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SEQUENCE NOTES

The *DIM1* Gene Responsible for the Conserved $m_2^6Am_2^6A$ Dimethylation in the 3′-Terminal Loop of 18 S rRNA is Essential in Yeast

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Biogenesis of cytoplasmic ribosomes universally involves methylation of ribosomal RNA. Little genetic evidence is available about the functional role(s) of this conserved post-transcriptional modification. The only known methylase gene involved in rRNA maturation is *ksgA* in *Escherichia coli*, which directs dimethylation of two adjacent adenosines ($m_2^6A_{1518}m_2^6A_{1519}$) in the loop of a conserved hairpin near the 3′-end of 16 S rRNA. This tandem methylation is the only rRNA modification common to pro and eukaryotes. Disruption of *ksgA* confers resistance to the aminoglycoside antibiotic kasugamycin without significantly impairing viability. Here we report the cloning of the *DIM1* gene encoding the homolog 18 S rRNA dimethylase in *Saccharomyces cerevisiae*. The yeast enzyme is evolutionary related to the ksgA protein. It carries a distinctive lysine-rich-N-terminal extension with a potential protein kinase C phosphorylation site. Like ksgA, DIM1 belongs to the erm family of prokaryotic 23 S rRNA dimethylases responsible for erythromycin resistance. Surprisingly, disruption of *DIM1* turns out to be lethal in yeast.

Keywords: ribosome biogenesis; 18 S rRNA; N^6,N^6-dimethyladenosine; methylase; translation

A KsgA⁻ *Escherichia coli* mutant (Suvorov *et al.*, 1988) resistant to the aminoglycoside antibiotic kasugamycin (Helser *et al.*, 1971, 1972) was used as host for cloning the *DIM1* gene by complementation with a yeast genomic library. Since antibiotic-sensitive candidates were to be screened by toothpick-replicating clones on kasugamycin, a completely stable host-vector system was required. Indeed, replicas from complemented clones would be scored as positives in case the vector would be lost in a fraction of the mother colony as a result of segregational instability under non-selective conditions prevailing on the master plate. We therefore introduced a second mutation (*pyrF*::*cat*) into the host strain by gene disruption (data not shown) in order to make it auxotroph for uracil. Segregational stability was then simply achieved by using pFL44 (*URA3*) as vector and growing transformants without uracil (Bach *et al.*, 1979). One kasugamycin-sensitive clone was found to harbor a plasmid encoding the expected dimethylase. Indeed, 16 S rRNA purified from this clone was shown to yield m_2^6A upon digestion to nucleosides followed by HPLC analysis, as opposed to the untransformed host (Figure 1).

The nucleotide sequence of the *DIM1* gene, together with the amino acid sequence of the encoded DIM1 enzyme, are given in Figure 2. It can be seen that the yeast dimethylase displays a distinctive lysine-rich-N terminus (5K/16 amino acids) with a potential protein-kinase-C-phosphorylation site (Woodgett *et al.*, 1986) (Ser14). The DIM1 protein is related to the ksgA enzyme (27% identity and 50% similarity) and both proteins belong to the *erm* family of dimethylases (van Gemen & van Knippenberg, 1990) which confer

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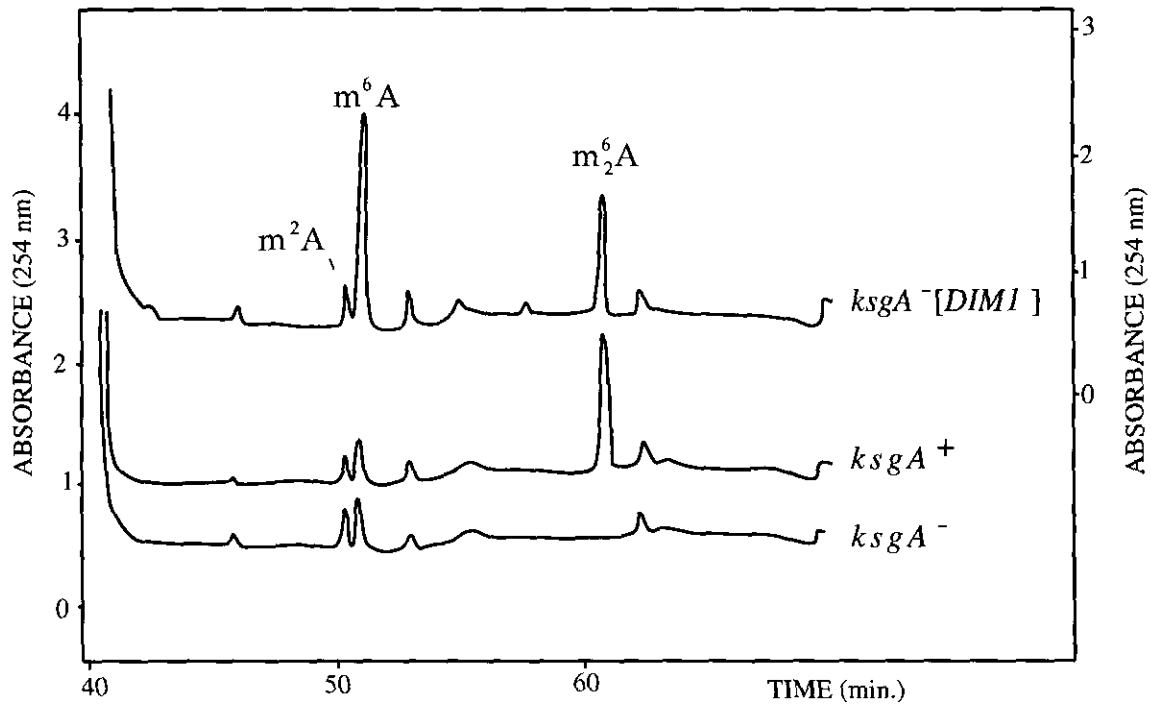

Figure 1. HPLC profiles of 16 S rRNA nucleosides in the *DIM1*-complemented *ksgA*⁻ *E. coli* mutant (upper; right ordinate) in comparison with (left ordinate) the untransformed *ksgA*⁻ host (lower) and the *ksgA*⁺ positive control (middle). A distinctive m^6_2A peak is observed upon complementation. Other indicated minor peaks are normally absent from 16 S rRNA and denote a slight contamination with 23 S rRNA (m^6A) and tRNA (m^2A, m^6A) at similar levels in all three samples. The drastic relative increase observed in m^6A abundance in the presence of *DIM1* reflects a substantial degree of monomethylation due to heterologous complementation of the *E. coli ksgA*⁻ mutation with the wild-type *DIM1* yeast gene. The *DIM1* gene was cloned by complementation of a *ksgA*⁻ *E. coli* mutant (Suvorov *et al.*, 1988) made auxotroph for uracil through two-step disruption of the orotidine-5′-phosphate decarboxylaxe *pyrF* gene. Briefly, a *cat* chloramphenicol resistance marker (Fuqua, 1992) was inserted *in vitro* into the *pyrF* gene (Turnbough *et al.*, 1987). The *pyrF*::*cat* construct was self-ligated and used as suicide Campbell-integration module. Integrants selected on chloramphenicol underwent spontaneous excision of the wild-type *pyrF* allele to yield 5-fluoro-orotate resistant (Boeke *et al.*, 1984) *pyrF*::*cat* disruptants which were authenticated by Southern and PCR analyses (not shown). The yeast genomic library was established by ligating *Sau*3A genomic fragments (2 to 10 kb) from the *Saccharomyces cerevisiae* FL100 strain (ATCC 28383) into the *Bam*HI-cleaved multicopy vector pFL44 (Bonneaud *et al.*, 1991). About 10,000 Ura⁺ transformants were selected on plates without uracil and toothpick-replicated on minimal medium supplemented with 50 μg/ml kasugamycin. One kasugamycin-sensitive clone was shown to harbor a recombinant plasmid encoding the expected dimethylase. This was demonstrated by extracting total RNA and isolating 16 S rRNA by sucrose gradient centrifugation. RNA was then hydrolyzed by nuclease P1 followed by bacterial alkaline phosphatase, and nucleosides were analyzed by HPLC (Desgrès *et al.*, 1989; Gehrke & Kuo, 1989). *DIM1* maps to the left tip of chromosome XVI since the overlaping clones 2778 and 4860 from the ordered yeast genomic library (gift from L. Riles and M. Olson) are positive (not shown).

erythromycin resistance to several bacterial species by m^6_2A methylation of 23 S rRNA (Figure 3).

To test whether DIM1 encodes an essential function, we introduced a *dim1*Δ::*URA3* null-mutation into a diploid *ura3*/*ura3* yeast strain using the one-step-gene-disruption procedure (Rothstein, 1983). Disruptive integration was checked by PCR† (Figure 4A) and Southern-blot analysis (not shown). Hemizygous diploid disruptants (*dim1*Δ::*URA3*/*DIM1*; *ura3*/*ura3*) were sporulated and tetrad analysis clearly showed that only the two Ura⁻ spores could give rise to viable colonies (Figure 4B). These results demonstrate that the *dim1*Δ::*URA3* allele is a recessive lethal, i.e. that the *DIM1* gene is essential in yeast.

Why is the conserved m^6_2A m^6_2A rRNA methylation dispensable in *E. coli*, and essential in yeast? It seems unlikely that yeast ribosomes lacking m^6_2A m^6_2A would be lethally impaired in protein synthesis, since the same mutation is virtually harmless to *E. coli* (van Knippenberg, 1986) and knowing the high degree of structural and functional conservation at that ribosomal site (Raué *et al.*, 1988). We cannot rule out that the DIM1 enzyme could methylate not only the twin adenosines in the 3′-terminal loop of 18 S rRNA, but also some other unknown, essential adenosine RNA residue. Rather, we favor

† Abbreviation used: PCR, polymerase chain reaction.

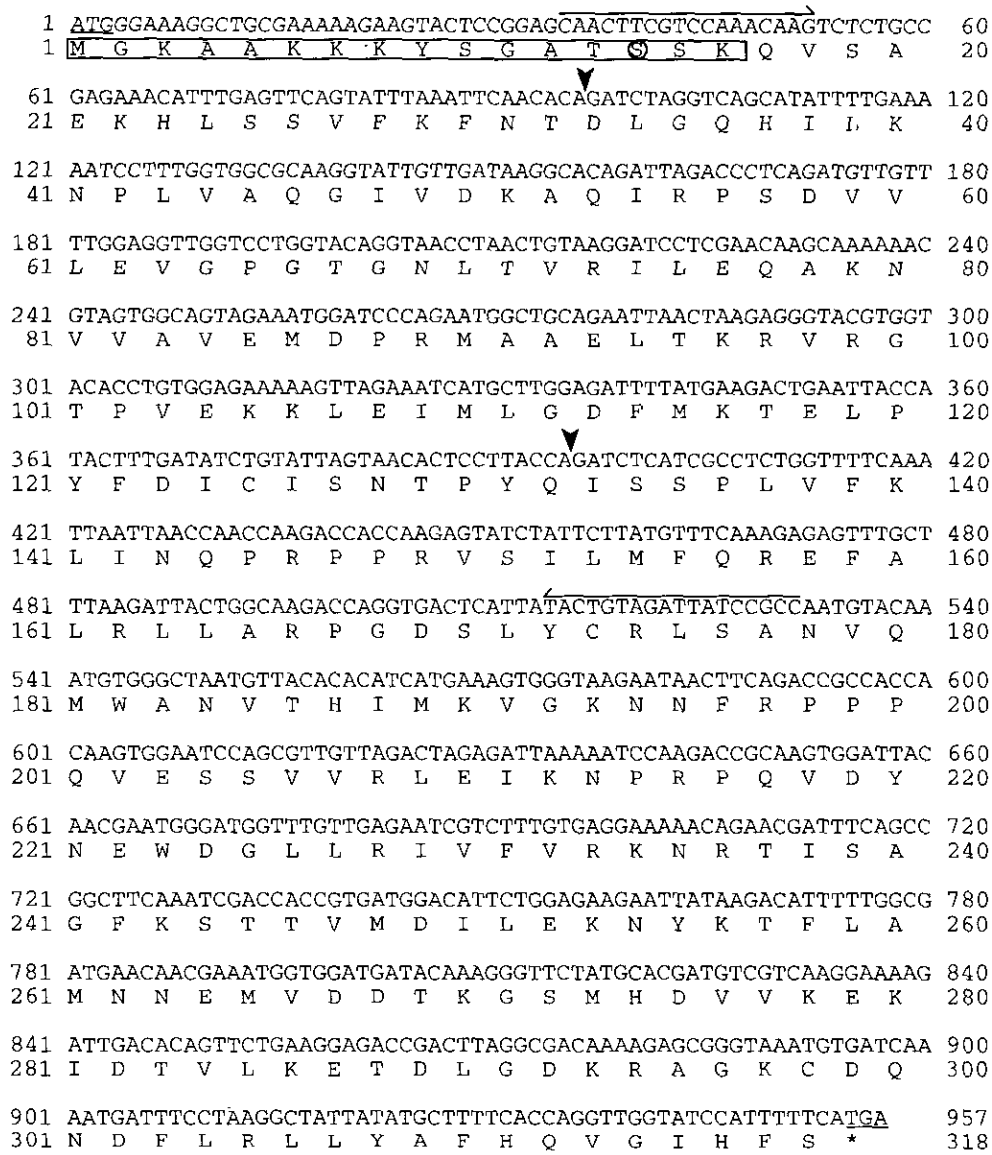

Figure 2. Nucleotide sequence (EMBL accession number L26480) of the *DIM1* open reading frame together with the amino acid sequence of the encoded DIM1 protein (predicted M_r: 35,927 Da). The lysine-rich N-terminal domain is boxed and its putative protein-kinase-C-phosphorylation site (Ser14) is circled. *Bgl*II cutting sites used in disruptive transplacement (see Figure 4) are indicated by arrowheads and oligonucleotides used in PCR amplification (see Figure 4) are overlined by half-arrows.

the view that the essential role of *DIM1* is somehow related to structural and functional constraints imposed by nucleo-cytoplasmic transactions taking place during ribosome biogenesis in the eukaryotic cell. More than 15 years ago, it was reported that the m_2^6A tandem methylation was a late step in rRNA maturation taking place in the yeast cytoplasm before 20 S pre-rRNA trimming to mature 18 S rRNA (Ehresmann *et al.*, 1971; Klootwijk *et al.*, 1972; Brand *et al.*, 1977). If methylation is a prerequisite for cleavage, then the *dim1* null-mutant would be blocked at a late, yet essential step of ribosome biogenesis and therefore be lethal.

The presence of a lysine-rich-N terminus in DIM1 is puzzling. This segment could address the enzyme to the nucleus (Garcia-Bustos *et al.*, 1991) in which case the cytoplasmic location of the late ribosome maturation steps should be re-examined. Or else, the enzyme could shuttle from cytoplasm to nucleus and then back to the cytoplasm bound to 20 S pre-rRNA, as recently reported in the case of ribosomal protein L5 and transcription factor TFIIIA which both mediate nuclear export of 5 S rRNA (Guddat *et al.*, 1990). Alternatively, the positively charged N-terminal domain could promote electrostatic binding of DIM1 to its site of action on the nascent 40 S-ribosomal subunit, possibly attaching to the RNA itself. Yeast elongation factors as well as aminoacyl-tRNA synthetases similarly bear lysine-rich amino-terminal extensions and the suggestion

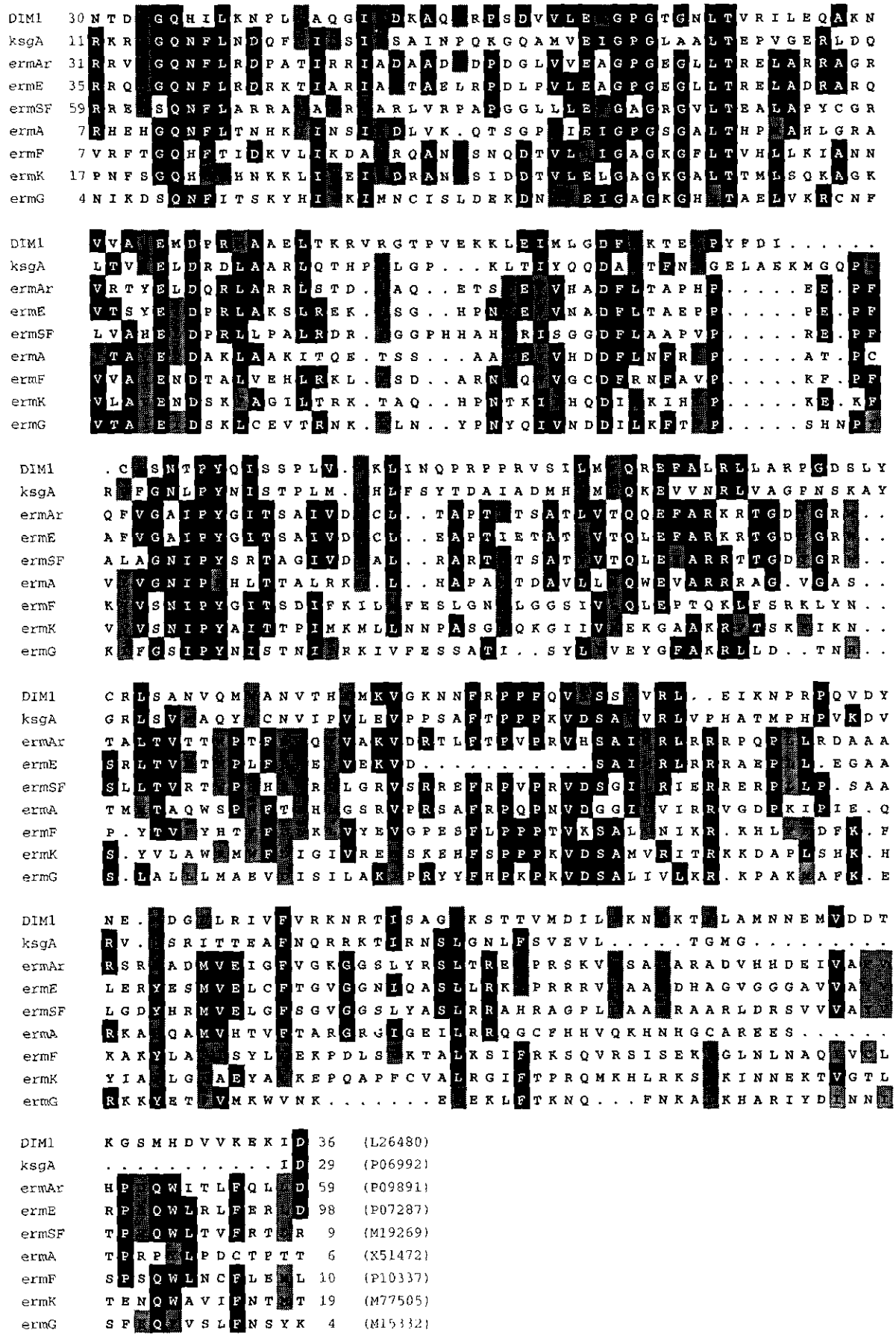

Figure 3. Sequence alignment of the DIM1 and ksgA dimethylases responsible for tandem m_2^6A m_2^6A dimethylation of the small subunit rRNA during normal ribosome biogenesis with a series of erm dimethylases conferring erythromycin resistance to several bacterial species through m_2^6A methylation of 23 S rRNA (GCG "Pile up" and "Pretty" softwares (Feng & Doolite, 1987)). Region 60 to 76 (DIM1 numbering) has been proposed as a putative SAM binding site (Ingrosso *et al.*, 1989). Identical and similar amino acids are highlighted in black and grey, respectively. Divergent N and C termini are not shown (missing amino acids are indicated by numbers before and after each sequence). EMBL accession numbers are given in parentheses.

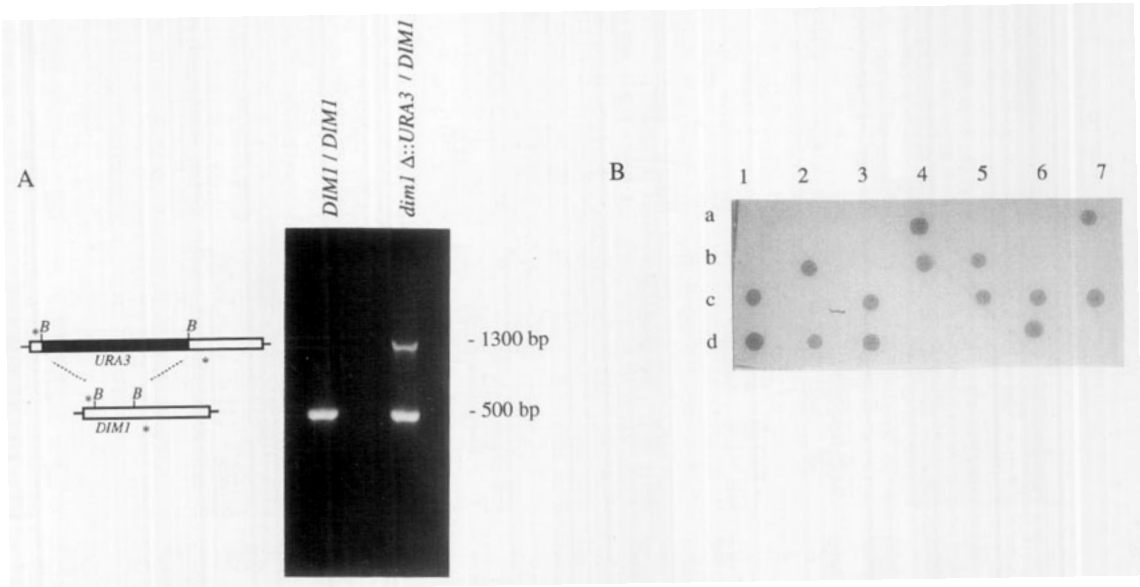

Figure 4. A, Agarose gel electrophoresis of PCR-amplified yeast genomic fragments from the wild-type homozygous control (*DIM1/DIM1*) and its hemizygous disruptant derivative (*dim1Δ::URA3/DIM1*). The restriction map of each allele is schematized to the left of the related PCR fragment (primers indicated by asterisks (*); *B* = *Bgl*II restriction sites). B, Analysis of seven tetrads (1 to 7) from the *dim1Δ::URA3/DIM1* hemizygous disruptant shown in panel A. Only two spores out of four (a to d) give rise to viable colonies. Six tetrads from another independent disruptant gave identical results. All viable colonies were shown to be Ura$^-$ (not shown). A 1-kb *Bgl*II DNA fragment containing *URA3* was substituted *in vitro* for the 0·3-kb internal *Bgl*II fragment (see Figure 2) of *DIM1*. This construct was introduced by electroporation (Becker & Guarente, 1991) into the *ura3/ura3* diploid strain YPH274 (Sikorski & Hieter, 1989) and transformants were selected on minimal medium. Hemizygous disruptants (*dim1Δ::URA3/DIM1*; *ura3/ura3*) were identified by Southern analysis (not shown) and by PCR amplification of genomic DNA using the primers indicated by asterisks and defined in Figure 2.

has been made that they could provide ionic interactions with polyribosomes and tRNA (Mirande, 1991). In any case, Ser14 could offer an opportunity for regulation of ribosome biogenesis through protein-kinase-C phosphorylation.

This paper is dedicated to the memory of the late Professor P. H. van Knippenberg. We are grateful to J. P. Cassart for his help in starting this project. We thank F. Lacroute for providing the pFL44 yeast library, L. Riles and M. Olson for the gift of the ordered lambda clones, X. De Bolle and H. Grosjean for helpful discussions, R-M. Génicot, M. Prevost and I. Becsei for technical assistance. D.L. holds a doctorate bursary from IRSIA. The *DIM1* sequence has been deposited in the EMBL Database under accession no. L26480.

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