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Genome organization of the linear plasmid, pSKL, isolated from *Saccharomyces kluyveri*

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Summary. We have determined the complete nucleotide sequence of the linear DNA plasmid, pSKL, isolated from *Saccharomyces kluyveri*. Sequence analysis showed that pSKL has a high (A+T) content of 71.7%, and that there are 10 open reading frames (ORFs) larger than 250 nucleotides. All 10 ORFs were shown to be transcribed in *S. kluyveri* cells by S1 nuclease mapping analysis. The localization of ORFs, direction of transcription, and the predicted amino acid sequences of each ORF were quite similar to that of pGKL2, one of the killer plasmids found in *Kluyveromyces lactis*. The amino acid sequences of the largest two ORFs (ORF2 and ORF6) have homology with several DNA polymerases and RNA polymerases, respectively.

Key words: Yeast – Linear plasmid – *Saccharomyces kluyveri* – *Kluyveromyces lactis* – Killer plasmid

Introduction

A linear DNA plasmid, pSKL, was isolated from the yeast *Saccharomyces kluyveri*. The 14.2 kb pSKL was found to have a protein associated with its terminal regions (terminal protein, TP) and inverted terminal repeat (ITR) sequences of 483 bp (Kitada and Hishinuma 1987). These structural characteristics are similar to those of extrachromosomal DNA molecules found in several organisms (Meinhardt et al. 1990), including adenoviruses (Friefeld et al. 1986) and bacteriophage ϕ29 of *Bacillus subtilis* (García et al. 1984), in which TPs and ITR sequences are believed to be required for replication of linear DNA molecules.

A killer strain of the lactose-fermenting yeast *Kluyveromyces lactis* was found to harbor two linear DNA plasmids, pGKL1 (8.9 kb) and pGKL2 (13.4 kb) (Gunge et al. 1981). The pGKL1 codes for three subunits of the killer toxin (Stark and Boyd 1986), an immunity determinant against the toxin (Gunge 1986; Tokunaga et al. 1987), and a protein, the predicted amino acid sequence of which is homologous to several DNA polymerases (Jung et al. 1987b; Fukuhara 1987; Volkert et al. 1989; Stark et al. 1990). On the other hand, pGKL2 is essential for the maintenance of pGKL1 (Gunge 1986) and for the expression of the immunity determinant (Tokunaga et al. 1987). The pGKL2 plasmid contains 10 ORFs (Tommasino et al. 1988). Two of them (ORFs 2 and 6) have sequence homology to DNA and RNA polymerase, respectively (Tommasino et al. 1988; Wilson and Meacock 1988). These enzymes seem to be essential for replication and transcription of the linear DNAs in the cytoplasm (Romanos and Boyd 1988).

The pSKL DNA does not hybridize to pGKL1 and pGKL2, but 15 out of 16 of the terminal nucleotides are identical to those of pGKL2, suggesting that the replication mechanisms of both plasmids are similar (Kitada and Hishinuma 1987). Since the *S. kluyveri* strain cured of the pSKL plasmid is normal in growth and morphology, the function of pSKL is unknown so far. To identify functions of the pSKL plasmid, we determined the entire nucleotide sequence of pSKL DNA, and found that its gene organization and the amino acid sequences of the predicted proteins from each ORF showed similarity with that of pGKL2, and that two of the ten ORFs were homologous to DNA and RNA polymerases from various organisms. These results suggest that pSKL and pGKL2 originated from a common ancestor.

Materials and methods

Strains. The yeast *S. kluyveri* UVC40 which contains a high copy number of the pSKL plasmid was isolated from the strain IFO1685 (Kitada and Hishinuma 1987) by UV irradiation and used for the isolation of the pSKL

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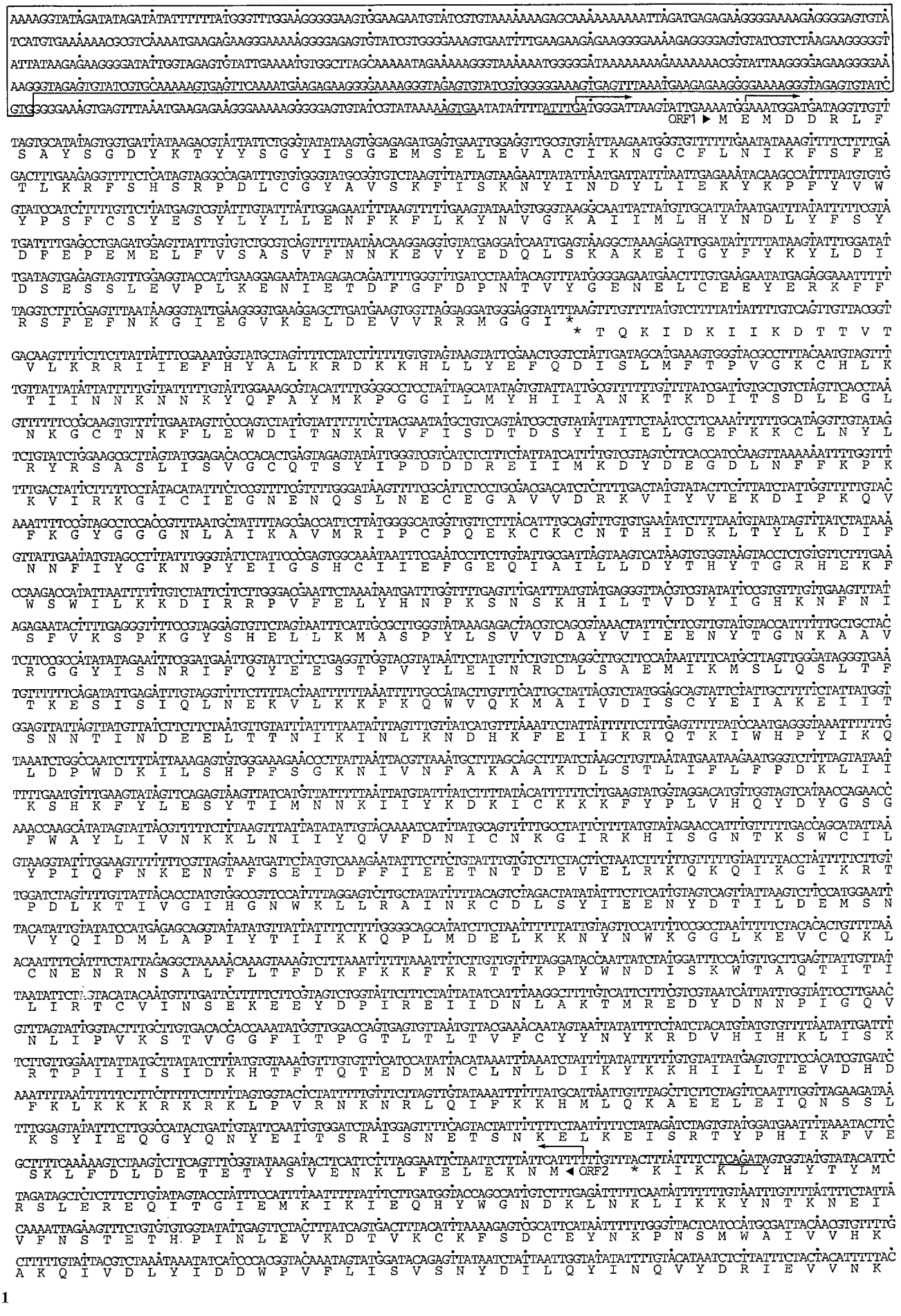
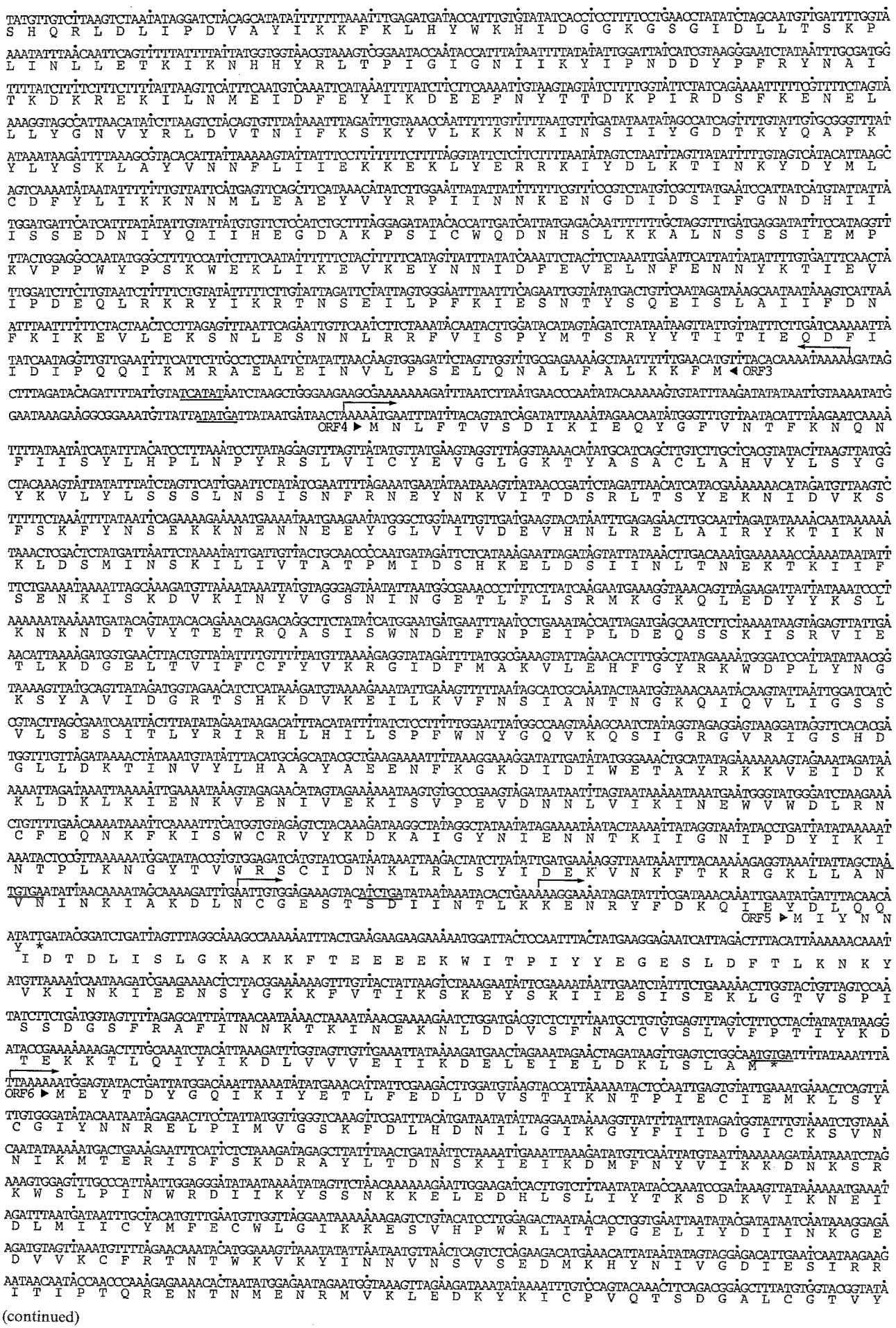


Fig. 1



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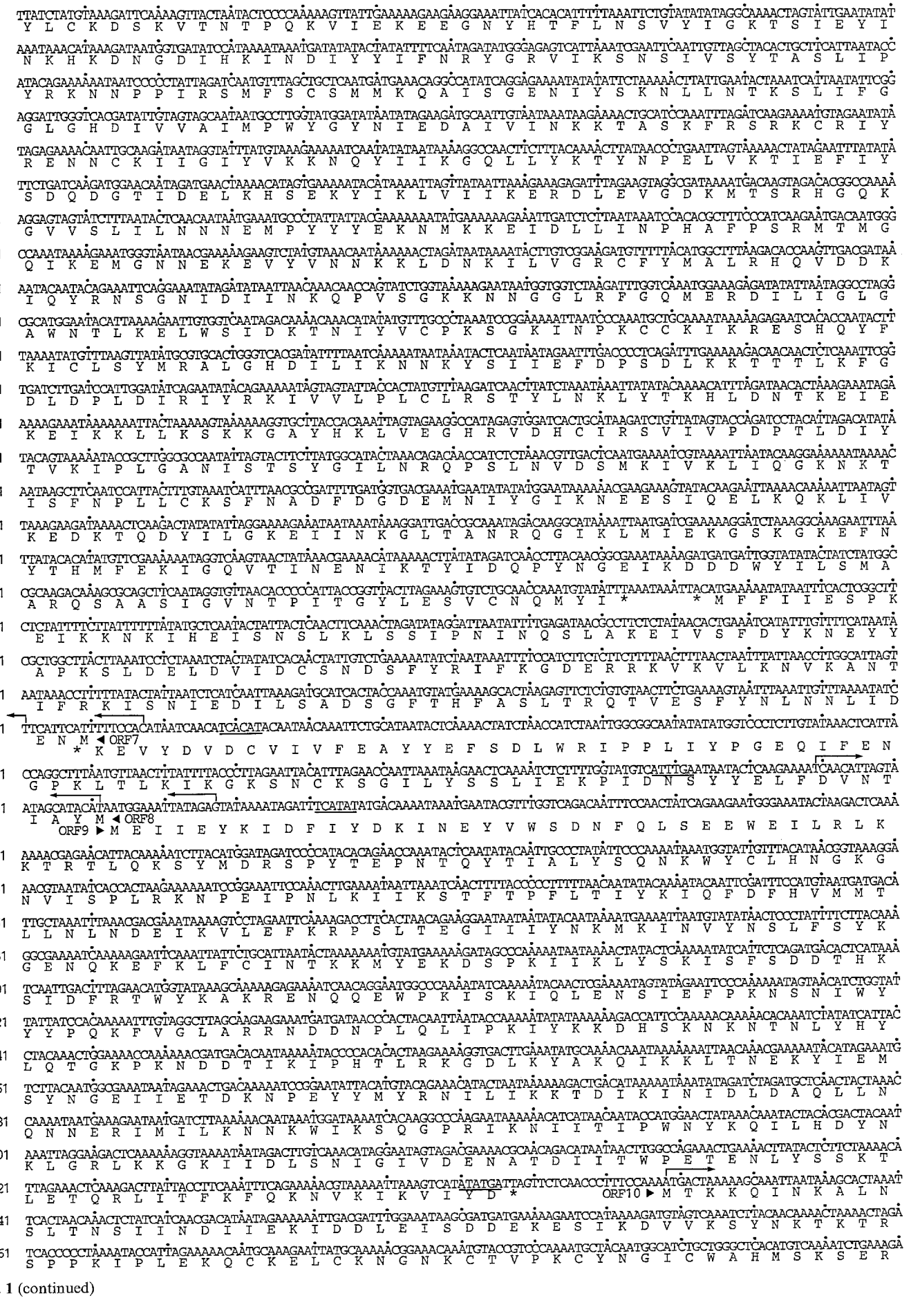

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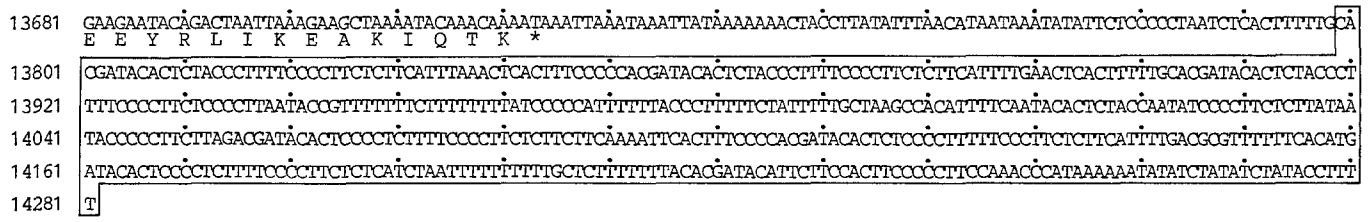

Fig. 1. Nucleotide sequence of the pSKL plasmid. The sequence of pSKL (14281 bp) is shown together with the predicted amino acid sequences of the 10 ORFs. The terminal 600 bp sequences have been reported previously (Kitada and Hishinuma 1987). The terminal 483 bp identified as inverted terminal repeat (ITR) sequences are *boxed.* The initiation sites and the directions of transcription of the ORFs are shown by *arrows*; the sites of translational termination are indicated by *asterisks*. The upstream consensus sequences (UCS) are *underlined.* The accession number of the sequence is X54850 in DNA Data Bank of Japan, EMBL, and GenBank

plasmid. *Escherichia coli* strain JM83 was used as a host for the subcloning of pSKL DNA.

DNA sequencing. The DNA fragments of pSKL produced with appropriate restriction endonucleases were subcloned into pUC13, pUC18, or pUC19. The DNA sequence was determined by the chain termination method (Sanger et al. 1977) with a sequencing kit from Takara Shuzo Co. using M1, M4, and RV oligonucleotide primers and [α-^{35}S]dCTP (Amersham).

S1 mapping. Total mRNA was isolated from *S. kluyveri* strain UVC40. S1 mapping analysis was performed as previously described (Inokuchi et al. 1987).

Miscellaneous methods. Manipulations of DNA were carried out by the methods of Maniatis et al. (1982). All enzymes were used according to the recommendations of the manufacturer. The nucleotide sequence was analyzed using the GENETYX software (Software Development Co., Tokyo), and amino acid sequences were compared with the NBRF data base.

Results

Nucleotide sequence of pSKL is highly homologous with that of pGKL2

We have previously established a restriction map of pSKL DNA using several restriction endonucleases, and subcloned all *Eco*RI fragments into *Eco*RI (for internal fragments) or *Eco*RI−*Sma*I (for terminal fragments) sites of pUC13 (Kitada and Hishinuma 1987). To analyze the gene structure of pSKL, the nucleotide sequence was determined directly using these nine *Eco*RI subclones or using plasmids containing shorter fragments after further subcloning. To determine the sequences of the junction regions and the order of *Eco*RI fragments, we further constructed subclones of pSKL DNA by using several restriction enzymes. Where there are no restriction sites available within about 300 bp, a series of deletion plasmids was obtained according to Henikoff (1984). The sequences were determined at least once on both strands and showed that the pSKL DNA was 14281 bp long (Fig. 1) and had a high (A+T) content (A, 36.6%; T, 35.1%; G, 15.3%; C, 13.0%), which is almost coincident with the value derived from buoyant density measurements (Kitada and Hishinuma 1987).

To determine potential protein coding regions, the nucleotide sequence of pSKL was analyzed for the position of stop codons in all possible reading frames. In this analysis, the pSKL sequence reveals 10 ORFs covering about 90% of the genome (Table 1). Several ORFs overlap (ORFs 1–2, ORFs 4–5, ORFs 7–8, and ORFs 8–9; see Fig. 1), as observed in pGKL1 and pGKL2, the killer plasmid of *K. lactis* (Stark et al. 1984; Hishinuma et al. 1984; Sor and Fukuhara 1985). Surprisingly, the orientations and locations of all ORFs were quite similar to those of pGKL2 (Fig. 2), although we could detect no obvious homology between pSKL and pGKL2 by Southern hybridization under the conditions of 50%

Table 1. The ORFs of pSKL and characteristics of the predicted proteins

	Nucleotide positions	Number of amino acids	Similarity[1] (%)
ORF1	578– 1276	233	42.5
ORF2	4275– 1279	999	61.7
ORF3	5977– 4286	564	61.5
ORF4	6170– 7924	585	66.8
ORF5	7908– 8384	159	62.9
ORF6	8408–11362	985	63.3
ORF7	11769–11374	132	52.3
ORF8	12011–11769	81	63.0
ORF9	12013–13386	458	53.5
ORF10	13408–13722	105	42.8

The analysis of the open reading frames revealed 10 predicted protein coding regions (ORF1–ORF10) in pSKL. The number of amino acid residues and the molecular weight of each predicted protein were calculated by assuming that each ORF is translated from the first ATG codon, except for ORF8 in which a predicted protein having 92 amino acid residues was originally found. However, based on S1 mapping, transcription initiates between the first and the second ATG codons. Therefore, the protein from ORF8 could be translated from the second ATG codon and comprise 81 amino acid residues instead of 92

[1] Percentages of identical amino acid residues with that of the corresponding protein from pGKL2 are shown. Gaps were inserted for maximum matching

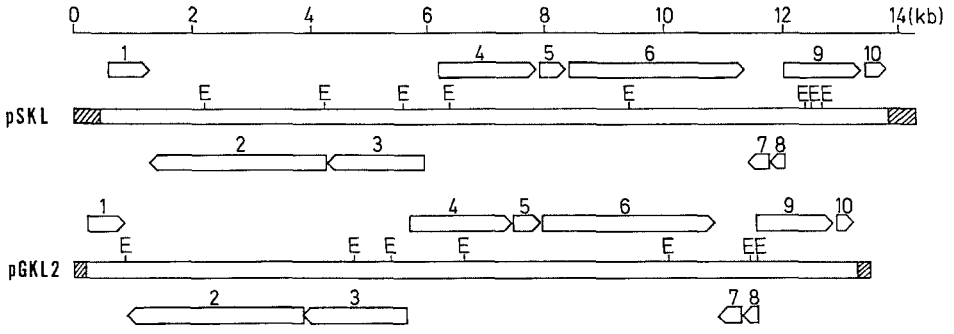

Fig. 2. Genome organization of pSKL and pGKL2. The location and the orientation of ORFs are represented by *arrows*. The *Eco*RI (E) sites are shown to facilitate orientation of the plasmid DNAs

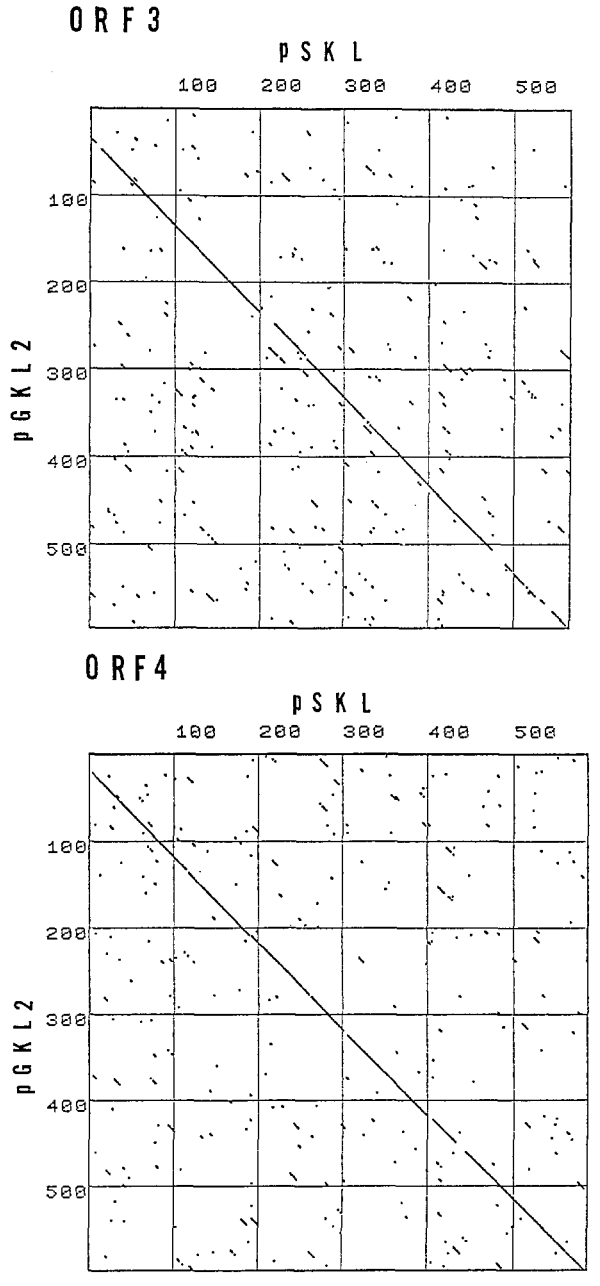

Fig. 3. Harr plot analysis of ORFs 3 and ORFs 4 from pSKL and pGKL2. Each dot was drawn when the minimum mean scores (Staden 1982) were more than 2.0 in 10 amino acid residues using the HARPLT program in the GENETYX software

formamide and 4× SSC (1×SSC is 0.15 M NaCl, 15 mM sodium citrate) at 30° C overnight (data not shown). Furthermore, the amino acid sequences of predicted proteins from the corresponding ORFs showed high degrees of similarity between pSKL and pGKL2 (about 42%–67%) (Table 1). Moreover, the homologies of the ORF products between pSKL and pGKL2 were found throughout the entire length of each protein. Two examples are shown in Fig. 3. These results strongly suggest that pSKL and pGKL2 are derived from a common ancestor.

Determination of transcriptional initiation sites of pSKL

In order to see whether these ORFs represent expressed genes, we examined the transcripts of pSKL by S1 nuclease mapping analysis. The probe DNA fragments were hybridized individually to total RNA from *S. kluyveri* UVC40 cells. Since the mRNAs from ORFs 2 and 9 were hardly detected by the use of total RNA, poly(A)$^+$ RNA was prepared to concentrate the mRNA, and was used for S1 mapping analysis. DNA fragments protected from digestion with nuclease S1 were detected in all ORFs, showing that the 10 ORF regions are transcribed in *S. kluyveri* cells (Fig. 4). Two or more bands were observed in the case of ORFs 1, 5, 7, and 8, suggesting that these genes are transcribed from multiple sites. The starting site of transcription deduced from the data of Fig. 4 is shown in Fig. 1. The 5′ untranslated regions of mRNA from pSKL are relatively short (0–24 nucleotides) except for ORF5. There are no ATG codons in the 76 bp upstream of ORF5. We originally predicted that a protein of 92 amino acid residues was encoded by ORF8, but the 5′ end of the transcript from ORF8 region was found to localize between the first and second ATG codon. Therefore, we concluded that its translation should start from the second ATG codon (positions 12011–12009), and that a protein of 81 amino acid residues is produced from ORF8 (Table 1).

ORF2 and ORF6 probably encode a DNA polymerase and an RNA polymerase, respectively

The amino acid sequences derived from the potential coding regions of pSKL were compared with those of

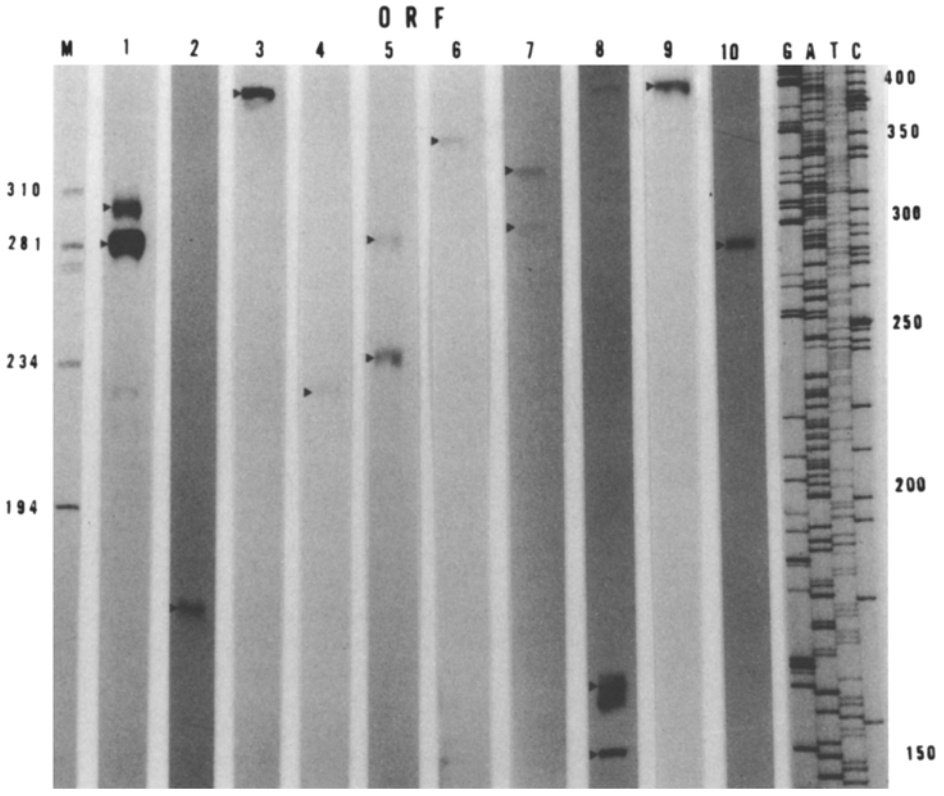

Fig. 4. S1 nuclease mapping of the transcripts of pSKL. The isolation of total cellular RNA from *Saccharomyces kluyveri* UVC40 cells and S1 mapping were performed as described previously (Kitada and Hishinuma 1987). One hundred micrograms of total RNA was used for the detection of mRNAs from ORFs 3, 4, 5, 6, and 9, and 200 μg of the RNA was used in the case of ORFs 7, 8, and 10. Poly(A)$^+$ mRNA (about 50 μg) was used in the case of ORF2. The temperature during hybridization was 37° C for ORFs 1–3 and 30° C for ORFs 4–10. The following DNA fragments were used as probes. ORF1, *Hin*fI(866)-left terminal(1); ORF2, *Bal*I(4100)-*Afl*II(4689); ORF3, *Eco*RI(5610)-*Nsi*I(6325); ORF4, *Eco*RI(6395)-*Bgl*II(5845); ORF5, *Asu*II(8114)-*Acc*I(7605); ORF6, *Xba*I(8760)-*Aat*II(8230); ORF7, *Ssp*I(11467)-*Hin*fI(12113); ORF8, *Ava*II(11858)-*Dra*I(12371); ORF9, *Eco*RI(12398)-*Nsi*I(11680); and ORF10, *Alu*I(13705)-*Hae*III(13288). The restriction enzyme sites shown first in each probe DNA were labeled at the 5′ ends. Transcripts hybridizing to the DNA fragments which were protected from digestion with S1 nuclease are indicated by the *arrowheads*. Lane M shows molecular weight markers in which ϕX174 DNA was digested with *Hae*III and labelled at the 5′ ends. The four lanes marked G, A, T, and C show samples obtained from the sequencing reaction using [$\alpha-^{35}$S]dCTP by the chain termination method (Sanger et al. 1977). The sizes of molecular weight markers are given in bp

		Region I		Region II		Region III	
		** ** ******* ****		**** * **** **		* **********	
pSKL	638	VYADVVSLYPSAMKLLEHSYG	773	VAKIALMGGGYGKFVQK	876	LEIIYSDTDSIFVRK	
pGKL2	633	VYADVVSLYPSAMKLLKHSYG	768	VAKIALNGGGYGKFVQK	874	IDIIYSDTDSIFVKQ	
pGKL1	638	LCLDVKSLYPASMAFYDQPYG	774	VILIIMSN-LWGKFAQK	860	AECIYSDTDSIFVHK	
ϕ29	246	MVFDVNSLYPAQMYSRLLPYG	381	LAKLMINS-LYGKFASN	450	DRIIYCDTDSIHLTG	
PRD1	217	KVYDVNSMYPHAMRNFRHPFS	338	FYKLILNS-SYGKFAQN	422	ERPLYCDTDSIICRD	
S1	485	YYYDVNSLYPSSML-DDMPIG	615	IYKITMNS-LYGRFGIS	711	DDCYYTDTDSVVVER	
pClK1	667	YYYDVNSLYPFASI-NDIPGL	793	IAKLILNS-LIGRFGMN	910	GTLYYTDTDSIVTDL	
Ad 2	536	YVYDICGMYASALT-HPMPWG	639	IAKLLSNA-LYGSFATK	864	LKSVYGDTDSLFVTE	
HSV	714	VVFDFASLYPSIIQAHNLCFS	809	AIKVVCNS-VYGFTGVQ	880	MRIIYGDTDSIFVLC	
EBV	581	LVVDFASLYPSIIQAHNLCYS	679	AIKCTCNA-VYGFTGVA	749	LRVIYGDTDSLFIEC	
Vacc	521	LIFDYNSLYPNVCIFGNLSPE	635	TYKIVANS-VYGLMGFR	720	FRSVYGDTDSVFTEI	

Fig. 5. Comparison of the potential polypeptide encoded by ORF2 of pSKL and various DNA polymerases encoded by other plasmids and viruses. The following sequences were compared: ORF2 of the pGKL2 plasmid (Tommasino et al. 1988); ORF1 of pGKL1 plasmid (Hishinuma et al. 1984; Stark et al. 1984), the bacteriophage ϕ29 of *Bacillus subtilis* (Yoshikawa and Ito 1982); the small lipid-containing bacteriophage PRD1 of *Escherichia coli* (Jung et al. 1987a); the URF3 of the maize mitochondrial plasmid S1 (Paillard et al. 1985); ORF2 of the mitochondrial plasmid pClK1 of *Claviceps purpurea* (Oeser and Tudzynski 1989); the viral DNA polymerases of adenovirus 2 (Gingeras et al. 1982), herpes simplex virus (Quinn and McGeoch 1985), Epstein-Barr virus (Baer et al. 1984), and vaccinia virus (Earl et al. 1986). The three regions presented here are identical to those reported by Tommasino et al. (1988). The residues identical or analogous to that of pSKL ORF2 at a given position are indicated in *boldface*. *Asterisks* indicate positions where residues are identical among three sequences or analogous among five sequences

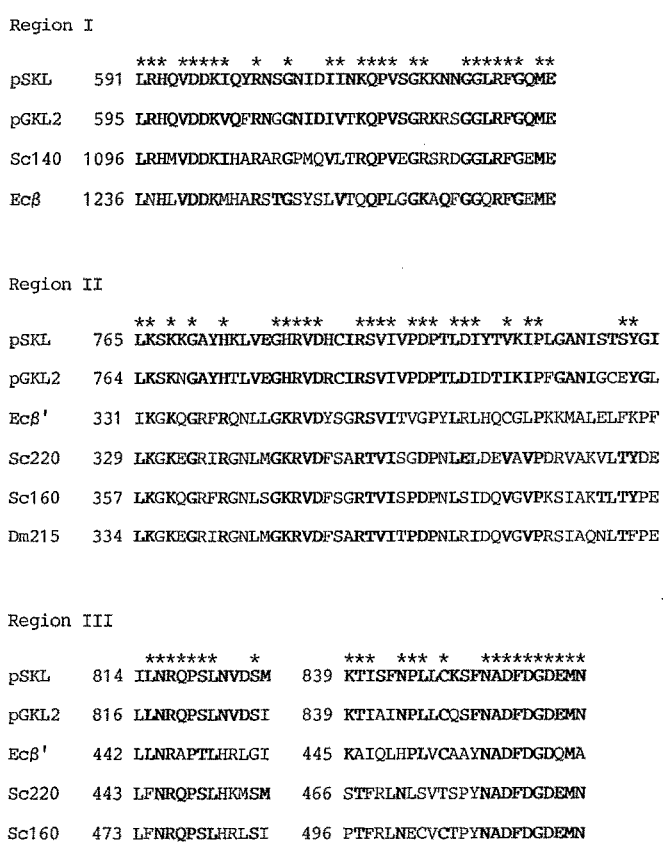

Fig. 6. The three regions of homology between the product of ORF6 of pSKL and several RNA polymerase subunits. The sequences compared are the ORF6 of pGKL2 (Tommasino et al. 1988), the 140 kDa RNA polymerase II of *S. cerevisiae* (Sweetser et al. 1987), the β subunit of *E. coli* RNA polymerase (Ovchinnikov et al. 1981), the β' subunit of *E. coli* RNA polymerase (Ovchinnikov et al. 1982), the 220 kDa RNA polymerase II of *S. cerevisiae* (Allison et al. 1985), the 160 kDa RNA polymerase III of *S. cerevisiae* (Allison et al. 1985), and the 215 kDa RNA polymerase II of *Drosophila melanogaster* (Biggs et al. 1985). The residues identical or analogous to that of pSKL ORF6 at a given position are indicated in *boldface*, and positions where residues are identical or analogous among three sequences are marked by *asterisks*

known proteins. The deduced amino acid sequence from ORF2 shows homology to several DNA polymerases, especially those of bacteriophages, viruses, and plasmids. Some examples are listed in Fig. 5. ORF2 contains the three main conserved domains described for various DNA polymerases, which are thought to be involved in nucleotide binding (Jung et al. 1987b). The first eight sequences listed in Fig. 5 are postulated or have been shown to use a protein as a primer. In addition, the relative spatial orientation of different domains within the proposed polypeptide and the expected molecular weight of about 100 kDa correspond well to viral and other plasmid-encoded DNA polymerases. Thus it is likely that ORF2 encodes DNA polymerase.

A comparison of the ORF6-derived amino acid sequence with that of known proteins showed homology in three regions with various RNA polymerases, as already shown by Wilson and Meacock (1988) for the ORF6 product of pGKL2 (see Fig. 6). Sweetser et al. (1987) indicated that nine regions (A to I) are homologous between the 140 kDa RNA polymerase II of *S. cerevisiae* and the β subunit of *E. coli* RNA polymerase. Indeed a sequence similarity between the region I and that of ORF6 of pSKL was observed. This region is also homologous with the β subunit of the chloroplast RNA polymerases of *Nicotiana tabacum* and *Marchantia polymorpha* as shown by Wilson and Meacock (1988). Furthermore, among the six regions (I to VI) which are homologous in the 220 kDa RNA polymerase II of *S. cerevisiae*, the 160 kDa RNA polymerase III of *S. cerevisiae* and the β' subunit of *E. coli* RNA polymerase (Allison et al. 1985), two of them (II and III) have similarities with the amino acid sequence of ORF6 of pSKL, and are also homologous to DNA polymerases of bacteria and bacteriophages (Allison et al. 1985), RNA polymerase II of *Drosophila melanogaster* and the β' subunit of *Marchantia polymorpha* RNA polymerase (Wilson and Meacock 1988). Thus, the predicted protein derived from ORF6 seems to be an RNA polymerase. It should be pointed out that the 22 carboxy-terminal residues of region II in RNA polymerases of *S. cerevisiae* was proposed to have a helix-turn-helix structure which is known as a DNA-binding domain (Allison et al. 1985; Ollis et al. 1985). The proline residue at the center of the "turn" structures are conserved in all RNA polymerases listed in Fig. 6.

Discussion

The complete nucleotide sequence of the linear DNA plasmid pSKL was determined. Analyses of the protein coding regions and the transcripts of pSKL showed that there are 10 ORFs and that all ORFs are transcribed in *S. kluyveri* cells. The genome organization and the structures of each protein derived from pSKL showed similarities with that of the killer plasmid pGKL2, suggesting that these plasmids evolved from a common ancestor, even though *S. kluyveri* and *K. lactis* are classified into different genera of yeast.

Although the terminal inverted repeat sequences of pSKL and pGKL2 are quite different, the terminal sequences are identical at 15 out of 16 positions. Since the terminal sequences seem to be important for the replication of the linear DNA molecules (Gutiérrez et al. 1988; Tamanoi and Stillman 1982), this similarity may suggest that the terminal protein of pSKL shares homology with that of pGKL2 in its structure and function. We believe that the terminal protein of pSKL plasmid is encoded by pSKL itself, similarly to adenovirus and ϕ 29. The amino acid sequences of the terminal proteins in ϕ 29 (Salas et al. 1978; Salas 1988) and adenovirus (Alestrom et al. 1982) have no similarities to any ORF products in pSKL. Since the molecular weight of the terminal protein of pGKL2 is about 36 kDa (Stam et al. 1986), this size of protein can be encoded by ORFs 1, 3, 4, or 9. Another possibility is that the terminal protein is produced by post-translational processing of the protein from ORF2 or ORF6 which are assumed to for

a DNA polymerase and an RNA polymerase, respectively (see above).

The killer gene encoded by pGKL1 was not transcribed accurately when the pGKL1 DNA was introduced on a circular replicating plasmid (Romanos and Boyd 1988). The pGKL plasmids seem to have novel promoters that are inactive on circular plasmids which replicate in the nucleus. The upstream consensus sequence (UCS) of ACTNAATATATGA (N; any base) in pGKL1 was found 13–18 bp upstream of the transcriptional initiation sites (Stark et al. 1984; Sor and Fukuhara 1985). In a search for the UCS sequence in the upstream regions of each ORF in pSKL, the above UCS was not found, but the shorter sequence, ATNTGA, was found 16–35 bp upstream from the transcriptional initiation site of all the ORFs (Fig. 1). The UCS of pSKL, which is similar to that of pGKL1 and pGKL2 (Wilson and Meacock 1988), may function as a promoter in the linear DNAs of yeast. The UCS of ORF2 in pGKL1 was shown in fact to direct cytoplasmic expression (Kämper et al. 1989). Stem-loop structures are located downstream of the UCS in pGKL1 (Stark et al. 1984; Sor and Fukuhara 1985) but these structures were not found in pSKL.

The finding that DNA polymerase of the linear plasmids in yeast (pSKL, pGKL1, and pGKL2) are highly homologous with the viral enzymes (Fig. 5), suggests that these plasmids are viral in origin, although we have not found the virus-like particle in yeast cells. The plasmid pGKL1 encodes the killer toxin whilst the immunity determinant genes are lost at low frequency during the maintenance of *K. lactis* cells. This may imply that *S. kluyveri* cells previously had two plasmids but now retain only pSKL. It would be interesting to investigate whether pSKL can functionally replace pGKL2 in the maintenance of pGKL1 and the expression of the immunity determinant.

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