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The 5' ends of yeast killer factor RNAs are pppGp

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ABSTRACT

The 5' nucleotides of the double-stranded RNAs of yeast killer factor have been isolated by digestion with pancreatic, T_1 and T_2 RNase followed by two-dimensional electrophoresis. They were identified by bacterial alkaline phosphatase and snake venom phosphodiesterase digestions. Both the larger double-stranded RNA (L, of 2.5 x 10^6 daltons) and the smaller double-stranded RNA (M, of 1.4 x 10^6 daltons) have the 5' end groups $ppp^{G}p$. These 5' ends are dissimilar to those of the double-stranded RNAs of animal viruses but may be characteristic of the 5' ends of the double stranded RNAs of fungal viruses.

INTRODUCTION

Killer factor is a cytoplasmically inherited genetic determinant in the yeast Saccharomyces cerevisiae[1,2] that confers upon its host cells ("killers") the ability to secrete several toxic glycoproteins that kill sensitive cells[3,4,5]. Killer factor is distinct from mitochondria[6,7] and is associated with two double-stranded RNAs (dsRNAs)[8,9], which are found intracellularly, separately encapsidated in virus-like particles (VLPs)[10]. These two dsRNAs are L, of 2.5 x 10^6 daltons, and M, of 1.4 x 10^6 daltons[9,11]. Mutants lacking M are unable to synthesize the killer toxins and are sensitive to their effects[11]. No mutants entirely lacking L exist, although some mutants have a much lower amount of L[11]. In a normal killer cell, there are about 1.5 x 10^4 killer VLPs, which consist of three polypeptides of molecular weights 75,000, 53,000 and 37,000 in addition to either L or M[12]. Cells entirely lacking M make VLPs containing the same polypeptides[12]. Thus L probably encodes the VLP polypeptides and M the polypeptides of the toxic glycoproteins. Particles containing L are separable, either by sucrose gradient centrifugation[10,12], or by CsCl equilibrium gradient centrifugation[14], from particles containing M. Particles containing L have a density in CsCl of 1.42[13,14] and particles containing M a density of 1.38[14]. No extracellular VLPs have been

detected[14]. Killer factor is thus similar to the fungal dsRNA viruses, which also generally lack an infectious cycle[15].

The best known of the fungal dsRNA viruses, the Penicillum stolinifer-um virus S, has a peculiar mode of replication, which involves the synthesis of both strands of each dsRNA within the parental particle[16], without the mRNA intermediate characteristic of reovirus. Intermediates in virus S replication are therefore particles with two molecules of dsRNA[16]. Reo-virus RNAs have one 5' end with the structure $m^7G^{5'}$ $ppp^{5'}$ GmpCp and one the structure ppGp[17]. The blocked 5' is on the plus (mRNA) strand[18] synthesized by the reovirus viral transcriptase, the unblocked 5' on the minus strand[17], synthesized on the plus strand template by the reovirus replicase[19]. Unlike reovirus viral RNAs, the 5' ends of killer factor RNAs are not capped, and are pppGp.

MATERIALS AND METHODS

Carrier-free ^{32}P as H_3PO_4 was obtained from New England Nuclear. Nucleoside 5' di- and tri-phosphates were from Sigma. Pancreatic ribonuc-lease (RNase A) was from Sigma, T_1 and T_2 RNases from Calbiochem, snake venom phosphodiesterase from Worthington and bacterial alkaline phos-phatase was a gift of E. Niles.

Isolation of ^{32}P-killer factor RNAs

Cells growing in 140 ml of low-phosphate YPD (1% yeast extract, 2% peptone, 2% glucose) were labelled with 50mCi of ^{32}P essentially as described by Rubin[20]. RNA and DNA were extracted from spheroplasts, essentially as described by Wickner and Leibowitz[21], or from whole cells by 0.5% w/v SDS, 50% v/v phenol in 50mM Tris-Cl, 1mM EDTA, pH 7.5. Killer RNAs extracted from whole cells were directly isolated by 3.5% polyacryla-mide slab gel electrophoresis or 1.4% agarose slab gel electrophoresis. Killer RNAs isolated from spheroplasts were first freed of rRNA by cellulose chromatography[21]. The bands were visualized by ethidium bromide staining or autoradiography, cut out, homogenized, and extracted overnight with 1mM EDTA, 10mM Tris-Cl pH 7.5, 50% phenol (v/v). Some preparations were purified by two cycles of electrophoresis: 3.5% polyacrylamide follow-ed by 1.4% agarose. All preparations appeared homogeneous on re-electro-phoresis and were more than 90% resistant to pancreatic RNase digestion in 2 x SSC (0.30 M NaCl, 0.03 M sodium citrate). Specific activities were generally 0.5 x 10^6 dpm/μg of RNA. Yields were about 4-10 x 10^6 dpm of L and 0.4-1 x 10^6 dpm of M from a "superkiller" strain of yeast (T158D SK1) with several times more M than present in the wild-type[11]. This strain

was a gift of G. Fink.

Isolation of 5' ends

The 5' ends of M and L were isolated by the two-dimensional electrophoresis procedure of Adams and Cory after complete digestion by T_1, pancreatic and T_2 RNase[22]. Electrophoresis in the first dimension was on cellulose acetate strips at pH 3.5 in the presence of 7M urea and in the second dimension on DEAE paper at pH 3.5. Contamination with ribosomal and tRNA was monitored by the presence of 2'-O-methyl modified dinucleotides (XmpNp) and modified mononucleotides[22].

Identification of 5' ends

Putative 5' ends were cut out of the DEAE paper, counted for ^{32}P by Cerenkov radiation, eluted and prepared for further digestion as described by Barrell[23]. They were subjected to further digestions with bacterial alkaline phosphatase or snake venom phosphodiesterase and the resultant digestion products fractionated by electrophoresis on DEAE paper or on Whatman 3MM paper at pH 3.5 in the presence of various marker nucleotides. Digestion and fractionation of digestion products were as described by Barrell[23], except that complete venom phosphodiesterase digestion of putative 5' ends was with 1-2 mg/ml of enzyme in 0.01 M Tris, 0.01 M $MgCl_2$, pH 8.9 for 4 hr at 37C.

RESULTS

A fingerprint of a combined complete T_1, pancreatic, and T_2 RNase digest of L dsRNA is shown (as an autoradiograph) in Figure 1. No 2'-O-methyl dinucleotides are present, indicating minimal contamination by rRNA in this preparation of L, which was isolated on a 3.5% acrylamide gel. L and M are better separated from the yeast 16S and 25S rRNAs on 1.4% agarose cells than on 3.5% acrylamide gels. Variable amounts of modified dinucleotides have been present in some preparations of L isolated on 3.5% acrylamide gels. The only unusual nucleotides present in most preparations of L are the unidentified modified mononucleotides present in the average molar amounts shown in Table 1. The material at the origin in Fig. 1 is RNA that was not in solution in the small volume (20 μ) applied to the cellulose acetate strip. This combined T_1, T_2 and pancreatic RNase digestion is equally effective with ^{32}P-yeast rRNA and the killer dsRNAs (greater than 99.9% of radioactivity rendered acid soluble).

The only putative 5' nucleotide is spot L1 (Figure 1). This has a mobility of 1.5 that of U_p in the first and 0.02 that of U_p in the

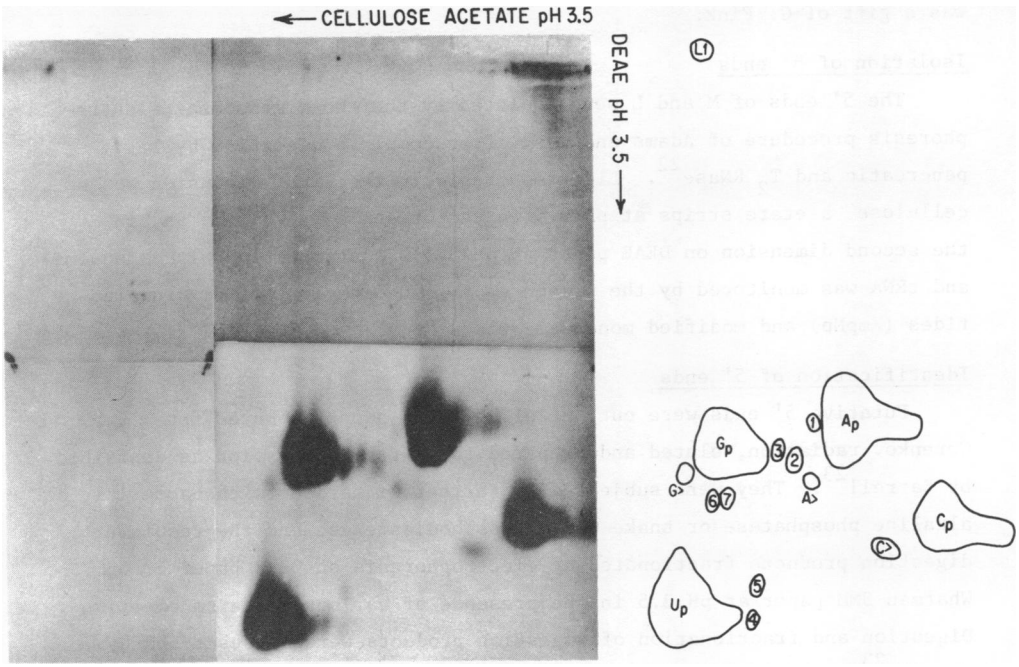

Figure 1. Two-dimensional electrophoresis of complete T_1, T_2 and pancreatic RNase digestion products of L (the larger killer-factor dsRNA). This sample of L was isolated on a 3.5% polyacrylamide gel.

second dimension. These mobilities are characteristic of mononucleoside tetraphosphates[23,24,25,26,27]. L1 is present at a molarity of about 2, assuming the structure pppXp (Table 1).

No nucleotides from combined T_1, pancreatic, and T_2 RNase digests of L have been observed to migrate in the position of N^6mA_p, or in the position of blocked 5' ends characteristic of animal cell mRNAs[22] or reovirus mRNAs[18] and viral plus strand RNAs[17]. These blocked 5' ends migrate approximately between G_p and U_p in the first dimension and about 0.1 times as fast as U_p in the second[22]. No oligonucleotides corresponding to the 5' ends of TMV RNA ($m^7GpppGp$)[24] or yeast mRNA ($m^7GpppGp$ and $m^7GpppAp$)[28] are present either. These have a mobility of 1.2 that of U_p in the first dimension and 0.09 that of U_p in the second[24]. All of the nucleotides (usually in submolar amounts) which could conceivably be blocked 5' ends were modified dinucleotides: i.e., none was susceptable to mild venom hydrolysis and all gave two radioactive products after alkaline phosphatase digestion, inorganic phosphate and a presumed dinucleoside monophosphate, of equal

Table 1. Molarities of nucleotides in T_1 + T_2 + pancreatic RNase digests

Nucleotide	Molarity	
	L	M
1	ND	0.046
2	0.15	0.15
3	0.10	0.70
4	ND	ND
5	ND	ND
6	2.1	ND
7	2.1	ND
L1	2.7	-
M1	-	2.4

The molarities given are the averages of 3-5 determinations. ND indicates nucleotides not present in RNAs isolated on 1.4% agarose gels but present in variable amounts in RNAs isolated on 3.5% acrylamide gels. Molarities were calculated assuming molecular weights of 2.5×10^6 for L, 1.4×10^6 for M, and 330 for an average nucleoside monophosphate.

radioactivity. In most preparations of L the only nucleotide migrating more slowly than A_p in the second dimension is L1.

In similar fingerprints of pancreatic, T_1 and T_2 RNase digests of M, there is a nucleotide migrating in the position of L1 (data not shown). M1 is also present at a molarity of about 2, assuming the structure pppXp (Table 1). The variability in the molarities of L1 and M1 is probably caused by variable transfer from the cellulose acetate strip of the first dimension to the DEAE paper of the second dimension. Transfer is from 50% to 90% complete under our conditions, but the four major mononucleotides, for example, rarely transfer with the same efficiency[22]. L1 and M1 have also been isolated by one-dimensional electrophoresis on DEAE-cellulose after T1, pancreatic and T2 digestion. They have the same mobility, and each has a molarity of about 2.

The putative 5' ends were subjected to mild snake venom phosphodiesterase digestion (0.005 mg/ml for 1 hr at 37C). This procedure did result in the near complete digestion of a 3'OH terminal oligonucleotide 12 nucleotides long. Spots L1 and M1 were not attacked under these conditions. The untreated nucleotides and the nucleotides reisolated after mild venom

phosphodiesterase treatment were subjected to bacterial alkaline phosphatase digestion[23]. Essentially all the radioactivity of each treated 5' nucleotide coelectrophoresed with inorganic phosphate after alkaline phosphatase treatment (Figure 2). Spot L1 is also hydrolyzed to inorganic phosphate by alkaline phosphatase (not shown). Several putative modified dinucleotides gave the expected dinucleoside monophosphates and inorganic phosphate after alkaline phosphatase treatment.

The structures of putative 5' ends were confirmed by complete venom phosphodiesterase digestion, as described in Materials and Methods. The resultant digestion products were fractionated by electrophoresis on DEAE cellulose at pH 3.5 in the presence of non-radioactive marker nucleotides, which are visualized by their ultraviolet absorbance (Figure 3). L1 gave mainly two digestion products, inorganic pyrophosphate and GDP. Non-radioactive guanosine-5'-diphosphate added to L1 after digestion (and located by ultraviolet absorbance) runs in the same position as the primary digestion product of L1. M1 in a similar experiment gave predominantly inorganic pyorphosphate and GDP. Given the specificity of T_1,T_2 and pancreatic ribonucleases, this diphosphate must have the structure pGp. These products of venom digestion are diagnostic for nucleoside tetraphosphates of the form pppXp[25,26]. Complete venom digestion of the nucleotide L1 also gave some inorganic phosphate and some GTP (Figure 3). This is probably the result of the 5' nucleotidase activity contaminating commercial preparations of venom phosphodiesterase[29]. It serves to further confirm the identity of L1, however. The same 5' end was also recovered from a combined T_1,T_2 and pancreatic RNase digest of the 10% trichloroacetic acid precipitable material resulting from T_1 digestion of L in 2 x SSC. Yeast ^{32}P-tRNA was rendered greater than 99% TCA soluble by a control T_1 digestion in 2 x SSC. We therefore conclude that the 5' ends of L and M are pppGp.

DISCUSSION

The 5' ends of yeast killer factor RNAs are pppGp. These 5' ends are found in the appropriate molar amounts. No blocked 5' ends have been isolated from any killer factor dsRNA preparation. The only nucleotides present in T_2 digests of L and M other than U_p, C_p, G_p, A_p and the above 5' phosphorylated nucleotides probably originate from contaminating rRNA, since they are present in submolar amounts in all preparations except those from acrylamide gels (where rRNA and L and M are poorly separated). The only exceptions are spots 6 and 7, which are present in an average of 2.1

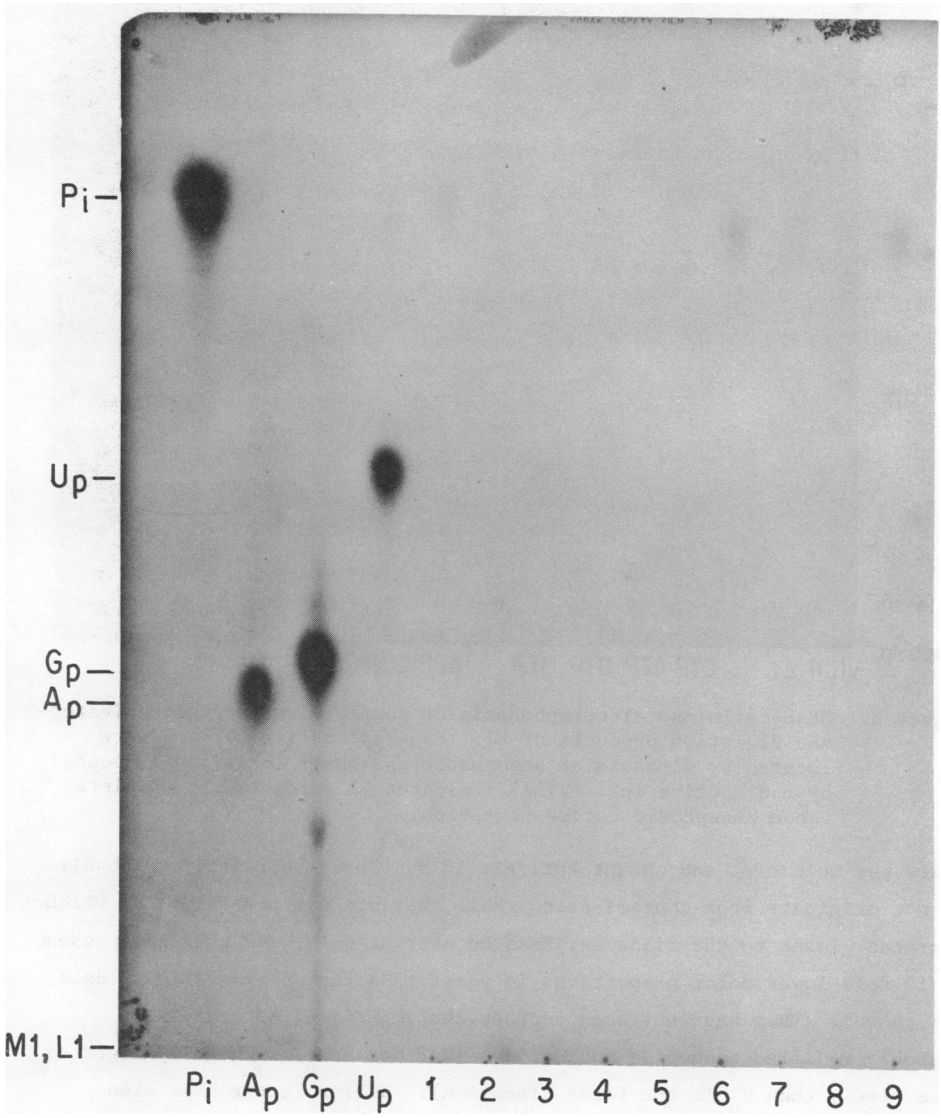

Figure 2. DEAE-cellulose electrophoresis of alkaline phosphatase digestion products. Spots 2 and 5 are unidentified 2'-O-me-dinucleotides from complete T_1, T_2 and pancreatic RNase digests of yeast rRNA (as controls) treated with alkaline phosphatase. Spots 1, 3, 4, 6 and 7 are modified mononucleotides, also from yeast rRNA, treated with alkaline phosphatase. Spot 8 is alkaline phosphatase treated M1. Spot 9 is undigested M1 re-isolated (by electrophoresis at pH 3.5 on DEAE-cellulose) after mild venom phosphodiesterase treatment and subjected to alkaline phosphatase treatment.

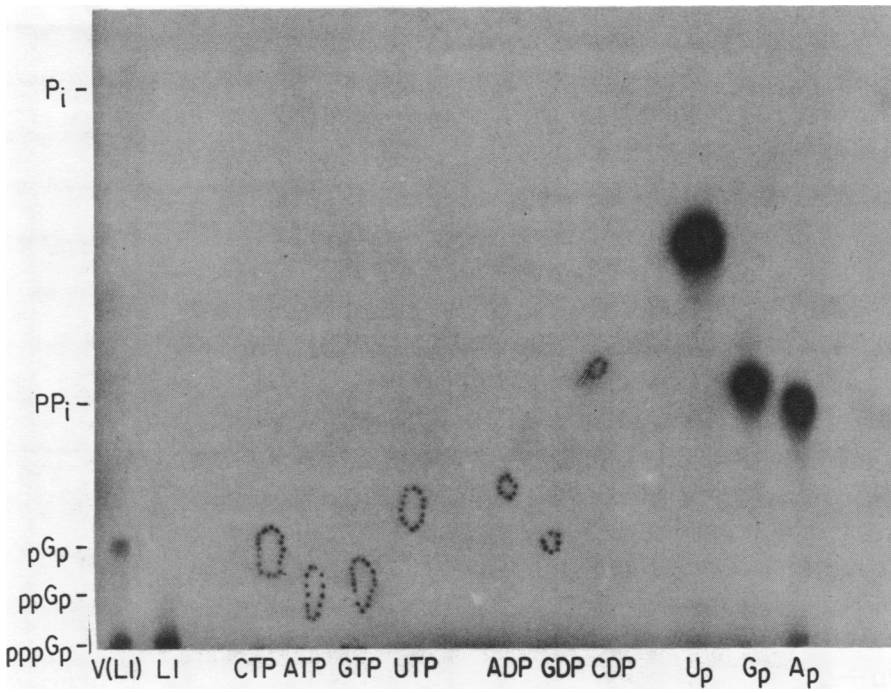

Figure 3. DEAE-cellulose electrophoresis of complete venom phosphodiesterase digestion products of L1. Non-radioactive markers were located by ultraviolet absorbance and their locations indicated by radioactive ink. V(L1) indicates L1 subjected to complete venom phosphodiesterase digestion.

moles per mole of L and absent entirely in M. These nucleotides probably do not originate from contaminating rRNA: they are not present in M, which migrates closer to the ribosomal RNAs on agarose gels, and they are present in 10-fold lower molar proportions in yeast rRNA than they are in L (data not shown). They have not been further characterized, but they are probably modified guanosine nucleotides (but not m^7G_p, since m^7G_p migrates more slowly than C_p in the first dimension). Killer factor RNAs also lack the 2'-O-methylation characteristic of nucleotides at the 5' ends of animal cell mRNAs[22] but lacking in plant virus plus strands[24].

The 5' ends of killer factor RNAs isolated from VLPs have not been characterized, since direct isolation of the labelled RNA is easier. Nevertheless, since essentially all of the dsRNA in yeast appears to be present in VLPs[12], we have every reason to believe that the 5' ends we have isolated are those present in the VLPs.

The presence of 5' terminal nucleoside triphosphates in L and M may

have significance for the mode of replication of the killer factor RNAs. Yeast mRNA has 5' termini of the form $m^7GpppGp$ or $m^7GpppAp$[28]. Killer factor mRNAs would be expected to have similar blocked 5' ends. Therefore, the absence of blocked 5' ends on the "viral" RNAs may indicate the absence of an mRNA intermediate in their replication. This would be consistent with the model for replication of fungal dsRNAs proposed by Buck[16], in which a viral replicase synthesizes progeny dsRNA within the parental particle, creating a progeny particle with two dsRNA molecules. The progeny particles are disrupted by host proteases; the freed dsRNA is liberated to the cytoplasm where it is transcribed, possibly by the still-attached replicase; and the freed dsRNAs are encapsidated to complete the cycle. In agreement with this model, the known fungal virus RNA dependent RNA polymerases are S-adenosyl-methionine independent[16], although no such polymerase activity has yet been demonstrated in yeast killer VLPs.

There remains the possibility that one strand of L and one strand of M do act as mRNAs and are intermediates in dsRNA synthesis, but that these mRNAs do not have blocked 5' ends. The plus-strand RNA of STNV (satellite tobacco necrosis virus), for instance, appears to lack a blocked 5' end and to be translated without being capped[30]. Similarly, poliovirus mRNA has the 5' terminus pUp[31]. There is also no evidence to indicate that the reovirus replicase requires a capped 5' on its substrate RNA[32]. Consequently, it will be interesting to determine if the 5' ends of polysomal mRNAs transcribed from L and M are capped, an experiment now in progress.

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