
A364A	**a** *ade1 ade2 lys2 tyr1 his7 ura1 gal1*, [KIL-K_1]	L-A-HN + L-(BC)	W	(34)
1019	**a** *leu1 kar1-1* [HOK-o] [NEX-o] [EXL-o] [KIL-o]	L-(BC)[c]	W	(6)
1020	α *his4-15 kar1-1* [HOK-o] [NEX-o] [EXL-o] [KIL-o]	L-(BC)[c]	W	(6)
2-14A	**a** clo$^-$ [HOK-o] [NEX-o] [EXL-o] [KIL-o]	L-o	W	This work
2-14B	α *lys1 trp1 pet18 mkt1 met8-1* clo$^-$ [HOK-o] [NEX-o] [EXL-o] [KIL-o]	L-o	W	This work
14-1B	α *lys1* clo$^-$ [HOK-o] [NEX-o] [EXL-o] [KIL-o]	L-o	T, W	This work
14-1B_4	14-1B [*rho*^{0}]	L-o	T, W	This work
37-4C	**a** *leu1 kar1-1* clo$^-$ [HOK-o] [NEX-o] [EXL-o] [KIL-o]	L-o[d]	T, W	This work
1920	**a** *arg9* [HOK] [NEX] [KIL-o]	L-A-HN	T, W	(20)
1066	α *pet18*	NT	NT	This work
1160(JM6)	**a** *ade2-1 lys1-1 his4-580 met8-1 SUP4-3 mkt1* [HOK-o] [NEX-o] [EXL-o] [KIL-o] [PSI]	L-o	T-o, W-o	(26)
1635	α *lys*(1, x) *aro7 mak3-1* [HOK-o] [NEX-o] [EXL-o] [KIL-o]	L-(BC)	W	This work

[a] [*rho*^{0}], Lacking mitochondrial DNA. The elimination of mitochondrial genome was accomplished by growth in the presence of ethidium bromide. All other strains are [*rho*$^+$]. [XXX], Presence of the cytoplasmic genome. [XXX-o], Absence of the cytoplasmic genome. [XXX-o(39°C)], Cured of [XXX] by growth at 39°C.

[b] L species are known for strains that have been analyzed by T_1 fingerprints (20). L-o, Absence of L ds RNA. L-(BC) indicates the strain contains an L species other than L-A, but it is not known whether this is L-B, L-C, or some other L. NT, Not tested.

[c] The L species contained in both strains 1019 and 1020 are sensitive to the clo$^-$ defect.

[d] Some subclones of 37-4C, when grown at 37°C (a permissive temperature for the cold-sensitive clo$^-$ defect), were L-o, indicating a complete loss of L. Other subclones had low levels of L. The L-o subclones were used in the cytoduction experiments, whereas the clones carrying low levels of L were used in the virus-like particle experiments (Fig. 7).

diploid strain 1065 on SD agar plates supplemented after autoclaving and cooling with DL-ureidosuccinic acid (100 μg/ml) and with nutrients required by the strains tested, but lacking uracil; the lawn grew only around [URE3-1] colonies, which take up ureidosuccinic acid and excrete uracil under these conditions (1), but not around [ure3$^+$] colonies.

ds RNA extraction and purification. RNA was either extracted from intact cells by the rapid method of Fried and Fink (9) or extracted and then purified by cellulose chromatography and analyzed on agarose gels.

In the rapid method, after washing in 50 mM sodium EDTA, the cells were treated for 15 min in 50 mM Tris–H_2SO_4 (pH 9.3) with 2.5% 2-mercaptoethanol, lysed in 0.1 M NaCl–10 mM EDTA–10 mM Tris-hydrochloride (pH 7.5)–0.2% sodium dodecyl sulfate, and extracted with 1 volume of phenol for 1 h. The nucleic acids of the aqueous phase were then precipitated with ethanol at −20°C. Ethanol precipitates were dissolved in Tris-acetate buffer and fractionated on 1% agarose gels. The other method consisted of extracting nucleic acids from spheroplasts. The cells were grown to the stationary phase in liquid medium and after washing were suspended in 2 ml of 1.2 M sorbitol, 0.3 ml of 1 M KPO_4 (pH 7.6), 0.03 ml of 2-mercaptoethanol, and 0.1 mg of zymolyase 60,000 per g (wet weight) of cells. After 30 min to 2 h of incubation at 30°C, the protoplasts were centrifuged at 5,000 rpm for 5 min and thoroughly suspended in 3 ml of 2% SDS, 0.3 M NaCl, 0.1 M EDTA, 0.2 ml of 1% bentonite, 1 ml of water, and 1 mg of proteinase K per g (wet weight) of cells. After about 20 h of incubation at 45°C, the proteinase K-digested material was extracted with phenol for 1 h at room temperature.

The nucleic acids were precipitated by adding 2 volumes of ethanol at −20°C. The ethanol precipitate was dissolved in 0.1 M NaCl–1 mM EDTA–50 mM Tris-hydrochloride (pH 7.3)–15% ethanol, loaded on a CF11 column (8), rinsed extensively, and eluted with the same buffer without ethanol. The peak fractions were concentrated by precipitation with 2 volumes of ethanol at −20°C overnight.

For labeling, the ds RNAs were purified by two cycles of chromatography on CF11-cellulose.

RNase and DNase treatments. The ds RNA fraction from cellulose chromatography of nucleic acids was adjusted to 5 mM $MgCl_2$ and digested at 37°C for 1 h under the following conditions. For DNase I digestion, 10 μg of DNase I (Worthington Diagnostics) per ml was used. After the incubation EDTA was added to a final concentration of 10 mM. For RNase A digestion, samples were digested with 100 μg of pancreatic RNase per ml, with or without 0.8 M NaCl. After treatment the RNase was inactivated as previously described (35).

RNA blot. The transfer of RNA to nitrocellulose filters was performed by the method of Thomas (22).

DNA preparation. Total yeast DNA was prepared from 200-ml cultures of cells. After spheroplasts were formed as described above, cells were suspended in 6.4 ml of 10 mM Tris (pH 7.4), 1 mM EDTA (pH 8.5), 0.32 ml of 2 M Tris, and 0.32 ml of 10% sodium dodecyl sulfate. The mixture was then heated at 65°C for 30 min; 1.6 ml of 5 M potassium acetate was added, and the mixture was left at 0°C for 1 h. After centrifugation in a Sorvall SS34 rotor at 20,000 rpm/min for 20 min at 4°C, the supernatant was precipitated with 2 volumes of ethanol at room temperature. The resulting pellet was suspended in 10 mM Tris (pH 7.4)–1 mM EDTA containing 100 μg of pancreatic RNase per ml. The sample was then treated with proteinase K and extracted with phenol. DNA was precipitated from the aqueous phase with ethanol.

Restriction enzyme analysis and DNA blots. Restriction enzyme digestion of DNA and electrophoresis were performed as described previously (30). After electrophoresis of

the digests on agarose gels, the DNA fragments were transferred to nitrocellulose filters (21).

Labeling of RNA and hybridization. The 5′ ends of RNA were labeled with [γ-^{32}P]ATP and T_4 polynucleotide kinase after alkali shearing (0.05 M Na_2CO_3, 60 min, 50°C) by the method of Goldbach et al. (11). Labeled RNA was hybridized to RNA and DNA blots under the following conditions: 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–50 mM $NaPO_4$ (pH 6.5)–1× Denhart solution, 42°C, 20 to 24 h. After hybridization the filters were washed four times for 5 min at room temperature in 2× SSC–0.1% sodium dodecyl sulfate and twice for 15 min at 50°C in 0.1× SSC–0.1% sodium dodecyl sulfate. The radioactive filters were then autoradiographed with Kodak X-ray film (XAR-5).

Purification of virus-like particles. A subclone of strain 37-4C containing a small amount of L-B ds RNA was grown at 37°C to amplify the T and W ds RNAs. The procedure of Welsh et al. (29) was used, with the following modification. Cells were treated with 0.1 mg of zymolyase 60,000 per g (wet weight) in buffer A containing 0.15 M NaCl before breaking in the French press as described previously (29). The final 10 to 40% sucrose gradient was run at 24,000 rpm for 14 h, and 23 fractions of about 1.6 ml each were collected and analyzed. RNA was extracted from 0.6 ml of each fraction. Virion RNA was solubilized by suspension in 0.1 M EDTA–1% Sarkosyl-containing bentonite (0.1 mg/ml), followed by extraction with 2 volumes of phenol. RNA in the aqueous phase was precipitated with 2 volumes of ethanol at −20°C. The precipitate was dissolved in 40 μl of 50 mM Tris-acetate buffer (pH 7.4) and loaded on a 1% agarose gel. RNA polymerase was assayed on 20 μl of each fraction as described previously (29) (see Fig. 7A and B).

To compare the particle association of L, T, and W ds RNA in crude extracts, 3 ml of the S16 crude supernatant fraction of Welsh et al. (29) was loaded on a sucrose gradient run as described above. A 0.3-ml sample of each fraction of the sucrose gradient was digested with proteinase K at 37°C for 3 h (200 μg of proteinase K per ml, 5 mM EDTA, 0.5% Sarkosyl). This was followed by two phenol extractions, and the aqueous phase was precipitated with 2 volumes of ethanol. The precipitates each were dissolved in 75 μl of 0.05 M Tris-acetate (pH 7.4), and half of each sample was loaded on a 1% agarose gel. A parallel gradient containing 50 μg of purified T and W ds RNAs (from strain 1773) was run. Half of each fraction was ethanol precipitated and dissolved in 50 μl of 0.05 M Tris-acetate (pH 7.4), and half of each sample was loaded on a 1% agarose gel (see Fig. 7C).

RESULTS

clo$^-$, a chromosomal defect resulting in loss of certain L-B and L-C ds RNAs. A cross of strains 1066 and 1160, done as part of an earlier study, unexpectedly showed 2 L:2 L-o segregation. Crossing one of the L-o segregants (strain 1815) with strains carrying only L-B (strain S140) or only L-C (strains 1686 and S161) or only L-A (various strains) showed frequent segregation of L-o clones only in the crosses involving the L-B strain S140. We call this chromosomal defect clo$^-$ (chromosomal L-o). The *pet18* and *mkt1* mutations, also segregating in the crosses, were unlinked to clo$^-$, and both L-B and L-C were maintained in pet18 segregants. Likewise, crossing a *mak10* L-o strain with strains carrying L-B and the L-C of strain S161 showed both these molecules to be independent of *MAK10*. Crossing clo$^-$ strains with K_1^+ or K_2^+ strains produced only K_1^+ and K_2^+ segregants. Apparently, neither M_1 nor M_2 ds RNA needs the CLO$^+$ genotype.

The cross 1815 × S140 between clo$^-$ L-o and L-B suggested that clo$^-$ was a two-gene defect (4 L-B:0 [four tetrads], 3 L-B:1 L-o [five tetrads], 2 L-B:2 L-o [5 tetrads]). To test this, each of the spore clones from a 4 L-B:0 tetrad was crossed with clo$^-$ L-o segregants from the 2 L-B:2 L-o tetrads. If clo$^-$ were due to defects in two chromosomal genes, then each of these four crosses should show 2 L-B:2 L-o segregation. In fact, only one of the crosses gave this result (Fig. 1), whereas two others showed 4 L-B:0 segregation, and the fourth showed multigene segregation. These results indicate that clo$^-$ is due to at least two (and probably more) chromosomal defects.

The backcrosses indicate that the clo$^-$ defect is thermosensitive: the loss of L-B occurred at 25°C and not at 30°C. We could not detect this effect of temperature in the original crosses because the *pet18* mutation, which confers temperature sensitivity for growth, was involved. It is also possible that in some strains the clo$^-$ defect affects copy number without producing a complete loss of L-B, because sometimes on returning a strain to a higher temperature (37 or 30°C), L reappears. Its failure to reappear in most cases indicates that it was completely lost; a mere decrease in copy number would presumable be reversible.

Cytoduction of the L from the *kar1* strains 1019 or 1020 into clo$^-$ L-o strains produced almost exclusively L-o cytoductants, indicating that these Ls also require CLO$^+$. We then constructed a *kar1* clo$^-$ L-o strain and tested by cytoduction the dependence of L-A, L-B, and the two L-Cs on CLO. Neither L-B, the L-C from 1686, nor the L-C from S161 was maintained in the *kar1* clo$^-$ strains, whereas L-A was maintained. L-B and both L-Cs are efficiently transferred by cytoduction into CLO$^+$ strains. This *kar1* clo$^-$ L-o strain can be used to test the L in any strain for its dependence on CLO.

Characterization of minor ds RNAs, T, and W. When *S. cerevisiae* ds RNAs purified by CF11 chromatography (see above) are analyzed by agarose electrophoresis, several

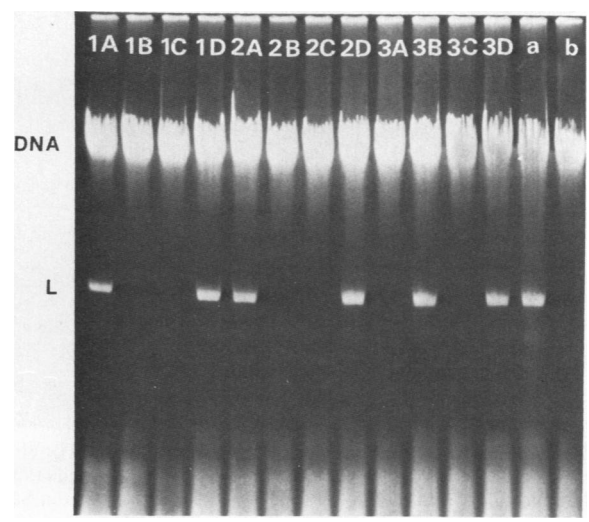

FIG. 1. The 2:2 segregation of clo$^-$; L-B segregation in one backcross (see text). Lanes: a, L-B parent; b, clo$^-$ L-o parent; 1A through 3D, L-B ds RNA composition of three tetrads. RNA was extracted from equal weights of each strain by the method of Fried and Fink (9) and electrophoresed on a 1% agarose gel.

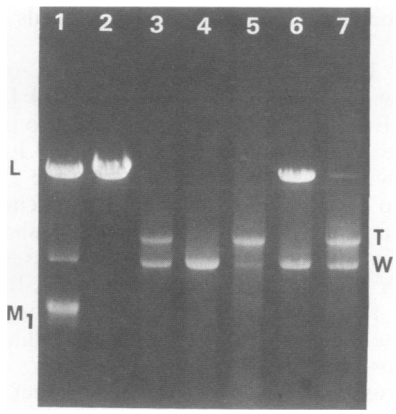

FIG. 2. Agarose gel electrophoresis (1%) of ds RNAs purified by chromatography on CF11 cellulose. Lanes: 1, strain A364A [L-A-HN, L-(BC), M_1, W]; 2, strain AN33 (L-A-E, L-C); 3, strain 1773 (T, W); 4, strain 2-14B (W); 5, strain 14-1B$_4$, an [*rho*^{0}] derivative of 14-1B (T, W); 6, strain 1019 (L-B, W); 7, strain 14-1B (T, W).

minor bands can often be seen in addition to the nucleic acids of the killer system (reference 35 and Fig. 2). To test the complete loss of L-B in the clo$^-$ strains, the ds RNAs of some of these strains were purified and concentrated on cellulose columns and analyzed with agarose gel (Fig. 2). This showed that two of the minor bands (called T and W) could be maintained without L.

T and W were degraded by treatment with pancreatic RNase at low salt, but not by treatment with pancreatic RNase at high salt or with DNase (Fig. 3). Under the high salt conditions used, pancreatic RNase degrades single-stranded rRNA. Thus, these two bands are ds RNAs, based on their ability to bind to the CF11 column and their sensitivities to nucleases. Their migration on agarose gels before and after denaturation indicates that the sizes of T and W ds RNAs are approximately 2.7 and 2.25 kilobase pairs, respectively, assuming they are linear and with native and denatured L and M ds RNA and 18S RNAs as standards.

Presence of T and W bands in different strains. Because of their low copy number, T and W are not detected by the rapid method of RNA preparation (9). To test for the presence of T and W in different strains of our collection such as *mak*, *ski*, or *kex* mutants, we labeled a mixture of T and W ds RNA as described above. The ds RNAs thus labeled were hybridized to Northern blots of ds RNA preparations of various strains.

The strains we tested fell into three categories with respect to their TW band composition: (i) the presence of T and W; (ii) the presence of W only; and (iii) the absence of both T and W. Out of 80 strains we examined, none carried T alone. Also, we could detect the W species in all of the *mak*$^-$, *ski*$^-$, and *kex*$^-$ strains tested. These experiments also show that there is no detectable cross-hybridization between T or W and any of the L or M ds RNAs.

Cytoplasmic inheritance of T and W ds RNAs. Crosses between strains having both T and W and strains having neither were performed. The tetrads from such crosses showed a 4:0 pattern of segregation for the presence of T and W (nine tetrads). This non-Mendelian inheritance has been confirmed by cytoduction. The *kar1* clo$^-$ strain 37-4C that was constructed possesses T and W ds RNAs only. The cytoplasm of this strain was transferred to the cytoplasm of a [*rho*^{0}] derivative of AN33 that is T-o W-o. All 14 cytoductants tested received both T and W ds RNAs (Fig. 4).

Heat inducibility of T and W ds RNAs. During this study we observed that in certain strains the amount of T and W can be increased when the cells are grown at 37°C, whereas the amount of L or M is unchanged. The increase is at least 10-fold, and as a result the T and W bands can be detected on ethidium bromide-stained agarose gels of rapid RNA preparations (Fig. 5). However, this heat inducibility was not observed for all strains. To see whether any nuclear control was involved in this phenomenon, two crosses between heat-inducible strains and noninducible strains were done

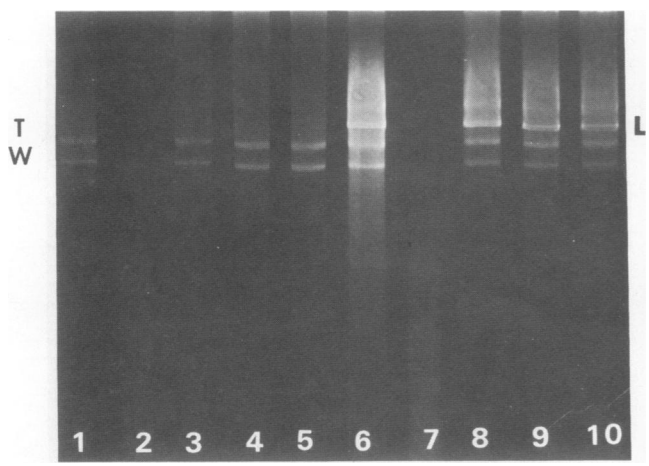

FIG. 3. Sensitivity to nucleases of the T and W bands. The eluate of the CF11 column of one strain having only T and W bands (strain 2-14A) and one strain having in addition L-B ds RNA (strain S140) were treated as described in the text before electrophoresis on a 6% acrylamide gel. Lanes: 1 through 5, strain 2-14A; 6 through 10, strain S140; 1 and 6, 10 μg of ds RNA incubated with RNase and 0.8 M NaCl; 2 and 7, incubation with RNase without NaCl; 3 and 8, incubation with 0.8 M NaCl; 4 and 9, incubation with DNase; 5 and 10, incubation without additions.

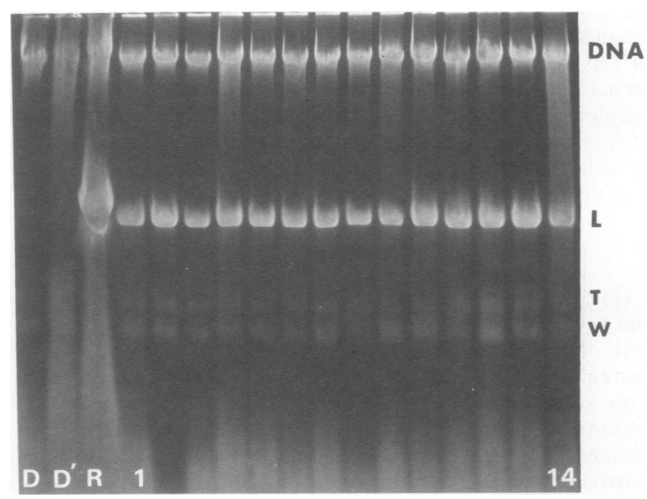

FIG. 4. T and W ds RNAs are cytoducible. D-D', Donor strain, 37-4C; R, T-o W-o recipient strain (a [*rho*^{0}] derivative of AN33) (see also Fig. 6, lane 2); 1 through 14, cytoductants. In this experiment we took advantage of the heat inducibility of the T and W bands to detect them in crude preparations (see the text). For all strains, ds RNA was extracted by the rapid method (9) and run on a 1% agarose gel.

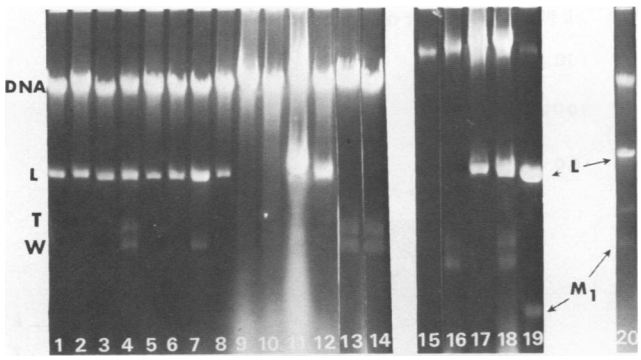

FIG. 5. T and W ds RNAs are heat inducible. Strains were grown at different temperatures, and the RNAs were extracted from equal weights of cells. Except for lanes 9 through 12, which were sixfold overloaded, equal samples were electrophoresed on 1% agarose gels. Lanes: 1 thourgh 4, strain S140 grown at 20°C (1), 25°C (2), 30°C (3), and 37°C (4); 5 through 8, strain 1019 grown at 25°C (5), 30°C (6), and 37°C (7) and cells coming from a culture at 37°C and then grown at 20°C (8); 9 and 10, strain 1773 grown at 25°C (9) and 37°C (10); 11 and 12, strain 1635 grown at 25°C (11) and 37°C (12); 13, [*rho*^{0}] derivative of strain 14-1B grown at 37°C; 14, [*rho*^{0}] derivative of strain 37-4C grown at 37°C; 15 and 16, strain 14-1B grown at 25°C (15) and 37°C (16); 17, same as 2; 18, same as 4; 19 and 20, strain A364A grown at 25°C (19) and 37°C (20). Strains S140 and 1019 show the inducible pattern, whereas strains 1773 and 1635 are noninducible. These noninducible strains have the same basal levels of T and W as do inducible strains, but no change is seen on shifting to 37°C.

(1773—T, W noninducible × 14-1B—T, W inducible, 1635—W noninducible × A364A—W inducible). In each case, the segregation was 4 heat inducible:0 (four tetrads for each cross). This result indicates that the plasmids themselves or some other nonchromosomal genetic element is responsible for the heat inducibility. When available, this temperature effect is very useful for the detection of the plasmids.

Homology of T and W ds RNA with each other or nucleic acids of the cell. We investigated whether there was sequence homology of the T and W ds RNAs with each other on the one hand, and with other nucleic acids of the cell on the other hand. Three different ds RNA preparations were labeled as described above and used as probes: one was the CF11 eluate of a strain having only T and W ds RNA (14-1B). The other two were T and W bands (from the same strain, 14-1B) separated by electrophoresis and excised from the gel, and the purified individual ds RNAs were labeled.

When Northern blots of ds RNAs were probed with T or W ds RNA, no cross-hybridization was detected between T and W and between T and W and the other ds RNAs, L-A, L-B, L-C, and M_1 (Fig. 6). Southern blots were made from restricted DNA purified from two strains: 14-1B, which contains T and W; and AN33, which lacks both ds RNAs. No sequences complementary to T and W were detected with nuclear, mitochondrial or 2μ DNA. Minor variations in the size of T and W were also seen from strain to strain.

Relation of T and W to other non-Mendelian genetic elements. Several other genetic elements and molecules showing non-Mendelian inheritance have been previously described. The T and W ds RNAs are not products of the mitochondrial genome since [*rho*^{0}] derivatives of strain 14-1B and 37-4C, produced by growth in the presence of ethidium bromide, still had both T and W ds RNAs. In addition, *pet18* strains, which are known to lack the mitochondrial DNA (14), possess both T and W ds RNAs. Neither the T nor the W ds RNA hybridized to 2μ DNA on Southern blots. Furthermore, whereas 2μ DNA is transferred by cytoduction at only 50% efficiency (15, 23), both T and W are transferred at 100% efficiency. Also, two strains known to carry 2μ DNA and four known to lack 2μ DNA (24) were tested for T and W ds RNAs, and all six strains were found to have only W.

[*psi*] is a cytoplasmic genetic element that increases the efficiency of ochre suppression (7). The [psi] phenotype is eliminiated in 100% of clones by growth of cells at high osmotic strength (18) or in 5 mM guanidine hydrochloride (25). Strain 14-1B was grown to single colonies on YPAD containing 5 mM guanidine, and two colonies were tested for the presence of T and W ds RNA. Both T and W remained in both colonies at their previous levels. The content of T and W ds RNA of two [*psi*$^+$] and three known [*psi*$^-$] strains were compared, and all were found to have W. These data show that T is not [*psi*], but do not unequivocally eliminate W as a possible site of the [*psi*] gene since the [*psi*$^-$] strains produced by guanidine or found among various laboratory strains are not known to lack the [*psi*] nucleic acid.

[URE3] is assayed by its making uptake of ureidosuccinate insensitive to nitrogen repression (1, 13). The [URE3] genetic element is efficiently transmitted by cytoduction, but only about half of meiotic segregants of a cross of [URE3] and [URE3-o] strains have the [URE3] element (1, 13). This is unlike the T and W ds RNAs, which are transmitted to all meiotic segregants. We also compared the T and W ds RNAs in two [URE3-1] and two [ure3$^+$] strains. All four strains had W and lacked T. This lack of correlation of [URE3-1] is, as in the case of [psi], limited by the facts that [ure3$^+$] strains may or may not lack the [URE3-1] molecule and that [URE3-1] was originally isolated as a mutant.

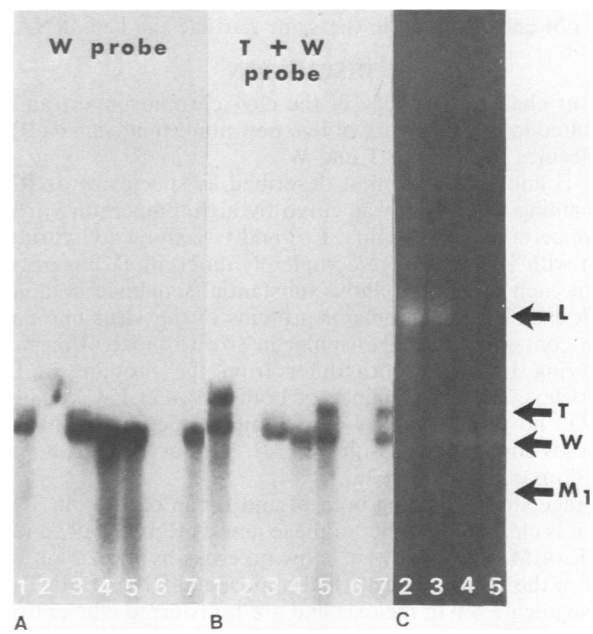

FIG. 6. Homology of T and W ds RNAs with each other and with other ds RNAs of the cell. The ds RNAs of different strains were denatured in 1 M glyoxal and then separated on 1% agarose gels and transferred to nitrocellulose. (A) Northern blot hybridized with labeled W ds RNA (see the text). (B) Northern blot hybridized with mixed probe. (C) Gel stained with ethidium bromide. Lanes: 1, strain S140 (L-B, T, W); 2, strain AN33 (L-A-E, L-C); 3, strain A364A [L-A-HN, L-(BC), M_1, W]; 4, strain 2-14B (L-o, W); 5, strain 1773 (L-o, T, W); 6, strain 1686 (L-C); 7, strain 14-1B (L-o, T, W).

20S RNA is a species synthesized by many strains under the growth conditions used for sporulation (acetate carbon source, no N_2 source) (28). The ability to produce 20S RNA is cytoplasmically inherited (10). Except for strain S228C (W, T-o, 20S RNA$^-$), the correlation of W with 20S RNA is good. Strains AN33, 200, and JM6 all lack W and lack the ability to make 20S RNA, whereas strains A364A, S37, and D585-11C and several *mak*$^-$ mutants tested all have W and can make 20S RNA under suitable conditions. We compared the amount of W in strain D585-11C grown in YPAD with the same strain grown under the conditions that induce 20S RNA. We found neither an increase of W ds RNA nor an induction of any RNA species detectable by the W probe. These data make it unlikely, but not impossible, that 20S RNA is related to W. None of the 20S RNA$^+$ strains examined had T.

Are T and W ds RNAs in virus-like particles? A subclone of strain 37-4C containing L-B, T, and W ds RNAs was grown at 37°C (to amplify T and W), and virus-like particles were purified as described previously (29) (see above). RNA polymerase activity sedimented in a peak at the usual position in the gradient for L-B-containing strains (20) coincident with a peak of L ds RNA (Fig. 7A and B). However, no T or W ds RNAs could be detected in the gradient. Although M ds RNA is even smaller than T or W ds RNA, it is found in such preparations (29).

To test whether T or W was in particles, a crude extract low-speed supernatant was applied to a sucrose gradient, and nucleic acids were extracted from the fractions. L was found in the usual location in the gradient. A peak of T ds RNA was found sedimenting more slowly than the L-containing particles, but faster than free T ds RNA in a parallel gradient (Fig. 7C and D). W ds RNA was not detected in the gradient. The results suggest that T and W are not encapsulated in the same particles as L ds RNA.

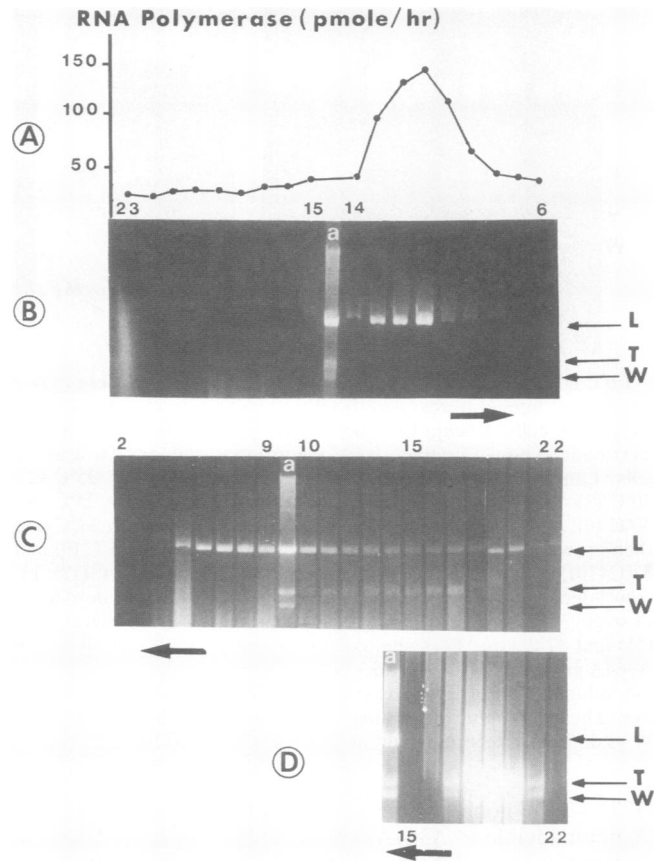

FIG. 7. Virus-like particles of T and W ds RNAs. Strain 37-4C was grown at 37°C. Virus-like particles were purified and analyzed as described in the text. (A) Samples of each fraction were assayed for RNA polymerase activity. (B) RNA was extracted from a sample of each fraction of the same gradient as in A and run on a 1% agarose gel (see the text). (C) Agarose (1%) gel electrophoresis of RNA from sucrose gradient fractions of a crude extract of strain 37-4C. (D) T and W ds RNA of strain 1773 purified by CF11 chromatography and run on a sucrose gradient identical to that in C. (a) ds RNA isolated from strain 37-4C. The arrows indicate the direction of sedimentation.

DISCUSSION

Our chance discovery of the clo$^-$ chromosomal trait has resulted in the discovery of two new non-Mendelian ds RNA molecules (plasmids), T and W.

L-B and L-C were first described as species of ds RNA remaining when L-A was cured by high temperature or lost from certain *mak*$^-$ strains. L-B and L-C show no hybridization with L-A and have completely different T_1 fingerprints from each other, but show substantial sequence homology with each other. The major proteins of the virus-like particles containing L-B are similar in size to those of particles carrying L-C, but both differ from the proteins in L-A particles. The maintenance of both L-B and L-C require a CLO$^+$ host as judged by cytoduction experiments. Meiotic crosses confirm this result for L-B, but not for L-C, a result we do not yet understand.

Since strains lacking both M and L can carry both T and W, it is clear that neither of these new ds RNAs is dependent on L or M. They likewise show no cross-hybridization with any of the Ls or Ms or with chromosomal DNA. Both T and W segregate 4:0 in meiosis and are transferred efficiently by cytoplasmic mixing. We find many strains carrying W alone and many carrying both T and W, but as yet none carrying T alone. It is thus possible that T depends on W.

We do not yet know how L-B, L-C, T, and W interact with the killer systems, if at all. All K_1 killer strains have W, and the few K_2 killer strains examined have T and W. It is thus possible that W is necessary for the K_1 phenotype and T and W are necessary for the K_2 phenotype, but more critical evidence will be needed to test this point. The heat inducibility of T and W is the inverse of the heat curability of M and L-A molecules. In some strains, the levels of T and W ds RNA are not heat inducible. The difference between heat-inducible and noninducible strains shows cytoplasmic inheritance, with all meiotic segregants being heat inducible. Inducibility may be a property of either the T and W species themselves or of some other nonchromosomal element. Preliminary experiments suggest that T and W are differently encapsulated from L ds RNA present in the same strain.

It seems likely that there are more than two different species of ds RNA smaller than L and larger than M, since many bands smaller than L are seen in various *mak*$^-$ strains (35), although the probes we have used in this study only detect two such species. Also, of the bands called ML_1 and ML_2 in the previous study (35), probably only one corresponds to one of the T and W bands described here. It will be necessary to isolate each of these species, probably by genetic means such as we describe here, to analyze fully the ds RNA molecules to be found in these strains.

The first studies of ds RNA in *S. cerevisiae* described two species in killers and one in nonkillers (2, 27). We now know that there are at least 11 ds RNA species distinguishable by a

combination of genetic and molecular techniques. A single strain may carry at least five of these. M_1, M_2, and M_3 ds RNAs determine the K_1, K_2, and K_3 toxins, respectively. Four forms of L-A are known carrying various combinations of the cytoplasmic genes [EXL], [NEX], and [HOK]. Elimination of L-A reveals the presence, in most strains, of either L-B or L-C, present in lower copy number and with different virus-like particle coat proteins than L-A. T and W are distinct from all of these and from each other.

It is possible that there are several virus-like or plasmid ds RNA systems in *S. cerevisiae* each with its own set of chromosomal genes needed for genome propagation. The L-A and M system needs *MAK* genes; the L-B and L-C system needs CLO; whereas T and W seem to need none of these. Three different types of encapsidation are also seen: molecules in the L-A coat are 160S, whereas L-B and L-C molecules may be found in either 160S or 80S particles with different coat proteins (20). Our preliminary studies indicate that T and W are not found in either size particle.

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