To study the interaction of an aminoacyl-tRNA synthetase with its cognate tRNAs, it is of interest to be able to design synthetic tRNA mutant genes, and to rapidly purify sufficient amounts of the corresponding tRNAs. A prerequisite is to show that the synthetase retains full specificity towards a wild type tRNA expressed from a synthetic gene. For this purpose, we have used an expression vector to overproduce and purify elongator tRNA\textsuperscript{met}.

To date, the most widely used rapid purification procedure, extraction from polyacrylamide gels, does not provide enough pure material to achieve fine characterizations, such as affinity constants measurements by the analysis of the intrinsic enzyme fluorescence quenching (1). We propose a rapid method for the purification of a functional tRNA\textsuperscript{met} based on the construction of a synthetic gene, the obtention of an overproducing \textit{E. coli} strain and a two-step FPLC driven purification from a crude extract.

A shuttle vector, pBSTNAV, (derived from Bluescript M13+KS) carrying a multisite cloning sequence EcoRI/Smal/PstI flanked upstream with a synthetic lipoprotein promoter and downstream with the \textit{rrnC} transcription terminator, both prepared from the pGFIB-1 phasmid (2), was designed (figure 1). The synthetic elongator tRNA\textsuperscript{met} gene was constructed by assembling six different overlapping oligonucleotides (figure 1) synthetised with the Pharmacia gene assembler and purified on a Mono Q column. A mixture of the 6 oligonucleotides (1.5 \textmu M each) was heated to 95 °C, slowly cooled and submitted to ligation during 1 hour at 37°C. Oligonucleotides 1 and 4 were kept unphosphorylated, to avoid the concatemerization of assembled genes during ligation. Agarose gel electrophoresis showed that the major ligation product corresponded to the tRNA gene. Cloning between the EcoRI and PstI sites of the shuttle vector was realised by performing ligation at 14°C, under the conditions defined by Dardel (3). To eliminate reconstructed vector molecules, the ligation mixture was then restricted by SmaI. After transformation of JM101 Tr cells (4), the screening of overproducing clones was achieved by determining the methionine accepting specific activity in crude tRNA minipreparations (5). Among the 20 clones randomly tested, 8 displayed a 10- to 20-fold higher activity, corresponding to a 30 to 50-fold overproduction of elongator tRNA, since initiator tRNA accounts for 70% of the total methionine accepting capacity in \textit{E. coli}.

Sequencing of the corresponding single-stranded DNAs showed that in each case, the construction was exactly as extracted.

Crude stripped tRNA was prepared from a 1 liter culture of the overproducing cells in LB medium (50 \textmu g/ml ampicillin), according to Zubay (6). 500 A\textsubscript{260} units were usually obtained, with a specific activity of 370 to 600 pmole tRNA\textsuperscript{met}/A\textsubscript{260} unit (i.e. 22 to 35 % purity). It was then loaded on a DEAE-Trisacryl M column (1.1 x 5 cm) equilibrated with a 0.2 M NaCl, 20 mM Tris-HCl (pH = 7.5), 8 mM MgCl\textsubscript{2}. tRNA was eluted by performing a 0.2 to 0.4 M NaCl linear gradient (2 ml/min; 0.24 M/h). The methionine accepting fraction contained 120 to 240 A\textsubscript{260} units with a specific activity of 670 to 1050 pmole/A\textsubscript{260} units (70% yield). After ethanol
precipitation, 60 $A_{260}$ units were dried, redissolved in 0.5 ml 10 mM ammonium acetate ($pH = 6.6$), 1.8 M ammonium sulfate, 10 mM MgCl$_2$ and loaded on a TSK-phenyl column (7.5 x 0.75 cm). tRNAs were eluted by performing a 1.58 M to 1.22 M reverse ammonium sulfate gradient (0.54 M/h; 0.7 ml/min). A typical chromatogram is shown below.

The pooled fractions were desalted by filtration through a GF05M-Trisacryl column (2.5 x 25 cm; 4 ml/min) equilibrated in 10 mM Tris-HCl ($pH=7.5$).

The total procedure allows purification of 1 to 2 mg $tRNA^{met}$ (overall yield=56%) to a more than 1400 pmol/$A_{260}$ unit specific activity in less than 48 hours (bacteria as starting material). The overproduced tRNA was fully aminoacylatable, as demonstrated by the methionine acceptance profile of the TSK-phenyl eluate. Indeed, the fraction corresponding to the maximal absorbance reached 1800 pmol/$A_{260}$. The Michaelis constant as well as the dissociation constant (1) of the final product for monomeric methionyl-tRNA synthetase have been determined ($K_m=0.8 \mu M; K_D=0.4 \mu M$) and compared to those of elongator $tRNA^{met}$ purified from a non-overproducing strain ($K_m=0.25 \mu M; K_D=0.1 \mu M$). In spite of the slight differences, possibly reflecting undermodification of the overproduced species, these results confirmed that methionyl-tRNA synthetase has retained full specificity towards the substrate of synthetic origin.

ACKNOWLEDGEMENTS
Dr J-M MASSON is acknowledged for the gracious gift of pGFIB-I.

REFERENCES
Extranuclear gene expression in yeast: evidence for a plasmid-encoded RNA polymerase of unique structure

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ABSTRACT

Strains of the yeast Kluyveromyces lactis that produce killer-toxin have been found to contain two linear dsDNA plasmids, k₁ (8.9 Kb) and k₂ (13.4 Kb). The four transcribed open reading frames of plasmid k₁ contain no recognisable yeast nuclear expression signals. Moreover, a toxin subunit gene fused with the lacZ gene of Escherichia coli is not detectably expressed when introduced to K. lactis or Saccharomyces cerevisiae on a nuclear vector, even when native k₁ and k₂ are present in the cell. This and other evidence is consistent with the hypothesis that k₁ and k₂ reside in an extranuclear location, and do not utilise the nuclear RNA polymerases I, II or III for transcription of their genes. Sequencing of plasmid k₂, which is thought to encode factors necessary for the maintenance or expression of k₁, reveals an open reading frame predicted to encode a 974 amino acid polypeptide with homology to several DNA-directed RNA polymerases. We suggest that this is a component of a novel plasmid-specific extranuclear gene expression system.

INTRODUCTION

Many strains of the budding yeast Kluyveromyces lactis contain two linear double-stranded A/T rich DNA plasmids, k₁ (8.9 Kb) and k₂ (13.4 Kb) that are associated with secretion of a multi-subunit protein toxin which inhibits the growth of certain sensitive yeast species. Structurally, the plasmids belong to a class of extrachromosomal linear elements which includes adenovirus, bacteriophage phi 29 plasmids of Streptomyces rochei and the S1/S2 mitochondrial plasmids associated with male sterility in maize. All of these molecules have inverted terminal repeat sequences (202bp and 184bp in the case of k₁ and k₂) and proteins covalently attached to the 5' end of each DNA strand (for k₁ and k₂ terminal proteins see reference 8). These may function as primers in the initiation of terminal DNA replication. In support of this hypothesis one of the four transcribed open reading frames of k₁ encodes a product with homology to the DNA polymerases of adenovirus and phi 29, and to a protein encoded by an ORF of the maize plasmid S11.

Plasmids k₁ and k₂ can be transferred to ρ₀ (rho zero) strains of...
Saccharomyces cerevisiae (which lack mitochondrial DNA) where they are maintained and express the killer phenotype. However, they do not become established in the presence of mitochondrial DNA. These observations, together with the high A/T content of the plasmid DNA, fluorescence staining of S.cerevisiae ρ° derivatives containing the plasmids and fractionation of yeast nuclei and cytoplasm by centrifugation techniques (reference 15, D.W. Wilson and P.A. Meacock unpublished observations) are all consistent with a cytoplasmic location for the plasmids. Plasmid curing experiments have shown that plasmid k2 can be maintained in the absence of k1, but plasmid k1 is unable to exist independently suggesting dependence upon k2-encoded products. Such factors may be concerned with the replication or segregation of k1, or for the expression of k1-encoded genes essential for k1 maintenance.

A variety of data suggest that these plasmids may utilise a novel system for gene transcription; viz. none of the ORFs of k1 is preceded by recognisable yeast nuclear promoter elements, although all four are preceded by a motif identical with, or closely related to, the sequence ACT(A/T)AATATATQA. This has been termed the Upstream Conserved Sequence or UCS9, and transcription is initiated approximately 14 bp downstream of this element (Romanos and Boyd, manuscript submitted). Toxin production cannot be detected from ρ° strains of S.cerevisiae which have been transformed with the coding region of plasmid k1 cloned into yeast 2 μm (micron)-based vectors (D.W. Wilson and P.A. Meacock, unpublished observations, reference 15).

Furthermore, Northern blotting reveals that when k1 DNA is introduced into K.lactis on nuclear vectors transcription of the k1 gene ORF2 (which encodes two toxin subunits) is initiated at a number of sites distinct from those used by native linear k1, and the transcript is prematurely terminated (Romanos and Boyd, manuscript submitted). Thus it appears that the yeast nuclear RNA polymerases I, II and III are unable to recognise and correctly transcribe the genes of these plasmids.

Here we demonstrate that expression of an ORF2/lacZ fusion gene, cloned into a yeast nuclear vector, cannot be detected in cells of K.lactis or S.cerevisiae which contain plasmids k1 and k2. This suggests that transcription of ORF2, and probably all plasmid-borne genes, occurs in an extranuclear cellular compartment. The provision of novel cytoplasmic expression factors could be one of the maintenance functions of k2, and to investigate this we have begun to determine the nucleotide sequence of this plasmid. The predicted product of one of the ORFs encoded by k2 has homology to two different subunits found within DNA-directed RNA polymerases, whilst...
another has homology to a vaccinia virus helicase necessary for specific viral transcription.

MATERIALS AND METHODS

Strains and Media

*Kluyveromyces lactis* IFO1267 (prototrophic \([k_1^+ k_2^+]\)) was obtained from the National Collection of Yeast Cultures, Food Research Institute, Colney Lane, Norwich, UK. *K. lactis* SD11 (*K. lactis lac4 trpl \([k_1^+ k_2^+]\)) was kindly provided by Prof. C. Hollenberg, and *K. lactis* ABK802 (prototrophic, \([k_1^0 k_2^+]\)) by Dr. A. Boyd. *Saccharomyces cerevisiae* SPK103 (a *his3-A trpl-289 ura3-52 leu2-3 \([k_1^+ k_2^+ L^+]\) \(\Phi^0\)) was prepared by cytoduction of a \(\Phi^0\) derivative of strain S150-2B (obtained from J. Hicks, Cold Spring Harbor Laboratory, New York) with JC25K \((\alpha ade2-1 his4-15 kar1-1 \([k_1^+, k_2^+]\)) obtained from Dr. M. A. Romanos. Yeast were propagated at 30°C using either YPD medium (1% yeast extract, 2% peptone, 2% glucose) or in minimal medium (0.67% yeast nitrogen base, 2% glucose) supplemented with nutrients as required. Plasmid constructions, bacterial assays for \(\beta\)-galactosidase activity, and superinfection for preparation of single stranded DNA made use of *E. coli* NM522 \((\lambda lacE' proAB) thi' supE hsd65 [F' proAB lacY ZAM15]^{17}\) (provided by Dr. A. Mileham), maintained on M9 minimal medium \(^{18}\) supplemented with 0.001% thiamine, in order to ensure maintenance of the \(F'\) episome and susceptibility to M13 and M13K07 phage infection. M13K07 helper phage was from Pharmacia, Uppsala, Sweden.

Enzymes

The Klenow fragment of DNA polymerase I, exonuclease III, and all restriction endonucleases used in this study were purchased from Bethesda Research Laboratories (BRL), Bethesda, Maryland, USA. T4 DNA ligase was purchased from Pharmacia, and Proteinase K from Boehringer (BCL), Mannheim, FRG. Zymolyase 100-T was from the Kirin brewery, Tokyo, Japan. All enzymes were used as recommended by the supplier.

Materials for Sequence Analysis

Deoxynucleotides and dideoxynucleotides were purchased from BCL. Acrylamide and bisacrylamide were "Electran" grade, purchased from BDH chemicals, Poole, UK. Ultrapure enzyme grade urea was from BRL. Sigmacote and \(N,N',N''\)-Tetramethylethylenediamine (TEMED) were obtained from Sigma, St Louis, Missouri, USA. Sequencing primers were 17 bp "universal primer" supplied by Pharmacia and custom-synthesised 17 bp oligodeoxynucleotides prepared by J. Keyte, Biochemistry department, Leicester University, using an Applied Biosystems DNA synthesiser. \([\alpha-35S]\)dATP, at 10 μCi μl\(^{-1}\) and 650 Ci
Nucleic Acids Research

Preparation of Plasmid k2 DNA

E. lactis ABK802 was grown in 1 litre of YPD broth to a density of 3x10^7 cells ml^{-1}, cells were pelleted and washed once in water, then resuspended in 50 ml of SED buffer (1.2 M Sorbitol, 20 mM EDTA, 50 mM Dithiothreitol) and incubated at 37°C for 20 mins. To the suspension was added 1 ml of 10 mg ml^{-1} Zymolyase 100-T in SED buffer and incubation continued until spheroplasting was complete. Spheroplasts were pelleted and resuspended in 20 ml 10 mM EDTA, 50 mM Tris.HCl pH 8.0, then Sodium N-lauryl Sarcosinate added to a final concentration of 2 %. To the lysate was added 1 ml of 10 mg ml^{-1} Proteinase K, the mixture incubated at 37°C for 1 h, then the lysate chilled on ice, mixed with 5 ml of 5 M NaCl and left on ice for 1 h. After centrifugation at 18000 rpm for 30 mins at 4°C in a Sorvall SS34 rotor the supernatant was extracted three times with an equal volume of phenol, then twice with chloroform. Extracted supernatant was mixed with 2 ml 3 M sodium acetate pH 5.5 and 50 ml absolute ethanol, placed at -80°C for 30 mins, nucleic acid pelleted and the pellet rinsed with 10 ml of 70 % ethanol. The pellet was briefly dried under vacuum, resuspended in 5 ml TE buffer (1 mM EDTA, 10 mM Tris.HCl pH 7.6) then nucleic acid fractionated according to size in a 10 %-40 % sucrose gradient, containing 1 M NaCl, 5 mM EDTA, 20 mM Tris.HCl pH 8.0, by centrifugation at 26000 rpm for 24 h at 26°C in a Sorvall AB27 rotor. Fractions containing plasmid k2 were identified by agarose gel electrophoresis, pooled and diluted with 2 volumes of water. DNA of plasmid k2 was precipitated for 1 h at -80°C after the addition of 1/10th volume of 3 M sodium acetate pH 5.5 and 2.5 volumes of absolute ethanol, then pelleted, the pellet rinsed with 0.5 ml of 70 % ethanol, briefly vacuum dried and resuspended in 200 μl TE buffer.

Quantitative Assay for β-Galactosidase Activity

Yeast transformants were grown to between 1x10^7 and 5x10^7 cells ml^{-1} in 25 ml minimal medium, imposing selection for presence of plasmid-borne markers. Optical density of the cultures at 600 nm was determined, and cultures plated on ice for 20 mins. An aliquot was removed and plated onto complete media in order to determine, by subsequent replica plating onto complete and selective media, the percentage of cells within the population which contained plasmids. Chilled cells were pelleted and resuspended in 1 ml 50 mM KH₂PO₄.

To 0.4 ml of the suspension was added an equal volume of 60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, 50 mM β-mercaptoethanol pH 7.0, then 10 μl 1 % SDS and Phenylmethylsulphonyl fluoride (PMSF) to a

8100
final concentration of 0.5 mM. The mixture was vortexed for 5 secs, incubated at 28°C for 10 mins then 30 μl chloroform added, the mixture vortexed again and incubation continued at 28°C for 10 mins. A 200 μl aliquot of 4 mg ml⁻¹ ONPG (freshly dissolved in 100 mM KH₂PO₄) was warmed to 28°C then mixed with the treated cells, a timer started and incubation continued at 28°C. At the first appearance of a yellow colour within the mixture the reaction was stopped, by addition of 0.5 ml 1 M Na₂CO₃, and time of incubation recorded. Cells were removed from the mixture and optical density of the supernatant recorded at a wavelength of 420 nm. Units of β-galactosidase activity were expressed as OD₄₂₀ min⁻¹ OD₅₇₈⁻¹, then multiplied by a factor of 1000. When the percentage of plasmid-containing yeast cells was known, assay values were corrected to that expected if 100 % of cells had contained plasmid at the time of assay. Bacterial transformants were assayed in exactly the same manner, but using 10 ml cell cultures. Since bacterial transformants were grown under antibiotic selection, all cells contained plasmids.

Generation of Nested Overlapping Deletions From Cloned k DNA DNA (5 μg) of a plasmid containing the 7.7 Kb Bam HI-Xho I fragment of k cloned within pBR322 was linearised by digestion with the restriction endonucleases Bam HI and Kpn I. The mixture was extracted with equal volumes of phenol and chloroform, and ethanol precipitated. The fragment was resuspended in TE buffer to a concentration of 1 μg DNA μl⁻¹.

For deletion of the 3'-recessed strand, 5 μl of linearised plasmid fragment were mixed with 27 μl water, 4 μl 10xExoIII buffer (10 mM MgCl₂, 660 mM Tris.HCl pH 8.0) and brought to reaction temperature by incubating at 37°C for 5 mins. Then 4 μl of 10 units μl⁻¹ exonuclease III were added. A 2 μl sample was immediately removed and mixed with 2 μl 10xExoVII buffer (300 mM KCl, 100 mM EDTA, 100 mM Tris.HCl pH 7.5) on ice. Samples were similarly taken at 30 second intervals for 5 mins, at which time 20 μl of prewarmed 1xExoIII buffer containing 40 units Exonuclease III were added to the incubation. Ten subsequent samples of 4 μl each were removed at 30 s intervals into 2 μl of ice-cold 1xExoVII buffer. The twenty samples were each diluted to 19 μl with water and mixed with 1 μl exonuclease VII diluted to 1.5 units μl⁻¹ in 1xExoVII buffer. The reactions were incubated at 37°C for 2 h to remove single-stranded regions of DNA. Samples were phenol extracted, chloroform extracted and ethanol-precipitated, resulting pellets were drained, vacuum dried and resuspended in 15 μl of 10 mM MgCl₂, 10 mM Dithiothreitol, 1 mM ATP, 100 μg ml⁻¹ Bovine Serum Albumin, 50 mM Tris.HCl pH 7.4, 50 μM dATP, 50 μM dTTP, 50 μM dCTP, 60 μM dGTP, 3 units T4
DNA Ligase, 0.5 unit Klenow fragment. Ligation was allowed to proceed overnight at 30°C. Twenty aliquots of competent E. coli NM522 were transformed with 5 µl of each ligation diluted by addition of 100 µl of 0.1 M CaCl₂. Transformants were picked and plasmid DNA prepared from 1.5 ml cultures by standard methods. Degree of deletion within each plasmid was determined by agarose gel electrophoresis of restriction digestion products.

Superinfection of pRMBL-containing E. coli
A 1 ml aliquot of M9 media, supplemented with 0.001% thiamine, 50 µg ml⁻¹ ampicillin, 0.2 % glucose, was inoculated with 10 µl of stationary phase culture of E. coli NM522 transformed with a deletion-derivative of a pRMBL/k₂ clone. The culture was grown at 37°C overnight, and a 20 µl aliquot used to inoculate 2 ml of 2xYT broth, 0.001 % thiamine, 150 µg ml⁻¹ ampicillin prewarmed to 37°C. This culture was shaken at 37°C until reaching an optical density of between 0.5 and 1.0 at a wavelength of 660 nm. A 1 ml aliquot of this culture was infected with M13K07 helper-phage at a multiplicity of 10 M13K07 plaque forming units/bacterial cell. The cell/phage mixture was shaken at 37°C for 1 h, then 400 µl added to 10 ml prewarmed 2xYT broth, containing 0.001 % thiamine, 150 µg ml⁻¹ ampicillin, 70 µg ml⁻¹ kanamycin. The culture was grown overnight at 37°C, cells pelleted and cell-free supernatants stored until required for template preparation.

DNA Sequencing
Sequencing was by the dideoxy method but using [α-³²P]dATP. All reactions were carried out at 50°C. Dideoxy-terminated fragments were electrophoresed upon a 6 % polyacrylamide/urea sequencing gel, in 1xTBE buffer (5.5 g 1⁻¹ boric acid, 0.93 g 1⁻¹ EDTA, 10.8 g 1⁻¹ Tris base). Electrophoresis was for 2.5 h or up to 7.5 h and gels dried under vacuum (without prior fixing) at 80°C for 90 mins then exposed to Kodak XAR-5 film for at least 12 h.

RESULTS AND DISCUSSION
Killer Plasmid Genes Are Not Expressed Within The Yeast Nucleus
Previous studies (reference 15. Wilson and Meacock, unpublished observations) have shown that k₁ toxin genes, cloned in circular autonomously replicating vectors, do not confer a killer phenotype upon their host. However, none of these experiments could be performed with yeast strains that also contained both k₁ and k₂ as native linear plasmids. Thus plasmid-encoded factors necessary for k₁ toxin gene expression may have been absent. In order to test, quantitatively, whether linear plasmid-encoded genes contained within nuclear vectors can be expressed when the endogenous plasmids k₁ and...
A translational fusion was made between the amino-terminal 36 codons of ORF2 and the lacZ gene of *Escherichia coli*. As the predicted lacZ-fusion product retains the ORF2 signal sequence it may become membrane associated. However analogous gene fusions between lacZ and the GAL2 permease of *S. cerevisiae* have been successfully assayed in yeast. The ORF2-lacZ gene fusion retained 1.8 Kb of *k1* DNA normally upstream of ORF2 and 1.5 Kb of *k1* DNA normally downstream of the ORF2 carboxy terminus. The fusion gene was transferred to the *S. cerevisiae* vector YRp7, to form plasmid YRpORF2. See Figure 1. During construction of plasmid YRpORF2, the ARS1 sequence, necessary for autonomous replication of YRp7 in *S. cerevisiae*, was removed. However the A/T rich *k1*-derived DNA fortuitously provides elements enabling
Table 1. Expression of an ORF2-lacZ fusion gene in yeast and E. coli

<table>
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<tr>
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Units of β-galactosidase activity expressed by various plasmids in strains of yeast and E. coli. For calculation of activity values and genotypes of strains, see materials and methods. SD11 carries an inactivating lesion within the endogenous K. lactis β-galactosidase, IFO1267 does not, but was grown under conditions which would not induce expression of the enzyme beyond basal levels. Plasmid pUCORPZ2 carries the ORF2-lacZ fusion expressed from the lac promoter of pUC19. YRpORFZ2 carries the ORF2-lacZ fusion cloned into the yeast high copy number vector YRp7. pLG669Z expresses the lacZ gene from the S. cerevisiae CYC1 nuclear promoter. pPA13 expresses lacZ from the cauliflower mosaic virus 35S promoter. YRp7 & KRp2 are, respectively, S. cerevisiae and K. lactis autonomously replicating plasmids which carry no lacZ gene.

autonomous replication of the plasmid in both S. cerevisiae and K. lactis.

Plasmid YRpORFZ2 was transformed into strains of K. lactis and S. cerevisiae which harboured linear plasmids k1 and k2, using a whole-cell transformation procedure. Transformed cells were permeabilised with chloroform, and β-galactosidase activity measured by spectrophotometric assay of the rate of hydrolysis of o-nitrophenyl-β-D-galactoside (ONPG). No enzyme activity could be detected in either yeast (Table 1). Detection of activity in E. coli strain W622, when transformed with YRpORFZ2 or with pUCORPZ2 (which expresses the gene fusion from the lac promoter) confirmed that, when expressed, the fusion protein did retain normal β-galactosidase activity. Also, other controls indicated that, under these conditions, it was
possible to detect β-galactosidase activity in yeast when expressed at high (plasmid pLO669Z) or low (plasmid pPA13 and non-induced IF01267) levels.

We suggest that expression of at least ORF2, and probably all of the k1 genes, therefore requires factors unavailable within the nuclear compartment, even when native linear k1 and k2 are also present in the cell.

**Plasmid k2 Encodes a Product With Homology to Two RNA Polymerase Subunits**

In order to investigate the novel extranuclear expression system which may be encoded by k2 we are determining the complete nucleotide sequence of this plasmid. DNA of plasmid k2 was prepared from *K. lactis* ABM02 [k1°,k2+] and digested to completion with the restriction endonucleases Bam HI and *Xho I*. This yielded two Bam HI-*Xho I* fragments, of 7.7 Kb and 5.5 Kb, which after modification of terminal restriction sites were cloned in both possible orientations into the sequencing vector pEMBL19+. An overlapping series of deletion-derivatives were prepared from the 7.7 Kb fragment, in both directions, using the exonuclease III/exonuclease VII deletion method. Deletion-derived clones were sequenced, and sequence data assembled manually using the University of Wisconsin genetics computer group (UWCG) programs.

Analysis of 7688 bp of continuous sequence data obtained from plasmid k2 reveals seven ORFs, with potential to encode products of 974, 579, 336, 158,
Fig. 3. Sequence of the ORF 974 coding region aligned with that of its predicted product, showing upstream DNA. The ORF was translated using the universal genetic code.

132, 112 and 103 amino acids. All seven ORFs exhibit the same codon usage as the ORFs of plasmid k1, all are preceded by UCS-like elements and the initiation codon of all ORFs is in a similar context (figure 2). We suggest that this indicates a common expression mechanism for the genes of plasmids k1 and k2. Alignment of k1 and k2 UCS elements leads to definition of a new minimal consensus UCS; (A/T)A(A/T/-)TNTQA, where - indicates the possible absence of a base, and N may be any base.
Table 2. Codon Usage of ORF 974 and $k_1$ ORFs

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Percentage codon usage. Average codon usage of the four open reading frames of plasmid $k_1$ are shown, compared with codon usage of ORF 974.

aa: Amino acid.

The complete sequence of this region of k$_2$ has been deposited within the EMBL database under accession number X07946. Analysis of the ORFs contained within it will be reported elsewhere (D.W.W., PhD thesis, University of...
Homology region I
Species & subunit
E. coli & FSCPYGLKTHILLVLDHRLKTFVSLYTOPLG
Mt. chl & FSCPYGLKTHILLVLDHRLKTFVSLYTOPLG
Mp. chl & FSCPYGLKTHILLVLDHRLKTFVSLYTOPLG
ORF974 & FSCPYGLKTHILLVLDHRLKTFVSLYTOPLG

Fig. 4. Homologies between product of ORF 974 and several RNA polymerase subunits. Origin of subunits abbreviated as follows: Mt chl, Nicotiana tabacum chloroplast. Mp chl, Marchantia polymorpha chloroplast. Sc, Saccharomyces cerevisiae. Da, Drosophila melanogaster. Numbers refer to location of final residue of homology region within corresponding polypeptide. Boxes and asterisks, respectively, indicate where a residue of the ORF 974 product is identical to, or conservatively different from, corresponding amino acids within two or more aligned RNA polymerase subunits. By this criterion, region A of ORF 974 product has 36.3% identity with region A of the other polypeptides. This rises to 53.8% if conservative substitutions are also taken into account. Region II shows 43.2% identity rising to 56.8%. Region III shows 48.3% identity rising to 58.3%.

Computer-assisted comparison of the predicted protein sequences of several RNA polymerase subunits with the ORF 974 product reveals an 80 amino acid region of homology (which we have termed region A) in common with the \( \beta \) subunits of RNA polymerases from E.coli\(^{27} \), the chloroplasts of tobacco Nicotiana tabacum\(^{28} \) and the chloroplasts of the liverwort Marchantia polymorpha\(^{29} \). This region is also homologous to one of nine conserved regions shared between the \( \beta \)-subunit of E.coli RNA polymerase and the 140 kD subunit of S.cerevisiae RNA polymerase \( \text{II}\(^{30} \). Additionally there are two other regions, of 43 amino acids and 59 amino acids, with homology to regions of the E.coli RNA polymerase \( \beta' \) subunit\(^{31} \) the M.polymorpha chloroplast RNA
Fig. 5. Structure of predicted ORF 974 product and several RNA polymerase subunits. Abbreviations as in Figure 4 legend. Homology regions A and I to VI are boxed. Dotted line: carboxy terminal repeat region of Sc Pol II.

polymerase $\beta'$ subunit, the 215 kD subunits of S. cerevisiae and Drosophila melanogaster RNA polymerase II and the 160 kD subunit of S. cerevisiae RNA polymerase III. These latter two regions of homology correspond to the RNA polymerase large subunit conserved regions II and III defined by Allison and coworkers. See Figure 4. However regions A, II and III are arranged differently within the ORF 974 product, as is shown in Figure 5.

We have been unable to detect any homology between the ORF 974 product and regions I, IV, V and VI common to S. cerevisiae RNA Pol II, Pol III and E. coli subunit $\beta'$. We have also failed to detect homology between the amino terminal 570 residues of the ORF 974 product and other RNA polymerase subunits. The ORF 974 product bears no primary sequence homology with the mitochondrial RNA polymerase of S. cerevisiae. This enzyme resembles the RNA polymerases of bacteriophage T3 and T7, and the predicted product of an ORF within the mitochondrial linear plasmid 82 of Zea mays. That the ORF 974 product is unrelated to this class of polymerases is consistent with an extramitochondrial location for the killer plasmids, as would be expected from their maintenance and expression within $\varphi^{0}$ strains of S. cerevisiae, and their capacity to encode proteins which enter the secretory pathway.

The apparent cytoplasmic location of $k_1$ and $k_2$ and their lack of recognisable expression signals implies that an RNA polymerase of novel properties may be required to transcribe their genes. We suggest that the ORF 974 polypeptide has a role in this task, perhaps via recognition of the UCS elements found 5' to all $k_1$ and $k_2$ genes. There may of course be additional plasmid-encoded or cellular proteins required for the process of transcription. In this regard we have detected homology between the product of a second $k_2$ ORF, ORF 579 (which is able to encode a polypeptide of 579 amino acid residues), and proteins D-569 and C-637 of the poxvirus vaccinia.
Vaccinia is a cytoplasmic DNA virus which carries its own gene expression system, and product D-569 has been identified as the DNA-dependent ATPase which may unwind the vaccinia duplex during transcription. Perhaps the product of ORF 579 performs a similar function for the killer plasmids. Like ORF 974, ORF 579 is preceded by a UCS-like sequence and its predicted initiation codon lies in an AAAATG context (figure 2). We note that a consequence of our interpretation of this data is that ORF 974 and ORF 579 products would be required for their own expression.

The roles performed by the various subunits of prokaryotic or eukaryotic RNA polymerases are poorly understood, although there is some evidence to suggest that the $\beta'$ subunit of E.coli RNA polymerase may have a role in DNA binding whilst the $\beta$ subunit may bind nucleotides. Because the product of ORF 974 shares homology with some of the regions found in both types of subunit, its structure is unique. Comparison of its biological activity with known RNA polymerases of conventional structure will lead to an increased understanding of the roles of each subunit and the conserved regions within them.

ACKNOWLEDGEMENTS

We thank Michael Romanos and Alan Boyd for communicating ORF2 transcript data prior to publication, and Michael Stark and Michael Pocklington for advice concerning interpretation of homology data. Plasmid pPA13 was a gift from Jennifer Richardson. This work was supported by the core research programme of the Leicester Biocentre, and the award of an SERC studentship to D.W.W.

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REFERENCES

Nucleic Acids Research
Since submission of this manuscript Tommasino et al. published the complete nucleotide sequence of plasmid k2 (Nucl.Acids.Res. 16, 5863-5878). Their predictions concerning the genetic organisation of k2 ORFs are in good agreement with our own, except that they have found ORFs 112 and 336 to be joined into a single ORF of 453 amino acids.