SUMMARY

A population of oligonucleotides co-purified with the dsRNA genomic segments of the killer virus of *Saccharomyces cerevisiae* during electrophoresis through agarose gels. These smaller RNA molecules must be separated from the viral genome in order to determine the structure of the dsRNA molecules. Sequence analysis of these isolated oligonucleotides showed that the population contained tRNA-like molecules, as well as 5S RNA, which are presumably encoded by the host cell genome.

Killer virus is a cytoplasmically inherited virus of *Saccharomyces cerevisiae*. Yeast cells harbouring the virus (killers) secrete a protein toxin which is lethal to cells that do not contain the virus (sensitives), but to which the killers are specifically resistant. The genome of the killer virus consists of two segments of dsRNA which are encapsidated in cytoplasmic virions in infected yeast cells (for reviews, see Bevan & Mitchell, 1979; Tipper & Bostian, 1984; Wickner, 1981). The genetic information for toxin and resistance is encoded on the M dsRNA (1830 bp) segment (Fink & Styles, 1972) which contains an internal 200 bp AU-rich region (Hannig et al., 1984; Fried & Fink, 1978). The other dsRNA segment, denoted LA (4980 bp), encodes the major capsid protein in which M and LA are encapsidated (Hopper et al., 1977). Other dsRNA molecules denoted LB and LC are present in the cytoplasm of some killer and non-killer yeasts. LB and LC are found in a different type of virion than are M and LA in some strains (Thiele et al., 1984a). Defective interfering mutants of killer virus with altered M dsRNA have been isolated. One of these altered dsRNA molecules, denoted S3, is 730 bp in length and contains only the 5'- and 3'-terminal sequences of M with the internal 1100 bp deleted (Fried & Fink, 1978; Thiele et al., 1984b).

A transcriptional polymerase activity, which catalyses the synthesis of full-length, positive polarity copies of the viral dsRNA genomic segments, co-purifies with killer virions (Bruenn et al., 1980; Hannig et al., 1984; Welsh et al., 1980).

It has been reported that the 3'-terminal regions of both strands of M, S3 and LA dsRNA contain extensive sequence heterogeneity (Bruenn & Brennan, 1980). These experiments were based upon sequence analysis of agarose gel-purified dsRNA. Other studies have shown that unambiguous sequences can be obtained for each dsRNA species after more extensive purification through several rounds of polyacrylamide gel electrophoresis (PAGE) (Thiele & Leibowitz, 1982; Thiele et al., 1984a, b), although trace heterogeneity cannot be excluded. We have found that these polyacrylamide gel electrophoretic steps remove a population of small oligonucleotides which remain associated with the dsRNA through the initial agarose gel electrophoresis. These oligonucleotides contain 3'-terminal sequences similar to those of yeast tRNAs, as well as 5S RNA.

Double-stranded RNA extracted from whole cells and purified by agarose gel electrophoresis (Thiele et al., 1982) contained a population of smaller oligonucleotides which were resolved on polyacrylamide gels. A similar population of oligonucleotide bands was detected upon analysis of preparations of M, S3, LA, LB and LC dsRNA. These bands were readily detectable upon...
Short communication

autoradiography of gels of RNA bearing 3'-terminal [5'-32P]pCp (Thiele et al., 1982), but were less visible by u.v. light-induced fluorescence in gels stained with ethidium bromide.

A similar population of oligonucleotides is found associated with dsRNA extracted from sucrose gradient-purified virions, purified as previously described (Thiele et al., 1984a; Welsh & Leibowitz, 1980). These virus particles were phenol-extracted to liberate the dsRNA to which 3'-terminal [5'-32P]pCp was then ligated. Electrophoresis on a 5% polyacrylamide gel revealed that the virion RNA preparation contained an oligonucleotide population similar to that present in dsRNA purified by agarose electrophoresis from phenol extracts of whole cells.

In order to test the effect of oligonucleotide removal on apparent dsRNA sequence heterogeneity, M dsRNA was subjected to ribonuclease T1 digestion (30 to 40 units/10 μg RNA in 20 mM-sodium citrate pH 5-0, 7 M-urea, 1 mM-EDTA) for 2 h at 37 °C, and the products were resolved by two-dimensional PAGE (Brennan et al., 1981; De Wachter & Fiers, 1972). Preparations of M dsRNA bearing 3'-terminal [5',32P]pCp were purified by either agarose gel electrophoresis or by two cycles of PAGE after agarose gel electrophoresis. The two-dimensional fingerprints of agarose gel-purified M dsRNA contained more than 50 spots, as previously reported (Bruenn & Brennan, 1980). Fingerprints of polyacrylamide gel-purified M dsRNA, on the other hand, contained less than ten digestion products, many of which were sensitive to further ribonuclease T1 digestion, indicating that they were incomplete digestion products of M dsRNA, which is less susceptible to enzymic digestion than is single-stranded RNA. The number of 3'-terminal T1 oligonucleotides derived from M dsRNA decreased markedly when the polyacrylamide gel-purified M dsRNA was analysed, indicating that the removal of the oligonucleotide species significantly reduced the apparent sequence heterogeneity of the dsRNA preparation.

The killer virion-associated transcriptase activity catalyses the synthesis of full-length positive-stranded copies of the genome (Hannig et al., 1984; Welsh & Leibowitz, 1980). In addition, smaller, incomplete copies of the positive strand which are presumably ‘pause products’ (Brennan et al., 1981) are found in reactions run at 37 °C. The electrophoretic mobilities of in vitro transcription products were compared with those of the oligonucleotides on a 5% polyacrylamide gel. The migration of the ‘pause products’ on the gel did not correspond to that of the dsRNA-associated oligonucleotides. In fact, the population of oligonucleotides migrated differently from all of the detectable transcriptional products, indicating that the origin of the oligonucleotides is probably not related to abortive transcription.

In order to characterize the oligonucleotides further, the sequences of several were determined. Individual oligonucleotides were isolated from preparations of agarose gel-purified dsRNA species which were reacted at their 3' termini with [5'-32P]pCp in the reaction catalysed by RNA ligase (Thiele et al., 1982), using a two-dimensional PAGE system (HsuChen et al., 1983), which separates the population into more than 50 individual spots. The fractionation pattern for M dsRNA-associated oligonucleotides is very similar to the pattern of LsLb- associated oligonucleotides, which is schematically shown in Fig. 1. LsLb refers to the L dsRNA species isolated from A364A x S7, which contains 90% Ls and 10% Lb (Sommer & Wickner, 1982).

The 3'-terminal nucleotide was analysed (Thiele et al., 1982) for 21 oligonucleotides excised from the gel (Fig. 1). Nineteen oligonucleotides contained a 3'-terminal A (all > 84% A), one a U (97% U, oligonucleotide no. 8), and one a C (78% C, oligonucleotide no. 6). Sequencing data were obtained for several of the oligonucleotides by either chemical (Peattie, 1979) or enzymic (Thiele et al., 1982; Donis-Keller et al., 1977) methods. All but one oligonucleotide (no. 8) thus analysed contained a 3'-HO-ACC terminus (see below).

The 3'-ACC sequence is characteristic of tRNA molecules. Three of the oligonucleotides had 3'-terminal sequences similar to those of yeast tRNA sequences (Fig. 2). Some of the sequencing gels for the oligonucleotides showed patterns expected for RNA species with modified nucleosides. For example, several nucleosides common in tRNA such as 5'-methylcytidine, thymidine and pseudouridine characteristically fail to be cleaved in the chemical or enzymic digestions (Krupp & Gross, 1983). The modified bases present in the authentic yeast tRNA species correspond to the missing bands on the sequencing gels, as can be seen in Fig. 2.
Fig. 1. Representation of preparative two-dimensional polyacrylamide (20%) gel fractionating L_ALB dsRNA-associated oligonucleotides bearing 3'-terminal [5'-32P]pCp (HsuChen et al., 1983). The first dimension (in 3 M-urea) of the gel ran from right to left; the second (in 8.3 M-urea) from top to bottom. Oligonucleotides were detected by autoradiography. Darkened, numbered spots represent oligonucleotides extracted from the gel (as described by Thiele et al., 1982) for further analysis. Minor variability of this pattern was noted among multiple preparations.

Fig. 2. Sequence of L_ALB dsRNA-associated oligonucleotides. The upper sequence of the first four pairs represents the 3' terminus of individual dsRNA-associated oligonucleotides. The letters (C or E) preceding each indicate the method of sequencing (chemical or enzymic, respectively); the numbers denote the oligonucleotide as indicated in Fig. 1. The lower sequence of each pair is that of the 3' terminus of a cellular RNA species. GLU is glutamic acid tRNA 4 (Singhal & Fallis, 1979); VAL is valine tRNA 9 (Singhal & Fallis, 1979); SER is serine tRNA 6 (Singhal & Fallis, 1979); 5S is yeast 5S RNA (Nazar & Wildeman, 1983). The 3'-terminal sequences of several other oligonucleotides for which no specific homologous tRNA was identified are also indicated. Left to right is 3' to 5'. X, No cleavage; +, weak cuts in C > U and A > G lanes; !, band compression, sequence is not readable; R, purine; ?, A or U, cuts in Phy M lane, U$_2$ lane is not readable; A$_m$, 1-methyladenosine; §, 5-methylcytidine; U$_m$, 2'-O-methyluridine; ψ, pseudouridine.
Oligonucleotide no. 8, which did not contain the 3'-ACC terminal sequence, appears to be identical to yeast 5S RNA. Fig. 2 also lists oligonucleotide sequences for which no specific homologous tRNA sequence was found.

Minor nucleotide analysis verified that the oligonucleotides contained modified and methylated bases as do tRNAs. Cells were grown in the presence of $^{32}$P. Uniformly radioactive dsRNA and the dsRNA-associated oligonucleotide population were individually isolated by PAGE and were digested to completion with ribonuclease T2 (37 °C, 1 h, 500 units/ml in 0-01 M-sodium acetate pH 4.5). The resultant nucleoside 3'-monophosphates were separated by two-dimensional thin-layer chromatography on cellulose plates (MN-300, Analtech, Newark, Del., U.S.A.) by the method of Nishimura (1979). A population of yeast tRNAs isolated by agarose gel electrophoresis of whole cell RNA extracts (Fried & Fink, 1978) and a population of L$_{ALB}$-associated oligonucleotides were analysed by this method. In both cases, the four unmodified nucleotides (Ap, Gp, Cp and Up) were present as the major species, with smaller amounts of methylated and otherwise modified nucleotides. Similar treatment of a population of M dsRNA-associated oligonucleotides resulted in an apparently identical pattern. However, similar analysis of polyacrylamide gel-purified M or L$_{ALB}$ ds RNA revealed only the four major nucleotides. Ribosomal RNA was also isolated from whole cells and purified by agarose gel electrophoresis. A population of oligonucleotides was also present in this RNA fraction. However, the migration on a 5% polyacrylamide gel of rRNA-associated oligonucleotides was different from that of the dsRNA-associated oligonucleotides.

The physiological function, if any, of the oligonucleotides associated with the killer virus dsRNA is unclear. They are associated with dsRNA extracted directly from purified virions. It is possible that they attach in some way to the outside of the viral capsid and, after phenol extraction, become associated with the dsRNA. It is also possible that the tRNA-like molecules are encapsidated with the dsRNA. Incubation of virions in the presence of RNase A under various conditions failed to result in any preferential degradation of the oligonucleotides relative to the dsRNA molecules. Further experiments are needed to clarify these points.

It is clear, however, that the association of the oligonucleotides with the dsRNA hinders structural analyses of the viral genomes. Initial attempts to determine the sequence of the genome of killer virus indicated apparent extensive sequence heterogeneity (Bruenn & Brennan, 1980). The existence of multiple L dsRNA species (Sommer & Wickner, 1982; Thiele et al., 1984a) only partially explains this heterogeneity. Only after tRNA-like molecules were removed by several rounds of PAGE were unambiguous sequences determined for each dsRNA species (Thiele et al., 1982, 1984a, b). Similar problems may be encountered in the purification of other viral RNA molecules.

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REFERENCES


SOMMER, S. S. & WICKNER, R. B. (1982). Yeast L dsRNA consists of at least three distinct RNAs: evidence that the non-Mendelian genes [HOK], [NEX] and [EXL] are on one of these dsRNAs. *Cell* 31, 429-441.


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