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Isolation and Characterization of *SUA5*, a Novel Gene Required for Normal Growth in *Saccharomyces cerevisiae*

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Manuscript received December 26, 1991
Accepted for publication April 10, 1991

ABSTRACT

We have identified the *sua5* locus as a suppressor of an aberrant ATG codon located in the leader region of the *cyc1* gene. The *sua5-1* allele enhances the iso-1-cytochrome *c* steady state level in the *cyc1-1019* mutant from 2% to ≈60% of normal (Cyc$^+$) and also confers a marked slow growth (Slg$^-$) phenotype. Suppression is not a consequence of altered transcription initiation at the *cyc1* locus. The *SUA5* wild-type gene was isolated and sequenced, revealing an open reading frame (ORF) encoding a potential protein of 46,537 Da. *SUA5* transcript analyses were consistent with expression of the predicted ORF and Sua5 antisera detected a protein with an apparent molecular mass of 44 kDa. *SUA5* was mapped to chromosome *VII*, immediately adjacent to the *PMR1* gene. Hybridization analysis revealed the presence of a related gene on chromosome *XII*. Neither the *SUA5* DNA sequence nor deduced amino acid sequence showed homology to any sequences in the data banks. Disruption of *SUA5* conferred the same Cyc$^+$ and Slg$^-$ phenotypes as the *sua5-1* suppressor, which is the result of a missense mutation, encoding a Ser_{107} → Phe replacement. In addition, *sua5* null mutants lack cytochrome $a \cdot a_3$ and fail to grow on lactate or glycerol medium. These results define *SUA5* as a new gene encoding a novel protein that is necessary for normal cell growth.

WE are engaged in a systematic analysis of mutants isolated as suppressors of a translation initiation defect in the leader region of the *CYC1* gene. The primary mutant, *cyc1-1019*, expresses ≈2% of the normal amount of iso-1-cytochrome *c* as the result of a single base-pair substitution that creates an aberrant ATG codon upstream and out-of-frame with the normal *CYC1* translation start codon (Hampsey *et al.* 1991). This ATG initiates a short open reading frame (ORF) that terminates two bases prior to the normal ATG start codon (see Figure 1). To date, eight different suppressor genes, designated *sua1-sua8* (suppressor of *u*pstream *A*TG) have been identified (Hampsey *et al.* 1991; Pinto, *et al.* 1992). These suppressors can be divided into two distinct classes based on their effects on transcription initiation at *cyc1*. The first, represented by the *sua7* and *sua8* suppressors, shifts transcription start sites downstream of normal. Both *sua7* and *sua8* dramatically diminish initiation at the normal positions −70, −62, −46 and −38 in favor of initiation at −28 and −14 (A of the normal ATG translation start codon is +1) (Pinto, Ware and Hampsey 1992; R. W. Berroteran, D. E. Ware and M. Hampsey, unpublished results). Therefore, these two suppressors bypass the primary mutation at the transcriptional level. The *SUA7* and *SUA8* genes have been isolated and characterized. Molecular analysis revealed that *SUA7* encodes a 38.1-kDa homolog of the human transcription factor TFIIB (Pinto, Ware and Hampsey 1992) and that *SUA8* is identical to *RPB1*, which encodes the largest subunit of RNA polymerase II (pol II) (R. W. Berroteran, D. E. Ware and M. Hampsey, unpublished results). These results define the TFIIB homolog and the largest pol II subunit as determinants of transcription start site selection *in vivo* and provide a genetic system to further investigate these functions.

In contrast to *sua7* and *sua8*, the *sua1-sua6* suppressors enhance iso-1-cytochrome *c* levels without affecting *cyc1* transcription start site selection and therefore compensate for the upstream ATG by distinctly different mechanisms. In this paper we describe the isolation and characterization of *SUA5*. The *sua5-1* suppressor restores growth of the *cyc1-1019* mutant on lactate medium and enhances β-galactosidase activity expressed from a plasmid-borne *cyc1-1019/lacZ* fusion (Hampsey *et al.* 1991). The effects of the suppressor are dramatic: the amount of iso-1-cytochrome *c* is enhanced from 2% to ≈60% of normal and the strain displays a marked slow growth phenotype (Slg$^-$). Interestingly, disruption of *SUA5* confers the same Slg$^-$ phenotype as *sua5-1*, but also results in loss of respiratory function while maintaining an intact mitochondrial genome. These results demonstrate that *SUA5* is required for both normal glycolytic growth as well as for respiratory competence. The function of the Sua5 protein is not yet known, although possible mechanisms of suppression are discussed.

MATERIALS AND METHODS

Yeast strains: The *Saccharomyces cerevisiae* strains used in this study are listed in Table 1. Construction of strains

TABLE 1

Yeast strains

Strain	Genotype	Source
T14	*MATα cyc1-1019 cyc7-67 ura3-52 leu2-3,112 cyh2*	HAMPSEY *et al.* (1991)
T14/pM107	MAT*α cyc1-1019 cyc7-67 ura3-52 leu2-3,112 cyh2* [pM107]	HAMPSEY *et al.* (1991)
T15	*MATα CYC1⁺ cyc7-67 ura3-52 leu2-3,112 cyh2*	BERROTERAN and HAMPSEY (1991)
YJN32-15B	*MAT***a** *cyc1-1019 ura3-52 his4-290 sua5-1*	This study
YJN51	*MAT***a** *sua5::SUA5-URA3 ura3-52 leu2-3,112 his4-519 ade1-100*	This study
YJN54	*MATα cyc1-1019 sua5-1::SUA5-URA3 cyc7-67 ura3-52 leu2-3,112 cyh2*	This study
YJN59	*MAT***a***/MATα cyc1-1019/cyc1-1 cyc7-67/cyc7-67 ura3-52/ura3-52 LEU2/leu2-3, 112 HIS1/his1-1 CAN1/can1-100 SUA5/sua5-1* (YJN192 × B-7462)	This study
YJN61	*MAT***a***/MATα ura3-1/ura3-52 leu2-3,112/leu2-3,112 ADE1/ade1-100 ADE2/ ade2-1 HIS3/his3-11,15 HIS4/his4-519 TRP1/trp1-1 CAN1/can1-100* (BWG1-7a × W303-1B)	This study
YJN62	*MAT***a***/MATα ura3-1/ura3-52 leu2-3,112/leu2-3,112 ADE1/ade1-100 ADE2/ ade2-1 HIS3/his3-11,15 HIS4/his4-519 TRP1/trp1-1 CAN1/can1-100 SUA5/ sua5::LEU2*	This study
YJN63	*MATα CYC1 cyc7-67 ura3-52 leu2-3,112 cyh2 sua5::LEU2*	This study
YJN64	*MATα cyc1-1019 cyc7-67 ura3-52 leu2-3,112 cyh2 sua5::LEU2*	This study
YJN192	*MATα cyc1-1019 cyc7-67 ura3-52 leu2-3,112 cyh2 sua5-1*	This study
MH3-A36	*MAT***a** *cyc1-5000 cyc7-67 ura3-52 his1-1 can1-100*	This study
D311-3A	*MAT***a** *lys2 his1 trp2*	F. SHERMAN
D789-2B	*MAT***a** *cyc1-362 his5-2 leu1-12 trp5-48 can1-100*	F. SHERMAN
B-7462	*MAT***a** *cyc1-1 cyc7-67 ura3-52 his1-1 can1-100*	F. SHERMAN
E280	*MAT***a** *his4-290 trp1 can1ʳ*	Cold Spring Harbor
DV147	*MAT***a** *ade2 can1ʳ* [*rho⁰*]	Cold Spring Harbor
BWG1-7A	*MAT***a** *ura3-52 leu2-3,112 his4-519 ade1-100*	L. GUARENTE
W303-1B	*MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100*	R. ROTHSTEIN

described in this paper is presented in the text. The *cyc1-1019* allele is the result of an A → G transition at position −18 that was generated by *in vitro*, site-directed mutagenesis of the cloned *CYC1⁺* gene. This mutation recreates the leader sequence short ORF (Figure 1) initially identified in the *cyc1-362* mutant (STILES *et al.* 1981). Transplacement of this construct into the normal chromosomal locus to create strain T14 was done as described previously (BERROTERAN and HAMPSEY 1991). T14 expresses ≈2% of the normal amount of iso-1-cytochrome *c*. The *cyc1-362* and *cyc1-5000* alleles contain the same upstream ATG as *cyc1-1019* and differ only in the sequence context flanking the ATG (HAMPSEY *et al.* 1991). The *cyc1-1* allele is a complete deletion of the structural gene (SINGH and SHERMAN 1978). The *cyc7-67* allele is a nonreverting, nonsuppressible allele of *CYC7*, which encodes iso-2-cytochrome *c*, thereby assuring that T14 revertants cannot arise by overexpression of iso-2-cytochrome *c*.

Growth media, genetic methods and nomenclature: Lat⁺ revertants of strain T14/pM107 were selected on YPDL medium following exposure to ultraviolet light (≈60% survival) (HAMPSEY 1991), and subsequently screened for elevated levels of both iso-1-cytochrome *c* (Cyc⁺ phenotype) and β-galactosidase activity as described previously (HAMPSEY *et al.* 1991). YPDL medium consists of 1% yeast extract, 2% peptone, 2% lactate and 0.05% glucose. "+13/Glu" and "+13/Lac" media are synthetic complete media containing either 2% glucose or 2% lactate as sole carbon sources. Synthetic complete and all other media were prepared according to SHERMAN (1991).

Standard methods were used for strain crosses, diploid selection, sporulation and tetrad dissections (SHERMAN and HICKS 1991). Phenotypes referred to in this paper are defined as follows: Lat⁻, inability to grow on lactate (YPL) medium; Cyc⁻, <5% of the amount of iso-1-cytochrome *c* contained in a *CYC1⁺* strain, as determined by spectroscopic analysis; Slg⁻, distinctly impaired growth on rich (YPD) medium at 30°; Ura⁻, failure to grow on Ura⁻ medium.

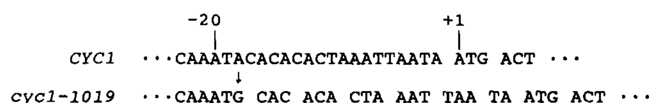

FIGURE 1.—The *cyc1-1019* allele. The *cyc1-1019* allele was derived from the *CYC1* wild-type allele by *in vitro* generation of an A → G mutation at position −18. This substitution creates an aberrant ATG start codon (underlined) that initiates a short ORF terminating (double underline) prior to the normal start codon at +1.

Spectroscopic assays: Iso-1-cytochrome *c*, cytochrome $a \cdot a_3$ and cytochrome *b* were assayed by low temperature (−196°), whole-cell spectroscopy as described by SHERMAN and SLONIMSKI (1964).

Recombinant DNA techniques: Recombinant plasmids were constructed by standard techniques (SAMBROOK, FRITSCH and MANIATIS 1989). Unless otherwise noted DNA fragments encompassing all or part of the *SUA5* gene are present in the yeast low copy-number vector pRS316 (SIKORSKI and HIETER 1989). Plasmid pJN10 was constructed by transferring the 3.6 kb partial *Hin*dIII fragment from pJN7 to the yeast integrating plasmid YIp5 (STRUHL *et al.* 1979). pJN34 was derived from pJN22 by Klenow fill-in of the *Spe*I site within the *SUA5* ORF. pJN41 was constructed for γ-transformation by transferring the *Bgl*II fragment encompassing the *SUA5* promoter and the *Hin*dIII fragment encompassing the *SUA5* terminator to the integrating plasmid pRS305 (SIKORSKI and HIETER 1989). pJN46 is the *GAL1-SUA5* fusion plasmid constructed by transferring the *Eco*RI-*Xba*I fragment from the M13 vector 18/SUA5/H-P (see next section), following mutagenesis to create the *Eco*RI site, to the vector pYES (Invitrogen Corp., San Diego,

California). Plasmids pJN47 and pJN49 were constructed by transferring to pRS316 the *SacI-SalI* fragments encompassing *sua5* from 18/SUA5/H-P, following mutagenesis to create, respectively, the missense mutation at codon 107 and the nonsense mutation at codon 139. Plasmid pM107 is a low copy-number vector carrying a *cyc1-1019/lacZ* fusion such that *lacZ* expression is controlled by the *cyc1-1019* promoter and leader region (HAMPSEY *et al.* 1991). Plasmid DNA was isolated from *Escherichia coli* (BIRNBOIM and DOLY 1979) and from yeast (HOFFMAN and WINSTON 1987) by procedures described previously. Yeast and *E. coli* transformations were done by the lithium acetate (ITO *et al.* 1983) and calcium chloride (MANDEL and HIGA 1970) procedures, respectively.

Site-directed mutagenesis: Mutations were created in the cloned *SUA5* gene by *in vitro*, site-directed mutagenesis according to the technique described by KUNKEL, ROBERTS and ZAKOUR (1987). The template was derived from 18/SUA5/H-P, which is M13mp18 carrying the 2.7-kb *HindIII-PstI SUA5* fragment. The TAA nonsense codon was generated at amino acid position 139 using the oligonucleotide oJN-71 (CCATTAATTTAAAGCCTTTG, 406–425); the *EcoRI* site used for fusion of the *GAL1* promoter to the *SUA5* coding region was generated using oligonucleotide oJN-75 (CCTGTTTAGAATTCCATT, position −29 → −12); and the *sua5-1* missense mutation at amino acid position 107 was reconstructed using oJN-76 (ATGTTTCATTCATTGATC, position 311 → 328). The numbers in parentheses correspond to the numbering system in Figure 6. All oligonucleotides were synthesized by Oligos, Etc., Inc. (Wilsonville, Oregon).

Southern hybridizations: Total yeast genomic DNA was isolated by the method of HOLM *et al.* (1986) and used for Southern analysis according to standard procedures (SAMBROOK, FRITSCH and MANIATIS 1989). The probe was the two 1.8-kb *HindIII* DNA fragments encompassing *SUA5* that had been labeled either with [α-^{32}P]dCTP using the Nick Translation Kit (GIBCO BRL, Gaithersburg, Maryland) or with the ECL Gene Detection Kit (Amersham Corp., Arlington Heights, Illinois). The contour-clamped homogeneous electric field (CHEF)-blot was obtained from TOM CUNNINGHAM (University of Tennessee Health Science Center, Memphis) and probed using the 1.0-kb *BglII* fragment internal to the *SUA5* ORF that had been labeled by nick-translation as described above. All hybridizations were done under stringent conditions at 68° in buffer containing 6 × SSC, 0.01 M EDTA, 5× Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS) and 0.1 mg/ml salmon sperm DNA.

DNA sequence analysis: DNA sequences were determined according to the dideoxy-terminator method (SANGER, NICKLEN and COULSON 1977) using the kit supplied by United States Biochemical (Cleveland, Ohio). Single-stranded template DNA was obtained from M13mp18 and M13mp19 derivatives containing restriction fragments from within the 2.7-kb *HindIII-PstI* fragment encompassing *SUA5*. Both DNA strands were sequenced using either the M13 universal primer or the *SUA5*-specific primer oJN-61 (5′-CATCTACATATGTTCCC, position 1448 → 1464). DNA from the *cyc1* locus of strain YJN192 was isolated and sequenced as described previously (HAMPSEY, DAS and SHERMAN 1986).

RNA methods: Total RNA was isolated from 10 ml YPD cultures grown to OD_{600} = 1.0 as described elsewhere (MCNEIL and SMITH 1985). Oligo(dT) columns (5′ → 3′, Inc., West Chester, Pennsylvania) were used to purify poly(A)$^+$ RNA. Northern analysis was performed by size fractionating 15 μg of total RNA in a 1.5% formaldehyde-agarose gel and transferring to Magna Nylon membranes (Micron Separations, Inc., Westboro, Massachusetts). Prehybridization and hybridization reactions were performed under the conditions recommended by the supplier. Transcription start sites at the *SUA5* and *CYC1* loci were determined by primer extension according to the procedure of MCNEIL and SMITH (1986). The *CYC1* primer was oMH-5 (5′-CTTAGCAGAACCGGCCTTGA, position 30 → 11) and the *SUA5* primer was oJN-66 (5′-GATTGATAGCGGGTTAAC, position 84 → 67). Both were end-labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase. The cDNA products were analyzed in 8% polyacrylamide DNA sequencing gels; size markers were DNA that had been sequenced with the same primers.

Preparation of Sua5 antiserum: Sua5 antiserum was raised using a TrpE-Sua5 hybrid protein isolated from *E. coli*. Plasmid pJN38 was constructed by transferring the 1.5-kb *StuI-PstI* DNA fragment of *SUA5*, containing the coding information for amino acids 96 to 426 (see Figure 6), to the *SmaI-PstI* sites of the expression vector pATH22. Antigen was prepared as described previously (KOERNER *et al.* 1991). Two New Zealand white rabbits were injected subcutaneously with 100 μg of purified hybrid protein emulsified in an equal volume of Freund's complete adjuvant (GIBCO BRL) and boosted at 2-week intervals with 100 μg of the hybrid protein emulsified in Freund's incomplete adjuvant. Sera were collected prior to the initial immunization and one week following the third boost. Titer and specificity of the antisera were determined by Western blot analysis.

Western blot analysis: Total protein extracts were prepared as described previously (OHASHI *et al.* 1982), size-fractionated in a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. Western blot analysis was performed as described (TOWBIN, STAEHLIN and GORDON 1979). The Sua5 protein was overexpressed by growing strain T15, containing plasmid pJN46 (*GAL1-SUA5* fusion), in rich medium containing 2% galactose as the carbon source.

Nucleotide sequence accession number: The EMBL accession number for *SUA5* is X64319.

RESULTS

Isolation and genetic analysis of the *sua5-1* suppressor: Strain YJN192 was isolated as a Lat$^+$ revertant of strain T14/pM107 on YPDL medium; subsequent screening on yeast X-gal medium revealed a blue colony phenotype, consistent with the presence of an extragenic suppressor of the *cyc1-1019* defect (HAMPSEY *et al.* 1991). Spectral analysis indicated an increase in cellular iso-1-cytochrome *c* content from 2% to ≈60% of normal (Cyc$^+$); β-galactosidase activity was enhanced from 2% to 12% of the amount detected in extracts of strain T14 carrying a control *CYC1*$^+$/*lacZ* plasmid. (The discrepancy between the two protein levels in YJN192 might reflect different stabilities of the two proteins.) YJN192 also exhibited a marked Slg$^-$ phenotype on rich medium (Figure 2). The diploid resulting from a cross between strains YJN192 (Cyc$^+$, Slg$^-$) and B-7462 (Cyc$^-$, Slg$^+$) exhibited clear Cyc$^-$ and Slg$^+$ phenotypes, indicating that the mutation(s) conferring Cyc$^+$ and Slg$^-$ in YJN192 is recessive. Analysis of the progeny obtained from both tetrad and random spore analysis of a cross between strains YJN192 and E280 (Cyc$^+$ Slg$^+$) yielded Cyc$^+$:Cyc$^-$ segregants at an overall ratio of 3:1 (88

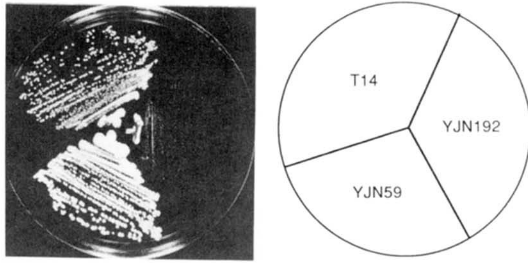

FIGURE 2.—The slow growth phenotype (Slg$^-$) associated with the *sua5-1* suppressor. Strains were grown for 3 days on YPD medium at 30°. The Slg$^+$ phenotype of YJN59 indicates that the mutation in strain YJN192 conferring Slg$^-$ is recessive. Relevant genotypes are T14, *MATα cyc1-1019 SUA5*; YJN192, *MATα cyc1-1019 sua5–1*; and YJN59, ***MATa**/MATα cyc1-1019/cyc1-1 SUA5/sua5-1*. The growth differential between strains T14 and YJN192 is much less pronounced after 10 days at 30° (not shown), indicating that the Slg$^-$ phenotype is not a consequence of growth arrest.

Cyc$^+$:32 Cyc$^-$), indicating that the suppressor is unlinked to the *cyc1* locus. Furthermore, all Cyc$^-$ segregants were Slg$^+$ and all Cyc$^+$ segregants displaying the suppressor phenotype (≈60% of normal iso-1-cytochrome *c*) were Slg$^-$, establishing cosegregation of the suppressor and the mutation conferring Slg$^-$. Dissection of a diploid constructed from a cross of YJN192 with MH3-A36 (*cyc1-5000 SUA5*) yielded exclusively 2 Cyc$^+$:2 Cyc$^-$ progeny, indicating a single gene suppressor. Together, these data define the *cyc1-1019* suppressor as a single nuclear gene, unlinked to *cyc1*, that cosegregates with the Slg$^-$ phenotype.

The relationship between the YJN192 suppressor and the previously defined suppressors of either *cyc1-362* or *cyc1-5000* (HAMPSEY *et al.* 1991) was defined by complementation analysis. Strain YJN192 (*MATα cyc1-1019 sua*$^-$), or its meiotic derivative YJN32–15B (*MAT***a** *cyc1-1019 sua*$^-$), was crossed with a representative of each of the *sua1-sua8* mutants (PINTO *et al.* 1992; HAMPSEY *et al.* 1991) and the resulting diploids were scored for their Cyc phenotypes. All *sua* suppressors isolated to date are recessive, therefore diploids displaying the Cyc$^-$ and Slg$^+$ phenotypes would indicate complementation and therefore define independent suppressor genes. Indeed, all diploids strains were Cyc$^-$ and Slg$^+$. We have designated this gene *SUA5* and the suppressor allele *sua5-1*.

Effect of *sua5-1* on the *cyc1* gene: The preceding genetic analysis of strain YJN192 indicated that the Cyc$^+$ phenotype is the result of a single gene suppressor of *cyc1-1019* that is unlinked to the *cyc1* locus and therefore does not involve any additional mutation at the *cyc1-1019* locus. This was confirmed by isolating and sequencing the *cyc1-1019* allele from YJN192, revealing that the upstream ATG is retained with no additional mutations (Figure 3A).

The *sua7* and *sua8* suppressors compensate for the upstream ATG of *cyc1-1019* by shifting transcription initiation sites downstream of normal, resulting in a major initiation site between the aberrant and normal ATG start codons (PINTO, WARE and HAMPSEY 1992; R. W. BERROTERAN, D. E. WARE and M. HAMPSEY, unpublished data). To determine whether *sua5* suppresses *cyc1-1019* in a similar manner the *cyc1* transcription initiation sites were mapped by primer extension (Figure 3B). In contrast to the results obtained with the *sua7* and *sua8* mutants, identical initiation patterns were found with strain D311-3A (*CYC1 SUA5*; lane 1), T15 (*CYC1 SUA5*; lane 2), T14 (*cyc1-1019 SUA5*; lane 3) and YJN192 (*cyc1-1019 sua5-1*; lane 4). This experiment establishes that the suppressor does not function either by altering the sites of transcription initiation or by transcript processing such that the upstream ATG is either eliminated or rendered less effective as a translation start codon.

The effect of the *sua5* suppressor on the level of the *cyc1-1019* transcript was determined by northern blot analysis (Figure 3C). The upstream AUG significantly diminished the steady-state level of the *cyc1-1019* transcript (*cf.* lanes 1 and 2), presumably due to decreased mRNA stability resulting from ribosomal dissociation following translation of the leader sequence short ORF. The *cyc1-1019* mRNA level was partially restored in either the *sua5-1* (lane 3) or *sua5::LEU2* (lane 4) genetic backgrounds. This demonstrates that suppression is not a consequence of a posttranslational effect on iso-1-cytochrome *c*, but does not distinguish between transcriptional or post-transcriptional events, including enhanced *cyc1* mRNA translation, since this might be expected to stabilize the transcript (see BROWN 1989).

Cloning the *SUA5* gene: The *SUA5* wild-type gene was cloned from a YCp50 yeast genomic library (ROSE *et al.* 1987) by its ability to complement the Slg$^-$ and Cyc$^+$ phenotypes associated with *sua5-1*. Eight Ura$^+$ Slg$^+$ transformants (scored after 2 days of incubation) were obtained from a total of ≈12,000 Ura$^+$ Slg$^-$ transformants (scored after 5 days). All eight Ura$^+$ Slg$^+$ transformants were also Cyc$^-$, containing <5% of the normal amount of iso-1-cytochrome *c*. When transformants were cured of plasmid DNA, both the Slg$^-$ and Cyc$^+$ phenotypes were restored. Plasmid DNA from all eight strains was shown to contain common 1.8-kb *Hin*dIII fragments. When strain YJN192 was retransformed to Ura$^+$ with one of these plasmids (pJNB2) the Slg$^+$ and Cyc$^-$ phenotypes were restored.

To delimit *SUA5* within the initial 13.7-kb DNA clone, DNA fragments were transferred from plasmid pJNB2 to the low copy-number plasmid pRS316 (SIKORSKI and HIETER 1989) and introduced into strain YJN192. Complementation of the *sua5-1* marker was determined by scoring both the Slg$^+$ and Cyc$^-$ phenotypes. Results indicated that the *SUA5* gene is contained within a 2.0-kb *Nhe*I-*Pst*I DNA fragment (Figure 4, pJN33).

Experiments were performed to establish the rela-

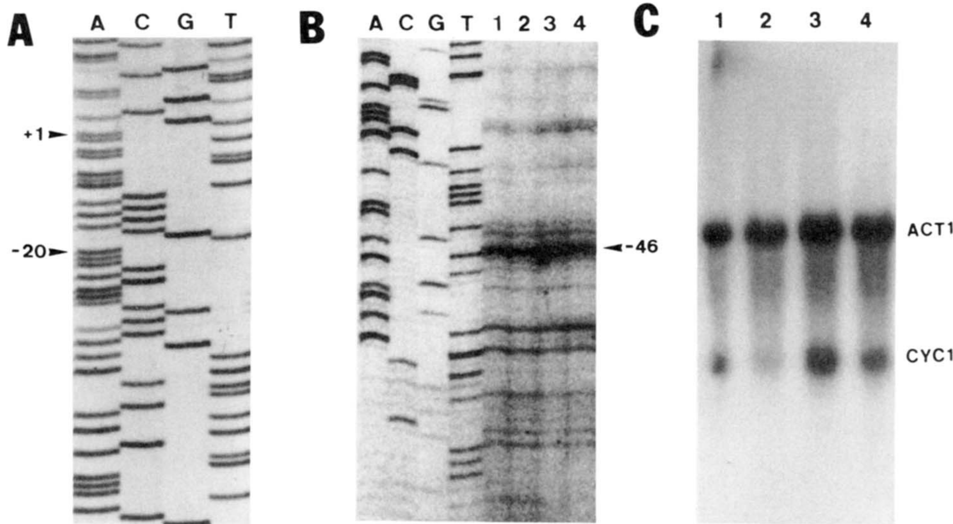

FIGURE 3.—DNA sequence and *cyc1* transcript analyses in *sua5* backgrounds. (A) The entire 246-bp *Xho*I-*Eco*RI fragment encompassing the *cyc1* promoter, leader and translation start site was sequenced from strain YJN192. A portion of that sequence is shown here, demonstrating no sequence changes relative to *cyc1-1019*, including retention of the upstream and normal ATG codons indicated at positions −20 and +1, respectively. (B) Transcription start sites at the *cyc1* locus were mapped by primer extension using total RNA. Primer oMH5, corresponding to the *CYC1* template strand from 30 → 11, was used to generate the sequence ladder (lanes A, C, G and T) and to synthesize cDNA, allowing for initiation sites to be directly determined from the DNA sequence. Lane 1, strain D311-3A (*CYC1 SUA5*); lane 2, strain T15 (*CYC1 SUA5*); lane 3, strain T14 (*cyc1-1019 SUA5*); and lane 4, strain YJN192 (*cyc1-1019 sua5-1*). The transcription initiation patterns are the same for all four strains, with the major initiation site at position −46 indicated. (C) Steady-state *cyc1* levels were determined by northern blot analysis. Lane 1, strain T15; lane 2, strain T14; lane 3, strain YJN192; lane 4, strain YJN64 (*cyc1-1019 sua5::LEU2*).

tionship between the cloned DNA and the genetically defined *sua5-1* locus. The genetic locus of the cloned DNA was marked by the *URA3* gene and followed through meiosis. Plasmid pJN10 (see Figure 9) was linearized at the unique *Bst*XI site and introduced into strain BWG1-7A (*SUA5 ura3*), selecting for Ura$^+$ transformants. One of these transformants, YJN51 (*SUA5::URA3 ura3*), was purified and crossed with strain YJN192 (*sua5-1 ura3*). The resulting diploid was sporulated and dissected. The Slg$^+$:Slg$^-$ and Ura$^+$:Ura$^-$ segregated 2:2 in all four-spore tetrads, indicating that pJN10 had integrated at a single site in the genome; moreover, the Slg$^+$/Ura$^+$ phenotypes and Slg$^-$/Ura$^-$ phenotypes cosegregated, establishing that the cloned gene is indeed *SUA5*.

***SUA5* genetic map position:** *SUA5* DNA was used as a hybridization probe to map its chromosomal location using a CHEF-blot of size fractionated, intact yeast chromosomes (CHU, VOLLRATH and DAVIS 1986). The strongest hybridization signal occurred at the position of the unresolved doublet corresponding to chromosomes *VII* and *XV* (Figure 5A). A weaker signal is also present at the position of chromosome *XII*, a result which is consistent with cross hybridization observed on Southern blots of restriction digested genomic DNA (data not shown). The probe in these experiments was the 1.0-kb *Bgl*II DNA fragment, which is internal to the *SUA5* coding region (see below). This indicates that *SUA5* might be a redundant gene. DNA sequence analysis (see below) subsequently established that *SUA5* is immediately adjacent to the *PMR1* gene, which had been mapped to the left arm of chromosome *VII*, linked to *lys5* and *aro2* (RUDOLPH *et al.* 1989) (Figure 5B).

Characterization of the *SUA5* gene: The DNA sequence of both strands of a 2065 bp fragment encompassing *SUA5* was determined. An open reading frame of 426 amino acids initiating at an ATG codon was identified within the 2.0-kb *Nhe*I-*Pst*I DNA fragment that complements *sua5-1* (Figure 6). This sequence encodes a potential protein with a molecular mass of 46,537 Da and pI of 6.9. The TACTAAC sequence, which is invariantly present at the branch site of yeast pre-mRNA introns (LANGFORD and GALLWITZ 1983), does not appear within *SUA5*; it can therefore be assumed that *SUA5* does not contain an intron. The codon bias of the ORF is 0.03, suggesting that Sua5 is a low abundance protein (BENNETZEN and HALL 1982; SHARP, TUOHY and MOSURSKI 1986).

Northern blot analysis of poly(A)$^+$ RNA, using strand-specific RNA probes, detected a single transcript of appropriate size and complementarity to encode the putative Sua5 protein (Figure 7A, lanes 1 and 2). The same blot was also probed with the *ACT1*

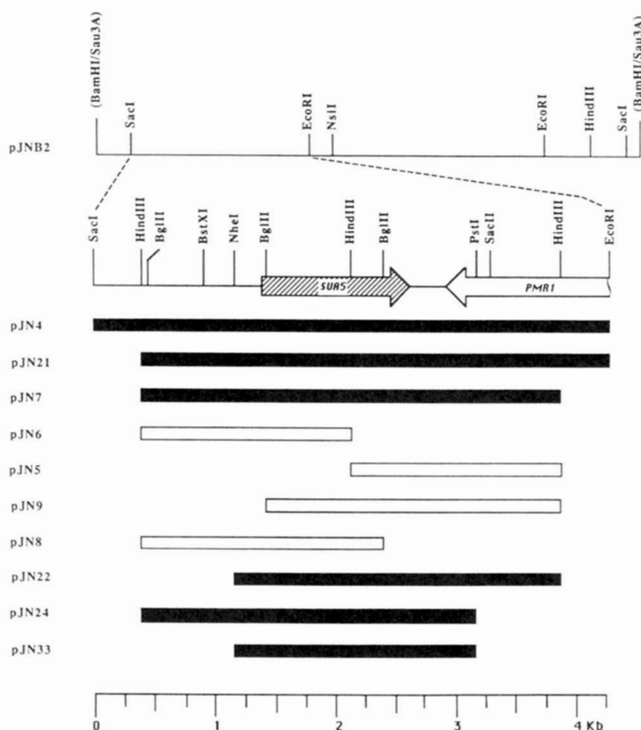

FIGURE 4.—Restriction map of the *SUA5* locus. The *SUA5* gene was cloned from a YCp50 yeast DNA library (ROSE *et al.* 1987) as a 13.7-kb *Sau*3A partial digestion fragment in the plasmid designated pJNB2. The indicated restriction fragments were subcloned into the low copy-number yeast vector pRS316 (SIKORSKI and HIETER 1989) to create the pJN series of plasmids, which were introduced into strain YJN192 by selecting for Ura prototrophy. Black bars indicate complementation of the *sua5-1* marker, establishing that the 2.0-kb *Nhe*I-*Pst*I fragment in plasmid pJN33 is sufficient to complement *sua5-1*.

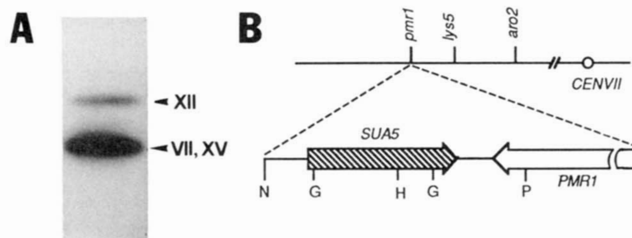

FIGURE 5.—The *SUA5* genetic locus. (A) A CHEF-blot of size-fractionated, intact yeast chromosomes was probed with the 1.0-kb *Bgl*II DNA fragment internal to the *SUA5* ORF (Figure 4). The secondary signal suggests that an *SUA5* homolog is present on chromosome *XII*. (B) DNA sequence analysis revealed that *SUA5* is immediately adjacent to *PMR1*, which had been mapped previously relative to the *lys5* and *aro2* markers on the left arm of chromosome *VII* (RUDOLPH *et al.* 1989). The orientation of the *SUA5* and *PMR1* genes relative to *lys5* and *aro2* is unknown. Restriction site abbreviations are N, *Nhe*I; G, *Bgl*II; H, *Hin*dIII; and P, *Pst*I.

gene and the expected 1.4-kb transcript was detected (Figure 7A, lane 3). Comparison of the *SUA5* and *ACT1* transcript levels indicated that *SUA5* is much less abundant than *ACT1* (<1% of *ACT1*), providing additional evidence that *SUA5* is expressed at a very low level. The 5′-ends of the *SUA5* transcript were mapped by primer extension analysis. A pattern representing multiple initiation sites was identified, with the major site at position −62 relative to the initiator ATG (Figure 7B). Thus, the *SUA5* locus is transcribed, producing a low abundance mRNA that is consistent in size and initiation sites with expression of the *SUA5* ORF.

Further evidence that the ORF encodes the predicted protein was obtained by constructing mutations within the *SUA5* coding region. A nonsense mutation was generated at position 416 (Figure 6) by *in vitro*, site-directed mutagenesis, from which plasmid pJN49 was created. Plasmid pJN24 carries the *SUA5* wild-type gene and is otherwise identical to pJN49. Both plasmids were introduced into YJN192 (*cyc1-1019 sua5-1*). Whereas plasmid pJN24 fully complemented both the Slg$^-$ and Cyc$^+$ phenotypes associated with *sua5-1*, pJN49 failed to complement either phenotype. A frameshift mutation was also generated by filling-in the unique *Spe*I site at position 900 (Figure 6). The resulting plasmid, pJN34, also failed to complement the *sua5-1* defect of YJN192. These results strongly support the premise that the Sua5 protein is represented by the identified ORF. Furthermore, the frameshift mutation at the *Spe*I site leaves intact the first 300 amino acids of Sua5, indicating that the C-terminal portion of Sua5 is essential for its function.

Antiserum to the Sua5 protein: Polyclonal antisera was raised against an *E. coli* synthesized TrpE-Sua5 hybrid protein. A Western blot of total cellular protein reacted with this antisera (Figure 8). Strain T15 (*SUA5*, lane 1) exhibits a signal at approximately 44 kDa that is absent from strain YJN63 (*sua5::LEU2*, lane 2). This signal is also seen from the strain YJN192 (*sua5-1*, lane 3), indicating that the protein is retained in the suppressor strain. Strain T15 transformed with a *GAL1/SUA5* fusion plasmid, pJN46, displays a slight increase in the 44-kDa antigen from a glucose-grown culture (lane 4) that is increased >500-fold from a galactose culture (lane 5). Combined with the results from the preceding experiments, these data establish that *SUA5* is transcribed and translated to yield a protein with a molecular weight similar to that predicted from the DNA sequence.

***SUA5* is a novel gene:** Neither the *SUA5* DNA sequence nor the deduced amino acid sequence showed significant similarity to any sequences in the GenBank (v. 70), EMBL (v. 29) or Swiss-Prot (v. 20) data bases. Furthermore, no obvious structural motifs that might suggest a function for the Sua5 protein were identified. These results define *SUA5* as a novel gene.

The sequence downstream of the *SUA5* ORF is identical to the yeast *PMR1* (*SSC1*) gene, which encodes a P-type ATPase (RUDOLPH *et al.* 1989). The polarity of *PMR1* is opposite to *SUA5*; consequently *SUA5* and *PMR1* are convergently transcribed. The genetic map position of the *PMR1* locus, determined by RUDOLPH *et al.* (1989), therefore establishes the *SUA5* map position (see Figure 5B).

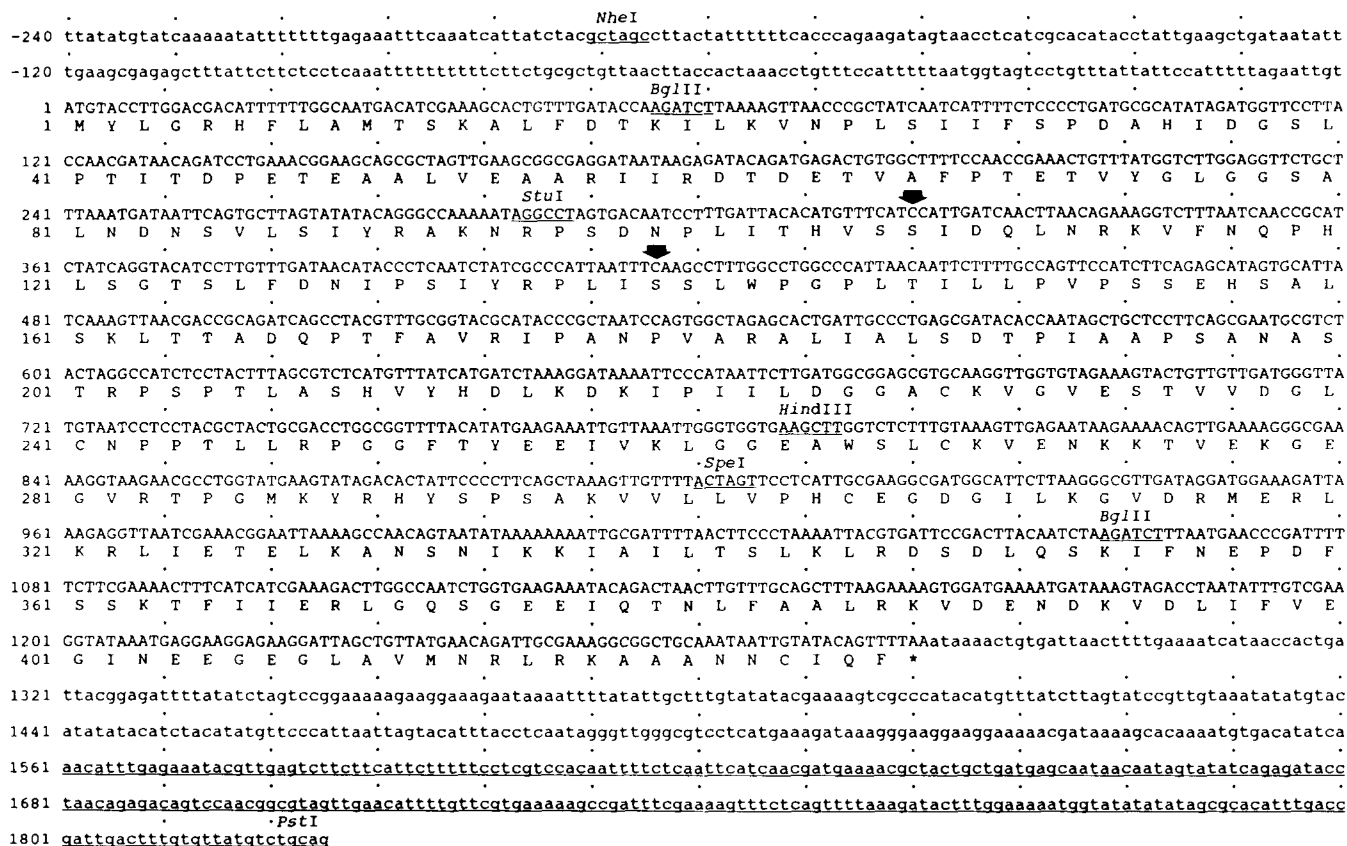

FIGURE 6.—The *SUA5* sequence. The DNA sequence of a 2065-bp region of the *SUA5* locus is shown, along with the deduced amino acid sequence of the Sua5 protein. Restriction sites referred to in the text are underlined. The positions of the missense and nonsense mutations referred to in the text are indicated by the arrowheads at nucleotides 320 and 416, respectively. The 3′-end of the *PMR1* gene located downstream of *SUA5* is also underlined.

The *sua5-1* allele: The *sua5-1* allele was cloned by the integration-excision method (WINSTON, CHUMLEY and FINK 1983) as depicted in Figure 9. Restriction fragments from within the 2.0-kb *Nhe*I-*Pst*I DNA fragment of pJN35 were transferred to M13mp19 and the entire region was sequenced. A single base-pair substitution, $C_{320} \rightarrow T$, encoding a $Ser_{107} \rightarrow Phe$ replacement, was found. This mutation was reconstructed by *in vitro*, site-directed mutagenesis of the *SUA5* wild-type gene. The resulting construct, carried on plasmid pJN47, failed to complement the Slg^- and Cyc^+ phenotypes associated with *sua5-1* in strain YJN192, confirming that both the suppressor and Slg^- phenotypes are the result of the Phe_{107} replacement.

Disruption of *SUA5*: The slow growth phenotype of *sua5-1* strains indicates that *SUA5* is important for normal cell growth. To determine whether *SUA5* is essential for viability a null allele was constructed by γ-transformation (SIKORSKI and HIETER 1989) of diploid strain YJN61 (*CYC1/CYC1 SUA5/SUA5*) as depicted in Figure 10. The resulting strain, YJN62, was sporulated and dissected. Four-spore viability was obtained (8 tetrads), with 2:2 segregation of the Slg^+:Slg^- phenotypes; moreover, the Slg^+/Leu^- and Slg^-/Leu^+ phenotypes cosegregated among all progeny. These data demonstrate that *SUA5*, although not essential for viability, is necessary for normal growth.

The *SUA5* locus of haploid strains T14 (*cyc1-1019 SUA5*) and T15 (*CYC1 SUA5*) was also disrupted by γ-transformation, resulting in Slg^- phenotypes in both cases. Spectroscopic analysis of strain YJN64 (*cyc1-1019 sua5::LEU2*) revealed ≈60% of the normal amount of iso-1-cytochrome *c*, identical to the Cyc^+ phenotype of original suppressor strain YJN192 (*cyc1-1019 sua5-1*). However, in contrast to YJN192, the absorption band characteristic of cytochrome $a \cdot a_3$ (605 nm) was completely absent and cytochrome *b* was significantly diminished. [The absorption spectrum for strain YJN64 is comparable to type VI depicted by SHERMAN and SLONIMSKI (1964).] This suggests that YJN64 is respiratory incompetent and that the Sua5 protein is required for biogenesis of functional mitochondria. Indeed, strain YJN63 (*CYC1 sua5::LEU2*) lacks cytochrome $a \cdot a_3$ and is phenotypically Lat^-, whereas the isogenic strain T15 contains cytochrome $a \cdot a_3$ and is Lat^+ (Figure 11). To confirm that respiratory deficiency is not a consequence of mitochondrial rho^0 induction in the absence of the *SUA5* gene, perhaps as an indirect consequence of the Slg^- phenotype, strain YJN63 (*sua5::LEU2*) was crossed with strain DV147 (*SUA5* rho^0). The resulting diploid, unlike either parent, exhibited a Lat^+ phe-

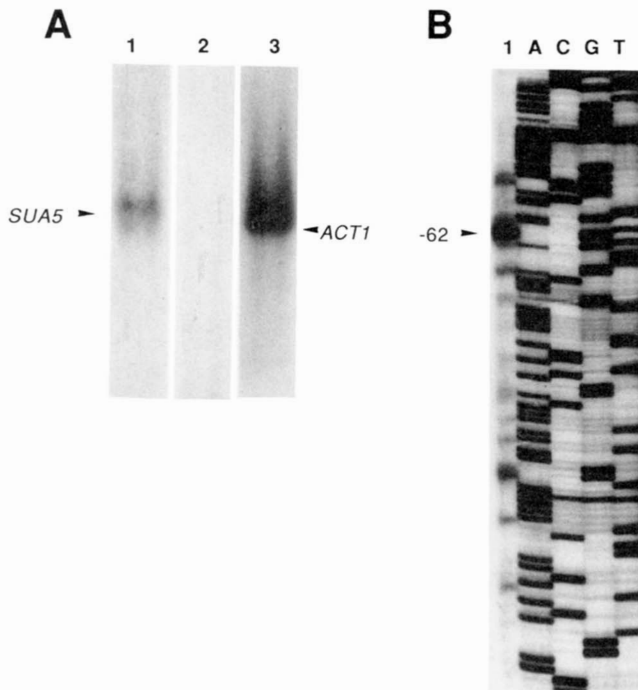

FIGURE 7.—*SUA5* RNA analysis. (A) Northern analysis of poly(A)$^+$ RNA from strain T15. The probes were strand-specific ^{32}P-labeled RNA corresponding to the *SUA5* template strand (lane 1), *SUA5* coding strand (lane 2), or *ACT1* template strand (lane 3). Lane 1 is the result of a 60-hr exposure compared to a 1-hr exposure for lane 3 using radiolabeled probes of comparable length and specific activity. From this information and relative band intensities the *SUA5* steady-state transcript level was estimated to be <1% of *ACT1*. (B) The transcription start sites at the *SUA5* locus of strain T15 were determined by primer extension. Primer oJN-66, corresponding to the *SUA5* template strand from 84 → 67, was used to generate the sequence ladder (lanes A, C, G and T) and to synthesize cDNA, allowing for the initiation sites to be directly determined from the DNA sequence (lane 1). A pattern indicating multiple initiation sites was found, with the most prominent site at position −62 (A of the ATG start codon is designated +1).

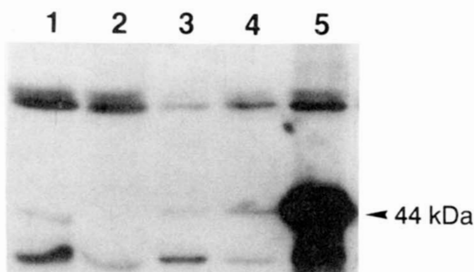

FIGURE 8.—Western blot probed with Sua5 antiserum. Protein samples were electrophoresed in a 10% SDS-polyacrylamide gel, transferred to nitrocellulose, blocked with bovine serum albumin and probed with polyclonal antibody (1:1000 dilution) raised against an *E. coli* synthesized TrpE-Sua5 hybrid protein. Lane 1, strain T15 (*SUA5*$^+$); lane 2, strain YJN63 (*sua5::LEU2*); lane 3, strain YJN192 (*sua5-1*); lane 4, strain T15 [pJN46] grown in 2% glucose; lane 5, identical to lane 4, except grown in 2% galactose. The apparent molecular mass of the Sua5 protein was determined to be ≈44 kDa using prestained molecular mass markers (not shown). The bands appearing below the 44-kDa marker in lane 5 are probably Sua5 degradation products. The bands appearing below 44 kDa in lanes 1–4 are not Sua5-specific since they are also present in the deletion mutant (lane 2).

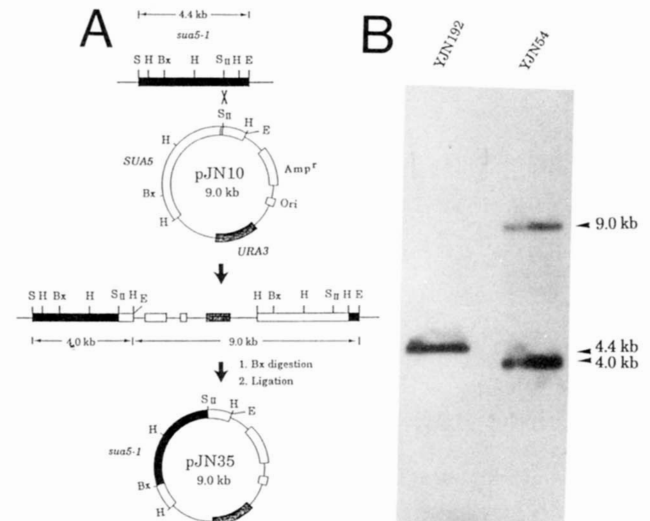

FIGURE 9.—Scheme for cloning the *sua5-1* allele. (A) Plasmid pJN10, containing the 3.6-kb *Hin*dIII DNA fragment encompassing *SUA5*, was linearized at the unique *Sac*II site and introduced into strain YJN192, selecting for Ura$^+$ transformants. Total DNA was isolated from one of these transformants (strain YJN54), digested with *Bst*XI, ligated, and introduced into *E. coli* strain HB101, selecting for ampicillin resistance. Plasmid pJN35, which is structurally identical to pJN10, was recovered. (B) Southern analysis confirming integration of pJN10 at the *sua5-1* locus. Total DNA from strains YJN192 and YJN54 was digested with *Sac*I and *Eco*RI and analyzed by Southern blot using the two 1.8-kb *Hin*dIII *SUA5* DNA fragments as the hybridization probe. Restriction site abbreviations are Bx, *Bst*XI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; S, *Sac*I; and S_{II}, *Sac*II.

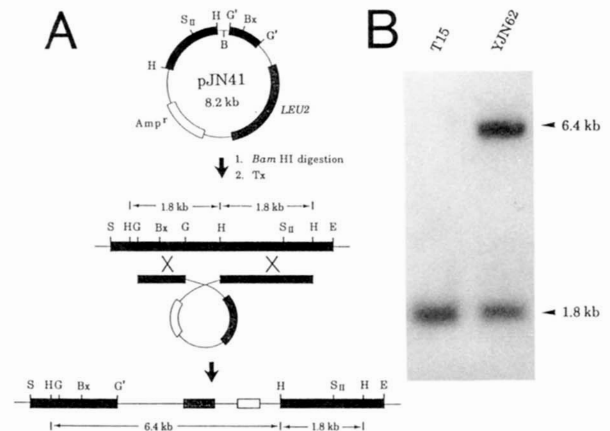

FIGURE 10.—Disruption of the *SUA5* gene. (A) Plasmid pJN41 (see MATERIALS AND METHODS) was linearized at the unique *Bam*HI site located between the two *SUA5* fragments and introduced into *SUA5*$^+$ strains (either diploid strain YJN61 or haploid strains T14 or T15), selecting for the plasmid-borne *LEU2* gene. (B) Southern blot analysis confirming disruption of *SUA5*. Total DNA from strains T15 and YJN62 were digested with *Hin*dIII and analyzed by Southern blot using the two 1.8-kb *Hin*dIII *SUA5* DNA fragments as the hybridization probe. Signals at 1.8 kb and 6.4 kb confirmed that a single copy of the *SUA5* gene had been disrupted in diploid strain YJN62. Restriction site abbreviations are B, *Bam*HI; G, *Bgl*II; all others are as defined in the legend to Figure 9.

notype. Furthermore, the Lat$^-$ phenotype of strain YJN63 can be rescued by transformation with the *SUA5* plasmid pJN7, which also restores cytochrome $a \cdot a_3$ to its normal level, but not by the *sua5* deletion

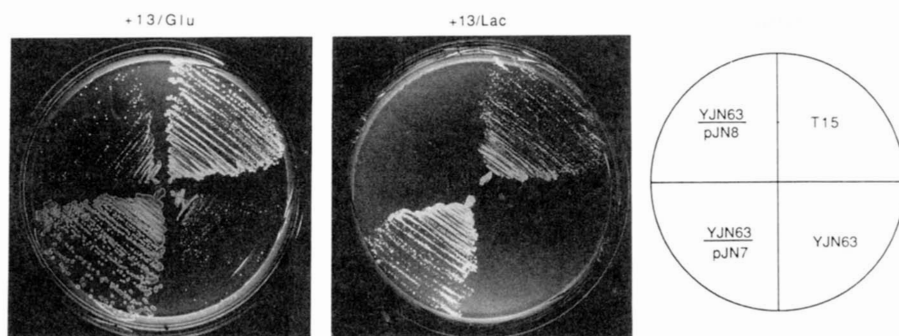

FIGURE 11.—Deletion of *SUA5* precludes growth on nonfermentable carbon sources. (A) Growth patterns on +13/Glu or +13/Lac media for isogenic strains T15 (*CYC1 SUA5*), YJN63 (*CYC1 sua5::LEU2*), YJN63/pJN7 (*CYC1 sua5::LEU2* [*SUA5*]), and YJN63/pJN8 (*CYC1 sua5*-Δ). Plasmids pJN7 and pJN8 are depicted in Figure 4. +13/Glu and +13/Lac are synthetic complete medium containing either 2% glucose or 2% lactate, respectively. Strains YJN63 and YJN63/pJN8 also fail to grow on +13/Gly medium, which contains 2% glycerol as the carbon source (not shown).

plasmid pJN8 (Figure 11). Clearly, the Lat^- phenotype associated with the *sua5* null allele is not the result of an indirect effect on the mitochondrial genome. Nonetheless, this phenotype might be a pleiotropic effect of the *sua5* deletion, such that the *SUA5* requirement for mitochondrial function would be indirect.

DISCUSSION

Identification of *SUA5*: The *SUA5* gene was uncovered based on the ability of a mutation at this locus to compensate for the deleterious effect of an aberrant ATG translation start codon in the leader region of the *cyc1-1019* allele. The *sua5-1* suppressor is the result of a missense mutation encoding a $Ser_{107} \rightarrow Phe$ replacement, which enhances iso-1-cytochrome *c* in the *cyc1-1019* background from 2% to ≈60% of normal and confers a marked slow growth phenotype on rich medium. Nearly identical Cyc^+ and Slg^- phenotypes are also conferred by an *sua5* null allele (*sua5::LEU2*), demonstrating that suppression is a consequence of loss of Sua5 function, a result consistent with the recessive nature of the *sua5* mutations.

The selection scheme used to uncover the *sua* genes is similar to the scheme used by DONAHUE and coworkers (1988) to uncover the *sui* class of suppressors. The principal distinction between the *sui* and *sua* systems is that the *sui* mutants enhance expression from the *his4* gene in the absence of the normal ATG start codon, whereas the *sua* suppressors compensate for an aberrant ATG located upstream and out-of-frame with the normal ATG at *cyc1*. Three different *sui* genes have been identified (CASTILHO-VALAVICIUS, YOON and DONAHUE 1990). Molecular cloning and DNA sequencing revealed that *SUI2* (CIGAN *et al.* 1989) and *sui3* (DONAHUE *et al.* 1988) encode eIF-2α and eIF-2β, respectively. *SUI1* encodes a novel translation initiation factor that functions, along with eIF-2, in recognition of the AUG start codon (YOON and DONAHUE 1992). Despite the similarities in these two systems, none of the *sua1-sua8* genes is allelic to *SUI1*, *SUI2* or *sui3* (PINTO, WARE and HAMPSEY 1992; I. PINTO, J. G. NA, R. W. BERROTERAN, D. E. WARE and M. HAMPSEY, unpublished results).

Structural features of *SUA5*: There are several notable features associated with the *SUA5* promoter/leader region. The most proximal TATA sequence is at position −239, which is 177 bp from the major transcription initiation site at −62 (Figure 6). This distance is greater than the 40–120 bp that generally separate the TATA box and initiation sites in yeast (STRUHL 1989). Furthermore, TATATG (−239 → −234) does not conform to the consensus TATAAA defined by CHEN and STRUHL (1988). It seems more likely that the *SUA5* promoter does not include a TATA element, accounting, at least in part, for the low level of *SUA5* expression. The promoter region contains several poly(dA:dT) sequences. The most extensive of these spans position −90 to −76 where 13 of 15 bp are dA:dT. This structural feature is associated with constitutive expression of yeast genes (STRUHL 1985) and may control *SUA5* expression.

A second interesting feature of the *SUA5* leader is the presence of an AUG sequence (position −37) downstream of the major transcription start site at −62 (Figure 6). This AUG initiates a short ORF upstream and out-of-frame with the *SUA5* ORF. According to the scanning model for eukaryotic translation initiation (KOZAK 1989) this sequence should preclude or diminish translation of the *SUA5* ORF from transcripts initiating upstream of this position. Although the sequence preceding the upstream AUG (UUUUA AUG) does not constitute a favorable context for efficient initiation (defined as AAAAA AUG, with the A at −3 most highly conserved (CAVENER and RAY 1991), neither does the sequence preceding the start codon of the *SUA5* ORF (AUUGU AUG). Nonetheless, the leader sequence AUG does not have a significant effect on *SUA5* expression since an *SUA5* construct containing an *in vitro* generated substitution of this sequence ($T_{-36} \rightarrow A$) fully complements both the Slg^- and Lat^+ phenotypes associated with either the *sua5-1* or *sua5::LEU2* mutants (J. G. NA and M. HAMPSEY, unpublished results).

There are no obvious structural motifs that suggest a function for the Sua5 protein. The C-terminal sequence ($-C_{423}$IQF, single letter code) conforms to the "CAAX" box (C is always cysteine, A is often aliphatic and *X* is any amino acid) common to the C termini of

proteins that become isoprenylated, including Ras and certain other GTP-binding proteins, nuclear lamins, and the yeast **a** mating factor (GLOMSET, GELB and FARNSWORTH 1990). This motif suggests that the Sua5 protein might be isoprenylated; however, an *SUA5* allele encoding a $Cys_{423} \rightarrow$ Ser replacement fully complemented all *sua5* mutant phenotypes (J. G. NA and M. HAMPSEY, unpublished results). Thus, despite the CAAX-box motif, Sua5 is apparently not isoprenylated.

The remaining four cysteines of Sua5 are located within a region that is enriched for glycines (13 of 89 residues from position 225–313 are glycine) and within this region the sequence $CKVX_4TVX_2G$ is repeated (positions 228–239 and 268–279). The potential significance of this region is not known.

Sua5 function: We do not know the function of the normal Sua5 protein. Transcript mapping experiments established that suppression of *cyc1-1019* does not occur by altering transcription start site selection (Figure 3B). The *sua5* suppressor is therefore mechanistically distinct from the *sua7* and *sua8* suppressors, both of which alter transcription start site selection at *cyc1* and other loci (PINTO, WARE and HAMPSEY 1992; R. W. BERROTERAN, D. E. WARE and M. HAMPSEY, unpublished results). Still *SUA5* could function at the transcriptional level by affecting the rate of *cyc1* transcription. Alternatively, *SUA5* could function at the posttranscriptional level by affecting (i) *cyc1* mRNA stability, (ii) AUG codon recognition by the scanning ribosome, or (iii) ribosomal reinitiation following translation of the short ORF. The elevated levels of the *cyc1-1019* transcript seen in the sua^- strains (Figure 3C) do not distinguish between these possibilities since enhanced translation of the *cyc1* mRNA might also be expected to increase transcript stability.

It is also possible that suppression of *cyc1-1019* is the result of a more indirect effect of the *sua5* mutation. For example, we do not know whether the Slg^- phenotype associated with *sua5* results from effects of the suppressor on expression of other genes or, conversely, whether suppression of *cyc1-1019* is a consequence of slow growth. One approach that we have taken regarding this concern is to isolate and characterize suppressors of the Slg^- phenotype conferred by $sua5^-$. These include Slg^+ Cyc^- revertants of YJN192 (Slg^- Cyc^+) resulting from single gene mutations that are extragenic to *sua5* (J. G. NA and M. HAMPSEY, unpublished results). We have identified one of these suppressors as an allele of *CYR1*, which encodes adenylate cyclase (KATAOKA, BROEK and WIGLER 1985). The involvement of adenylate cyclase in growth rate regulation suggests that suppression of *cyc1-1019* by *sua5* might therefore be an indirect consequence of slow growth. Moreover, this result suggests that *SUA5* might be a previously unidentified gene involved in the Ras-adenylate cyclase signal transduction pathway. In this case it would be extremely interesting that *SUA5* is also required for certain mitochondrial functions. The *sua5* genetic system, the cloned *SUA5* gene, and antisera to the Sua5 protein are currently being used in experiments designed to reconcile the seemingly disparate phenotypes associated with *sua5* mutations and the potential for *cyr1* mutations to at least partially compensate for these defects.

We are especially grateful to DAVID MCNABB (Louisiana State University Medical Center) for valuable advice regarding preparation of Sua5 antisera. We thank DAVID GROSS (Louisiana State University Medical Center) and DOUG JOHNSON (University of Vermont) for critical reading of the manuscript; TOM CUNNINGHAM (University of Tennessee Health Science Center, Memphis) for the CHEF-gel blot; TOM DONAHUE (Indiana University) for plasmids containing the *SUI* genes; PHILIP HIETER (Johns Hopkins University) for the pRS series of plasmids; and JEFF BECKER (University of Tennessee, Knoxville) for pointing out the CAAX box motif. This work was supported by U.S. Public Health Service grant GM39484 from the National Institutes of Health and by an American Cancer Society Junior Faculty Research Award to M.H.

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Communicating editor: M. CARLSON