LAG2, a gene that determines yeast longevity

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***Saccharomyces cerevisiae* has a limited life span, measured by the reproductive capacity of the individual cell. Several genes that are differentially expressed during the yeast life span have been isolated. One of these genes, LAG2, has been characterized for its role in longevity. LAG2 is preferentially expressed in young cells. It encodes a predicted 680 amino acid protein with a putative transmembrane helix. The sequence does not show significant similarity to any other DNA or protein sequences in the databases. Deletion of LAG2 in a haploid strain did not affect growth, but it resulted in a 50% decrease in the mean and maximum life span. When LAG2 was overexpressed, the mean and maximum life span of the yeasts was extended by about 36% and 54%, respectively. These results indicate that this is a longevity-assurance gene in yeast.**

Keywords: *Saccharomyces cerevisiae*, ageing, life span, longevity genes

INTRODUCTION

Evidence for a genetic component of longevity, acting in concert with environmental factors, has accumulated from studies in several organisms. In the nematode *Caenorhabditis elegans*, recombinant inbred lines with an increased mean and maximum life span have been obtained (Johnson, 1987). A mutation in the *C. elegans* *age-1* gene results in a 70% increase in the mean life span and a 110% extension in the maximum life span (Friedman & Johnson, 1988). More than a doubling of the life span is achieved by mutation of *daf-2* (Kenyon *et al.*, 1993). Selective breeding of outbred *Drosophila* strains has resulted in a postponement of senescence (Luckinbill *et al.*, 1984; Rose, 1984). Environmental influences modulate the expression of the extended-longevity phenotype (Buck *et al.*, 1993). At the cellular level, human diploid fibroblasts that normally undergo clonal senescence in culture become immortalized following transformation with the simian virus 40 (SV40) T-antigen in the presence of a second genetic event (Wright *et al.*, 1989). Four complementation groups for fibroblast immortalization have been identified (Pereira-Smith & Smith, 1988). The dominance of the senescent phenotype has been demonstrated in cell hybridization studies (reviewed by

Goldstein, 1990), and the influence of a 'senescence factor', functioning to limit the replicative capacity, has been adduced from RNA micro-injection experiments (Lumpkin *et al.*, 1986). The operation of a senescence factor has also been revealed in yeast (Egilmez & Jazwinski, 1989).

We are using the yeast *Saccharomyces cerevisiae* as a model to investigate the genetic basis of longevity. Each yeast cell has a finite life span, measured by the number of cell divisions and not by chronological age (Mortimer & Johnston, 1959; Muller *et al.*, 1980). The mother cell becomes older with each division, while the daughter (bud) generally starts anew, with the capacity for a full life span. Passage through this life span is accompanied by several morphological and physiological alterations (reviewed by Jazwinski, 1993). Among these changes are an increase in generation time and cell size, accumulation of bud scars and specific alterations in gene expression. Although the marking of the cell surface by bud scars was initially proposed to limit the life span (Mortimer & Johnston, 1959), subsequent observations render this possibility unlikely (Jazwinski, 1993). The same conclusion can be extended to the increase in cell size (Jazwinski, 1993; Kennedy *et al.*, 1994). Furthermore, it has been found that controlled expression of the oncogene *v-Ha-ras* in yeast results in a near doubling of the mean and maximum life span (Chen *et al.*, 1990), demonstrating that a single genetic change can affect yeast longevity irrespective of cell size and bud scar accumulation. Here, we describe a yeast gene, *LAG2*, that plays a role in determining the yeast life span.

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The GenBank/EMBL accession number of the nucleotide sequence reported in this paper is U50334.

METHODS

Strains and growth conditions. The *S. cerevisiae* strain SP1 (*MATa leu2 ura3 trp1 ade8 can1 his3 gal2*) was provided by M. Wigler (Cold Spring Harbor Laboratory, New York); strains X2180-1A (*MATa SUC2 mal mel gal2 CUP1*) and XDF1-3D (*MATa ade2 his3 trp1 ura3*) were from the Yeast Genetic Stock Center, Berkeley, California. Cells were grown on YPD medium (2%, w/v, peptone, 1%, w/v, yeast extract, 2%, w/v, glucose) at 30 °C. Strain XDF1-3D was crossed with X2180-1A. Zygotes were isolated by micromanipulation and grown at 30 °C for 2 d. Diploid cells were sporulated on sporulation medium (Sherman *et al.*, 1986) and tetrads were dissected by micromanipulation on YPD. One of the spores yielded strain XAC-14D (*MATa ADE2 HIS3 trp1 URA3*), which was used for meiotic mapping. Transformed strains were selected on synthetic complete (SC) medium lacking particular nutrients (Sherman *et al.*, 1986). This medium was also used for selecting mapping strains and in the meiotic mapping studies.

Escherichia coli strain DH5 α F' (Stratagene) was used for molecular cloning. Bacterial strains were grown in LB medium (1%, w/v, tryptone, 0.5% yeast extract, 1%, w/v, NaCl) at 37 °C. Cells transformed with plasmids pUC118 or pUC119 or their derivatives were selected on LB medium containing 100 μ g ampicillin ml⁻¹. Solid media contained 1.5% (w/v) agar.

Isolation of a full-length *LAG2* clone. Several DNA clones were isolated by differential hybridization, selecting for genes preferentially expressed in young and old yeast cells (Egilemez *et al.*, 1989). The *LAG2* gene was isolated from a yeast genomic library, constructed in plasmid pBR322. This clone was used to screen for a full length clone in a λ EMBL3 yeast genomic library (D'Mello *et al.*, 1994) by standard procedures. Two overlapping clones, a 12 kb *SaI* fragment and a 14 kb *EcoRI* fragment contained the *LAG2* gene, and were used for conventional DNA sequencing (Sanger *et al.*, 1977). Standard recombinant DNA techniques were used throughout (Ausubel *et al.*, 1987).

DNA sequencing. *LAG2* sequences were localized in subclones of the two λ EMBL3 clones by Southern analysis using the original *LAG2* clone in pBR322 as a probe. A 2.4 kb (*XbaI*–*SaI*) fragment was subcloned from the 12 kb λ EMBL3 insert, using the *XbaI* restriction site within the yeast DNA and the *SaI* site located in the λ EMBL3 vector, into the multiple cloning site of pUC118 and pUC119 (Vieira & Messing, 1987). A 4.0 kb *EcoRI* fragment, contained within the 14 kb λ EMBL3 insert, was subcloned into the *EcoRI* site of the multiple cloning site of pUC118 and pUC119. A restriction map was constructed and contained the entire 2040 bp ORF, 1 kb upstream region and 478 bp downstream region (Fig. 1). Subcloned *LAG2* DNA fragments were sequenced using the M13 universal primers and synthetic primers (Oligos ETC) or primers prepared by The Biotechnology Core Laboratories, LSU Medical Center, that were complementary to known *LAG2* sequence. Sequencing reactions were performed according to the standard dideoxy chain-termination method (Sanger *et al.*, 1977). Single-stranded DNA was prepared by infecting transformed *E. coli* DH5 α F' cells, carrying plasmids with inserted yeast sequence, with the defective phage M13KO7. Both DNA strands of the *LAG2* yeast DNA were sequenced using the Sequenase 2.0 dideoxy chain-termination kit (USB), and some portions were sequenced more than once. Computer programs available within the PCGENE analysis package (Intelligenetics) were used for sequence analysis, homology and motif searches, and predictions of structure.

Genetic mapping. The *LAG2* gene was mapped to a yeast chromosome by probing a CHEF blot with a 0.9 kb *LAG2* DNA fragment containing the ORF. *LAG2* was marked for

Fig. 1. Restriction map and sequencing strategy of *LAG2*. The 4.0 kb DNA fragment (*XbaI*–*EcoRI*) containing *LAG2*, obtained from the λ EMBL3 library, is illustrated (X, *XbaI*; H, *HindIII*; E, *EcoRI*; P, *PstI*; St, *StuI*; Xo, *XhoI*). Subclones were constructed by inserting restriction fragments into pUC118 and pUC119. Sequence data were obtained using the M13 universal primer (solid arrows) or primers synthesized from known *LAG2* sequence (dashed arrows). The coding region of *LAG2* (ORF) is shown with direction of transcription indicated by the arrow.

meiotic mapping (Guthrie & Fink, 1991). A 1.4 kb *EcoRI* DNA fragment containing *TRP1*–*ARS1* was inserted into the *EcoRI* site of *LAG2*. The introduction of the *TRP1* marker into strain SP1 was accomplished by single-step gene disruption (Guthrie & Fink, 1991). The *LAG2::TRP1*–*ARS1* disrupted strain was mated with XAC-14D and zygotes were grown at 30 °C for 2 d. This cross was used to map *LAG2* with respect to the genetic markers *ADE2* and *HIS3*. Diploid cells were sporulated and tetrads dissected by micromanipulation on YPD agar. *LAG2* was further localized on chromosome XV by probing nitrocellulose membranes containing recombinant DNA yeast genomic contigs (Olson *et al.*, 1986).

***LAG2* deletion.** The yeast *LAG2* gene marked deletion was constructed by insertion of the 827 bp *TRP1* gene and deletion of 1098 bp of coding sequence. This deletion construct left 90 bp of 5' sequence and 852 bp of 3' sequence of the *LAG2* coding region remaining. The yeast haploid strain SP1 was transformed with a linear DNA fragment containing the *LAG2*Δ::*TRP1* deletion construct. Insertion of the *TRP1* marker was verified by Southern blot analysis and Northern blot analysis demonstrated this to be a null mutation.

Overexpression of *LAG2*. A 4 kb *EcoRI* DNA fragment containing the bulk of the *LAG2* ORF was subcloned into the *EcoRI* site of the yeast shuttle vector pBM150 (Johnston & Davis, 1984), containing the *GAL1*–*GAL10* promoter. This placed the *LAG2* ORF downstream of the galactose-inducible *GAL10* promoter, 4–5 nucleotides from the *GAL10* transcription start site. Correct orientation of *LAG2* was determined by Southern blot analysis. The constructed fusion eliminated the translational start codon at position 1 in the *LAG2* ORF and provided the next available translation start at position 87 in the *Lag2* protein sequence. Yeast strain SP1 was transformed with the *GAL10::LAG2* construct. Transformants were cultured on SC medium minus uracil to maintain the plasmid. Cells were grown to stationary phase in medium with either 2% (w/v) raffinose for endogenous expression of *LAG2*, 2% (w/v) glucose to repress *GAL10::LAG2*, or in 4% (w/v) galactose to induce high levels of expression of the *GAL10::LAG2* fusion. Induction of *GAL10::LAG2* was verified by Northern blot analysis. Life span determinations were performed by streaking a loop of cells from an overnight culture onto SC medium minus uracil, plus 4% galactose, 2%

glucose or 2% raffinose. Overexpression of *GAL10:LAG2* was delayed until generation 12, when endogenous levels of *LAG2* expression had declined, by first growing cells on SC medium minus uracil with 2% raffinose. Cells that budded 12 generations were transferred to SC medium minus uracil containing 2% raffinose and 4% galactose to induce *LAG2* overexpression and micromanipulated further for life span determinations. The control cells were transferred in parallel to the same medium but with only 2% raffinose as carbon source. Raffinose had no effect on *GAL10:LAG2* induction by galactose, as determined by Northern blot analysis.

Life span determinations. A Nikon Labophot microscope with a 40 $\times$ long-working-distance objective and a micromanipulator attachment was used to determine the yeast life span, as described previously (Egilmez *et al.*, 1989). In brief, a loop of cells from an overnight culture was streaked along one edge of a slab of YPD medium and placed on a 1 $\times$ 3 inch glass slide. Thirty to forty-five cells were micromanipulated away from the streak and placed on designated spots on the agar slab. Generation zero (unbudded) cells were separated from isolated budding mother cells and used for life span determinations. The buds emerging from these initial generation zero cells were removed at each successive cell division and discarded until the mother cell lysed or ceased to divide for several days and lost refractility. The age of each mother cell at death was determined by the number of buds that emerged from that cell, and it was expressed in generations. Mean population life spans were compared using Student's *t* test.

RESULTS

DNA sequence

Several clones of genes preferentially expressed in either young or old yeast cells were isolated by differential hybridization (Egilmez *et al.*, 1989). These clones were isolated from a yeast genomic library constructed in plasmid pBR322, which contained approximately 1 kb inserts. One of the clones hybridized to a 2.1 kb transcript that was preferentially expressed in young yeasts by a factor of five (Egilmez *et al.*, 1989). Expression declined during the life span, reaching a plateau at 11 generations. This clone was used to screen for full length clones in a λ EMBL3 yeast genomic library. The gene encoding the 2.1 kb, age-dependent transcript, obtained this way, was designated *LAG2*. Two overlapping clones, possessing a 12 kb *SalI* fragment and a 4 kb *EcoRI* fragment, containing the *LAG2* gene, were subcloned into pUC118 and pUC119 for DNA sequencing (Fig. 1). The *LAG2* transcription unit was localized by Northern blot analysis using the subclones as probes, and its age-dependent expression was verified. The entire predicted ORF of *LAG2* with an additional 1.0 kb upstream and 478 bp downstream was sequenced from both strands (Fig. 2). The PCGENE analysis package (Intelligenetics) was used for DNA and protein sequence analysis, as well as homology searches. A single, long ORF of 2040 bp was predicted. A translation initiation codon is indicated at position 1 (Fig. 2), 24 bp downstream from the single transcription start site, which was identified by S1 nuclease mapping and primer extension analysis (data not shown). A putative TATA-box element was located at position -75 to -61, while a putative CCAAT-box (-146 to -135) was predicted 122 bp upstream of the transcription

start site and 71 bp 5' of the TATA-box. A transcription termination consensus sequence was not identified. A codon bias index (Bennetzen & Hall, 1982) of 1.32 suggests that *LAG2* is highly expressed in the cell. The *LAG2* gene is present in a single copy within the haploid yeast genome. Southern hybridization of yeast genomic DNA, digested separately with five different restriction enzymes that do not cut within the *LAG2* gene, and hybridized with a *LAG2* ORF probe, demonstrated a single band in all cases (data not shown).

Comparison of the *LAG2* sequence (DNA and protein) to sequences present in current databases did not reveal any similarities. However, a *LAG2* probe hybridized to human genomic DNA on Southern blots under moderately stringent conditions. The *LAG2* probe also hybridized to DNA from other mammals and some invertebrates, indicating potential sequence similarity to DNA in a number of organisms (data not shown).

Mapping of *LAG2*

The *LAG2* gene was assigned to chromosome XV by probing a CHEF blot, separating yeast chromosomes, with a DNA fragment containing the ORF. A 1.4 kb *EcoRI* DNA fragment containing *TRP1-ARS1* was inserted into the *EcoRI* site of *LAG2* as a marker for meiotic mapping. The disruption in haploid cells did not affect viability or growth. The *LAG2::TRP1-ARS1* (*MATa trp1 ade2 his3*) strain was mated with a *MATa trp1 ADE2 HIS3* strain to map *LAG2* with reference to *ADE2* and *HIS3* on the right arm of chromosome XV. Diploid cells obtained were sporulated and subjected to tetrad analysis (Guthrie & Fink, 1991). The distance of *LAG2* from *ADE2* (88.2 cM) and *HIS3* (88.5 cM) suggested a lack of linkage of *LAG2* to these markers.

To locate *LAG2* on chromosome XV, a probe containing the ORF was hybridized to nitrocellulose membranes containing recombinant clones of *S. cerevisiae* genomic contigs (Olson *et al.*, 1986). The *LAG2* probe hybridized to a single contig located on the left arm of chromosome XV. *LAG2* was further localized on the chromosome by dot blot analysis of three recombinant clones (ATCC 70141, 70025, 70482) from the corresponding region. *LAG2* DNA hybridized strongly to clone 70141, weakly to clone 70482 and not at all to 70025. The two clones 70141 and 70482 overlap by approximately 1.3 kb. The weak hybridization to clone 70482 by *LAG2* indicates that most of the gene is located in clone 70141, and it spans the overlap between these two clones. Therefore, the *LAG2* gene is located on the left arm of chromosome XV, between *SUP3* and *ARG1*. The *SUP3* and *ARG1* genes are located on the left arm of chromosome XV, 14.0 and 42.0 kb from the centromere, respectively (Olson *et al.*, 1986).

Predicted protein

The predicted protein sequence of *LAG2* was analysed using the PROSITE program (Bairoch, 1990). The *LAG2* gene encodes a protein of 680 amino acids with a

-1336	TCTAGACAACCTCGTAACCCTGAGCAAATTTAATCAAAAGCTTAGTAGAACTTTGATTTTCGTAACCCAAAAGTTT	
-1260	CGTCCCATTAGCATAATGTTTCAAGGCGTGCTTCACTTTGACCATAAGCGTCTCCTTGGGCTTGGCAGGAGCATCGGTGGATGT	
-1176	TGGAGCTACGGGCTTCGTAACACTTTTCGACTTATCAGTAGAATAAAATCTCAAAGATAAAGGATAGTTGGGCTGTACTCCTTG	
-1092	CGCTCTCAAAGGCCAATGACACGAGGGAATAGTGGAAAGCTATTAATCTAGGGCCTTGATTTTTAACAAAATACAAGCTTGCTTG	
-1008	ACGTCTTGTACACATGACGCTCTTGATGCGAAATCAACATCTTAGTATATATCTGTAGTGATAGAGATAGCGTATATATGCG	
- 924	CTGCCTGAAATGCACTGCGCTGTTTATATCATCACCGTGCGCAATGACTGATCCATTTATTTGGAGATGATTCAACGAATAATTT	
- 840	TTCAACGCGGGATTATCGCCCAAGACGGGGTCTGTTGAGGGTAATACCGTGGCTAGCTTTATGGCTTGTGAAACTATTGCCAGT	
- 756	TATGTGGTTACGTATAATCTACCGTATGTGTGTGTATTTATTTATGTAGGTTGCTAATGCTTTGGTGTATCGTTTAAAGGAAC	
- 672	TTGGACGCAACCCCTCCACGGGTGCCAGGGCTTGAATCTCTTTCAATCGTGCTATTTTGGCGGACTCTGGGTACACCTTATAC	
- 588	CCCTTGTTTCTATGGTTAAACCAATTGAATCTCCAGCAGAGCTCGTGAGCAAACTACTGCGCCGAAGCCGAGCATCATGCCGTTG	
- 504	AGGAAGGGCAAAAGTAGGTTGATGGAGCAGAGCCTACAAAGGATACAAGGCTCTGCACAAGTGGGCTCTCGTGGGCACTCACT	
- 420	GTGTTCTGCACCTCCATACAGTCTTTGTCTTCTGATTCTGACATCTGACACGCTCTCCAGAATCCCAACCTCTGTATATTT	
- 336	CTGTCTTGTGGCAGTTTCGTAAGAAGGGGGTGATGTTTCTAATATTACAGTGATGTTCTATTGTTCAAGTAAAGTGTATGGCT	
- 252	GTTTCTTATGGCGCGGATATACATCGTTATAAGTATTACATCTCGTAACGCATTCTCTGTTTCAAGTGGTGGGGAGGCCGAT	
- 168	GGTAGTGGATAATAACGCTTTTATGGGCCATTGGTATTACTATTTATCTTCTGTTATAATAAATAGTACACGTTGCAGACATATT	
- 84	TGGAGGAGAGTTTTTAAAGTACTGTTGGGGTGCTCTTGCTGTGCATTGTAAGTAATCGTGTCCAGTGGCCGAGTGGTTAAGCG	
	*	
1	ATGCCCTGCTATTTCTCAGAAAAGCAATTAGGCATTGGGTTTACCTGCGCAGGTTGGAATCCTGTCTGTGACGCATTCTTTT	
	METProCysTyrPheLeuArgLysAlaIleArgHisTrpValLeuProAlaGlnValArgIleLeuSerValThrHisPhePhe	28
85	TTTGTGAATCTTGCTTCTTATCCTTCTACAATCCTTTCTGTGACATTTATTGAATCATCTCTACAAAATTTTTTACACATAGCT	
	PheValAsnSerCysPheLeuSerPheTyrAsnProPheValThrPheIleGluSerSerLeuGlnAsnPheLeuHisIleAla	56
169	AAGGTTAGCTTGTCTACATTACACAAGTGGTAGCAATCGTTTACATTGCACCTCAGCTATATCAACATCACGTTCTACCGCAA	
	LysValSerLeuPheTyrIleThrGlnValValAlaAsnArgLeuHisCysThrGlnLeuTyrGlnHisHisValLeuProGln	84
253	CTAGTAATGAGCTTGCACATTAGTAAACTGATTGAGCAATACTGTTCCACTAAGGACAATGACCTTAAATATATGCTTTTAAGG	
	LeuValMetSerLeuHisIleSerLysLeuIleGluGlnTyrCysSerThrLysAspAsnAspLeuLysTyrMetLeuLeuArg	112
337	CAAACTTTAAATTAACGACATAGAGGATGAGCTGGCTCCTCTTGTTAACGAATTGCTGCTCCCGGTATTGGTCAAGAACAA	
	GlnAsnPheLysIleAsnAspIleGluAspGluLeuAlaProLeuValAsnGluLeuLeuLeuProValLeuValGluGluGln	140
421	GACATGGAAATCTCAACCTAGTATCGTTTCAAGTGCTCCCGATTAGTTCTTTCTATGATCAGCGACCTGCAGCTGCTCAG	
	AspMetGluIleLeuAsnLeuValSerPheGlnValLeuProAspLeuValLeuSerMetIleSerAspProAlaAlaAlaGln	168
505	TTGGGATGGGTAATAAGCTTGATATGCGACCCCTTTATTAACCAAAGCATGATACATGCAAATCGTTCTTTTGTCTGATTGAG	
	LeuGlyTrpValIleSerLeuIleCysAspProLeuLeuAsnGlnSerMetIleHisAlaAsnArgSerPheValLeuIleGlu	196
589	ACACTAAGAAATGTGTTACAAAAAATGAAAACCTCCCTCATTAGATTACCACCAACCAGTAAACAGTTTCATTAGAGTTTATC	
	ThrLeuArgAsnValLeuGlnLysIleGluAsnSerProHisLeuAspTyrHisGlnProValAsnSerSerLeuGluPheIle	224
673	TCCAAATTTATTGTTGAAATGAAAAGGCATATGTGTGATGTGGATGCTGCGCAGTTTCACATTCACTTTCTGAAAGCAATATG	
	SerLysPheIleValGluMetLysArgHisMetCysAspValAspAlaAlaHisValSerHisSerLeuSerGluSerAsnMet	252
757	CTAATTTACATAGAATCCCTTAATCTATTGCTAAAATTTAGCTTCTTTTCAGACGCGGCAAGTCCTTCAGTAATGGTCACACTG	
	LeuIleTyrIleGluSerLeuAsnLeuLeuLeuLysPheSerPhePheSerAspAlaAlaSerProSerValMetValThrLeu	280
841	CCCTTTGATATTTGAATGACGTTTTCACAATTGCGCAGGACTATTCTGCTACGAATACAAATGAAAGCATTGATAGGATCACA	
	ProPheAspIleLeuAsnAspValPheThrIleAlaGlnAspTyrSerAlaThrAsnThrAsnGluSerIleAspArgIleThr	308
925	GAAAAATTACTTTGACTTCTACGCAATTAACCTCATCCGGTGGACCTGGAATTTATGCCCCAAAATGAAGTACAATACTTTA	
	GluLysLeuLeuLeuThrSerThrGlnLeuThrHisProValAspLeuGluAsnLeuCysProLysMetLysTyrAsnThrLeu	336
1009	GCAGCAGTTTCTCGAATATGGTACAAATTTGGCCCCATAGTCGATAAATTTACTAACCAGGCTATTACCGGTTCTATTCCCC	
	AlaAlaValSerArgIleTrpTyrLysPheGlyProIleValAspLysLeuPheThrAsnArgLeuLeuProValLeuPhePro	364
1093	CCGCAATGGGAGAAGAATGTAACGTGGAAGATGTTCTCGAAATAGTTTCATACTTCCATCCATATTTTCTATTTCGTAGGCTA	
	ProGlnMetGlyGluGluCysAsnValGluAspValLeuGluIleValHisAsnPheHisProTyrPheSerIleArgArgLeu	392

Fig. 2. For legend see facing page.

1177	AAGGATAATAGGCCTCTATTGTCAGATTGCGACTATTAGCCAGCTAAGAGAGGGTTTGGTTTGGTATGCTCAGCATATTAACGAC	
	LysAspAsnArgProLeuLeuSerAspSerThrIleSerGlnLeuArgGluGlyLeuPheGlyMetLeuSerIleLeuAsnAsp	420
1261	TCGCTGACAAGGACGCAAAATGAAAACGACCATGGTAGCGACAATCTGATCGACAGTGACGACGGATTGGCTCAGACAACGAT	
	SerLeuThrArgThrGlnAsnGluAsnAspHisGlySerAspAsnLeuIleAspSerAspAspGlyPheGlySerAspAsnAsp	448
1345	CCTGAACAACAAGCGTACCTCGATGAACCTGATCAGAAGGTTATGACGAAAACATGTATGACGGTGATACGGATGACGAAGAC	
	ProGluGlnGlnAlaTyrLeuAspGluLeuValSerGluGlyTyrAspGluAsnMetTyrAspGlyAspThrAspAspGluAsp	476
1429	GCAGATGATATAAACGTTGAAAAAATGATGAAGCCACCAAGACATTACAGAAAACGAAAAATCCTTTTAACTCTTTCTGAG	
	AlaAspAspIleAsnValGluLysAsnAspGluAlaThrLysAspIleThrGluThrAsnLysIleLeuLeuIlePheSerGlu	504
1513	TTACATTATCCTCAGGAGGAGCGCTTCTCTGAACTGTTGGTTGAGTTGCAGACTAAGATCGCTATAAACACATCCTTAATTGAT	
	LeuHisTyrProGlnGluGluArgPheSerGluLeuLeuValGluLeuGlnThrLysIleAlaIleAsnThrSerLeuIleAsp	532
1597	AAAATTCTTTCAAAAGAGACAACAGAATTACCCACTCACATGGGGAAATCGCAGATTGAAACGAAATCTAAATGAAGTGAA	
	LysIleLeuSerLysGluThrThrGluLeuProThrHisAsnGlyGluIleAlaAspLeuAsnGluIleLeuAsnGluValLys	560
1681	GGCAATAAACCAATTAGAAAAACGTTATTTTTGTCAGTTACGCGATCTTGTCTGCAATCCGGTCCGAATTGTCTGTTCTA	
	GlyAsnLysProIleArgLysAsnValIlePheValSerTyrAlaIleLeuSerLeuGlnSerGlySerGluLeuSerValLeu	588
1765	CAGCTATCAATAGAGGTTATTGATCATCTGCTGGTTAAAAATCATTCAAATAACATCACTCGTGGCGAGCAATTTCAATTGATT	
	GlnLeuSerIleGluValIleAspHisLeuLeuValLysAsnHisSerAsnAsnIleThrArgGlyGluGlnPheGlnLeuIle	616
1849	AAGTTGATATTACCCCTCTAAAAACAAATAAATCTTTTATTGATACATTAAGCAGGAAATTTTACCCAAAAATTTGATGCA	
	LysLeuIleLeuProHisLeuLysThrAsnLysSerPheIleAspThrLeuLysAlaGlyAsnPheThrGlnLysIleAspAla	644
1933	AGGAGTGACCTTAAGAACAATGATTCTTTCTCTACTACTACAATATTCCCACTGGATTACTTATGCTAGGTGAGATTTTACCA	
	ArgSerAspLeuLysAsnAsnAspSerPheSerThrThrThrIleProThrGlyLeuLeuMetLeuGlyGluIleLeuPro	672
2017	CTATTGCGCGATATCTGTAAGGGATAAGGATCTTGGGGTGAGAGATCTGTCCTTCCAATTGCTGGACCAAATTTCTACGCACATA	
	LeuLeuArgAspIleCysLysGly---	
2101	CTATAATTACCTAATTGGTATCGATTGGGAATGGTATAAAGATGACTTCTATAAGTTTTCGAAGAACTTGTATTAAAAAGAC	
2185	ATAAATACCAACCTCTTGCTACAATTTCTCCTTATCTCCCTCATGACTAGATAGTTTATACCAAAATACATCATCGATCTTTT	
2269	TTTTTCTTATAGTTTGTGTCATCAACATGTTTTTGCCCGTATTAATACACAATAATCTATCTCTCAGGTAGGGAGAAGAAAA	
2353	CTAAATATACATAGCACCTCACACAGGATGTCAAACTATCGTCTTATCCTCATGCCGCCGATTTTATAAATATGGAAGAACC	
2437	GCCAAATCTAAAGAGTTTTTCGATGATCTTTGTGCCGTCCTAATTTACTCAAAAGACGTTTTTCCAAACTCTCGAAGATCGA	
2521	CGCATTATTGTGAGGCTTTAAATTATCTAGGAAAAAGCTTCCTGTTGTGTGTCTAAATG	

Fig. 2. Sequence analysis of LAG2. The LAG2 DNA sequence and predicted amino acid sequence. The PCGENE analysis package (Intelligenetics) was used for all DNA and protein sequence analysis, as well as homology searches. An ORF of 2040 bp was predicted following sequence analysis, consistent with the size of the transcript (2.1 kb). The putative methionine initiation codon, numbered 1, is located 24 bp downstream from the single transcription start site (asterisk). A potential TATA-box element was located at position -75 to -61 (single underline), and a putative CCAAT-box was found at position -146 to -135 (double underline). A translation stop codon was predicted at position 2041 (dashes).

calculated molecular mass of 77 540 Da and an isoelectric point of 4.62. Potential phosphorylation sites were identified, particularly towards the C terminus (Fig. 3). Nine potential protein kinase C phosphorylation sites (100, 197, 308, 355, 388, 495, 625, 632, 639), 11 casein kinase II phosphorylation sites (43, 100, 219, 246, 425, 445, 472, 529, 581, 608, 628), 3 possible tyrosine sulfation sites (463, 468, 507) and 11 potential N-glycosylation sites (182, 189, 218, 301, 419, 527, 602, 606, 626, 637, 651) were predicted. To analyse potential transmembrane domains, three programs were used, RAOARGOS (Rao & Argos, 1986), HELIXMEM (Eisenberg *et al.*, 1984) and SOAP (Kyte & Doolittle, 1982; Klein *et al.*, 1985). A single transmembrane domain (656–671) was predicted by all three programs. Membrane-associated helices were identified by the HELIXMEM program and included a surface domain

(starting at residue 35) and three globular protein domains (starting at residues 157, 265, 571). A PEST region (432–483) was identified, possibly targeting the protein for rapid turnover (Rogers *et al.*, 1986). A potential mitochondrial transit peptide (1–29) (Gavel & von Heijne, 1990) was also predicted. The presence of this sequence is not always an accurate indicator of mitochondrial import.

Deletion of LAG2

Over one-half of the ORF of LAG2 was deleted starting at the *EcoRI* site, 90 bp downstream of the translation initiation codon. The deleted sequences were replaced

Fig. 3. Lag2 protein sequence motifs. Protein signatures predicted by the PROSITE program (Bairoch, 1990) are: 9 protein kinase C phosphorylation sites (○); 11 casein kinase II phosphorylation sites (■); 3 predicted tyrosine sulfation sites (□); and 11 N-glycosylation sites (X). A potential PEST region was also predicted (*). The membrane-spanning region of the protein (open box), a hydrophobic surface domain (hatched box) and globular regions (filled box) are indicated. A potential mitochondrial transit peptide 1–29 (▲) is predicted at the N terminus of the protein.

Fig. 4. Life span of yeast cells with a marked deletion of *LAG2*. The age of the cells was determined by the number of buds that emerged from each cell and was expressed in generations. The deletion of *LAG2* (*LAG2Δ::TRP1*) resulted in a reduction in both the mean (10 generations; $P \leq 0.0001$) and maximum (17 generations) life span, compared to the mean (18) and maximum (28) life span of the isogenic control. □, *LAG2* deletion strain; ■, parent strain.

with *TRP1*. This marked deletion resulted in a null mutation in *LAG2* in haploid cells, as determined by Northern blot analysis. However, this mutation had no visible effect on the appearance and growth of the cells. The life span of cells with a deleted *LAG2* gene (*LAG2Δ::TRP1*) was determined by counting the number of buds produced by each cell (Fig. 4). The deletion of the *LAG2* gene resulted in a 50% decrease in the mean ($P \leq 0.0001$) and maximum life span, compared to the mean and maximum life span of the control. Similar results were obtained in two experiments with two independent clones. The *LAG2Δ::TRP1* strain displayed no loss in cell viability. Lag2 does not appear to be essential for vegetative growth and division, because it can be deleted in haploid cells without a loss in viability. This strain continued to display the characteristic asymmetric segregation of replicative potential between mother and daughter. Thus, the sole defect in the strain appears to be a decrease in longevity, suggesting that *LAG2* is a longevity-assurance gene.

Further phenotypic analysis was performed to gain insight into the function of *LAG2*. No difference in growth was observed in the cold (4 °C), following heat shock (55 °C)

or in hyperosmotic medium (1.5 M sorbitol) between *LAG2Δ::TRP1* and the control. In addition, no difference in sensitivity to nitrogen starvation, pH or salt was found. Yeast cells with *LAG2* deleted were unable to grow at 37 °C, unlike the parent strain. Both the deleted *LAG2* strain and its parent were able to utilize fermentable carbon sources (glucose, galactose, or raffinose) to the same extent. However, the deletion strain was unable to utilize non-fermentable carbon sources, such as potassium acetate or glycerol. These results, in addition to a predicted mitochondrial transit signal, may suggest a possible mitochondrial association. However, the deleted *LAG2* strain, unlike the control, failed to grow in 5 mM caffeine, a 3',5'-cAMP phosphodiesterase inhibitor, indicating a possible association with the cAMP pathway.

Overexpression of *LAG2*

To examine further the effect of this gene on yeast longevity, *LAG2* was overexpressed. The ORF of *LAG2* was placed under the control of the yeast *GAL10* promoter in a single-copy yeast vector [pBM150(*GAL10:LAG2*)]. *LAG2* was overexpressed by induction of the *GAL10:LAG2* fusion with 4% galactose, as verified by Northern blot analysis. A small increase in initial mortality was observed, following induction with galactose from the beginning of the life span (Fig. 5a). This was followed by an increase in longevity in the surviving population, as compared to the life span of cells transformed with the pBM150 vector alone. Chen *et al.* (1990) demonstrated that controlled expression of v-Ha-ras, using the galactose-inducible promoter *GAL10* on the pBM150 vector, resulted in a near doubling in the mean and maximum life span compared to cells containing the vector alone. At higher levels of expression of v-Ha-ras, the life span of transformed cells was not extended and may be due to a down regulation of the signal rather than a decrease in cell viability. The cells that survived the initial deleterious effect had a significantly longer mean life span (20%; $P = 0.007$) than the control (Fig. 5b). Early in the yeast life span, the endogenous expression of *LAG2* is high, decreasing as the cell ages (Egilmmez *et al.*, 1989). This raises the possibility that the deleterious effect of induced

Fig. 5. Life span of yeast cells overexpressing the *LAG2* gene. (a) Life spans of cells overexpressing *LAG2* from the *GAL10* promoter ($\square$) and cells with endogenous *LAG2* expression ($\blacksquare$) were compared. The life span was determined on medium containing galactose to induce overexpression of the *GAL10:LAG2* fusion at generation zero (unbudded) cells. Cells containing the fusion, but grown on glucose to repress *GAL10*, were used as controls. (No difference in life span between cells grown on glucose compared to galactose have been observed.) Cells overexpressing *LAG2* had a mean life span of 20 generations and a maximum of 42 generations compared to the controls with a mean of 18 and a maximum life span of 30. (b) Cells that survived the initial increase in mortality made their presence obvious at about generation 12, when the endogenous levels of *LAG2* expression had declined (Egilmez *et al.*, 1989). The life span of cells surviving this initial mortality and alive after generation 12 were replotted from the data in (a). The mean and maximum life spans of surviving cells were significantly longer. They were 24 ($P = 0.007$) and 42, respectively, compared to a mean of 20 and maximum of 30 generations for control cells. (c) Life spans were determined initially on medium containing raffinose (uninduced cells). Cells were induced to overexpress the *LAG2* gene at generation 12 (arrowhead) on medium to which galactose was added, and life span determinations were continued. Induction of *LAG2* expression later in the cell life span resulted in a significantly longer ($P \leq 0.0001$) mean life span compared to the uninduced control. The mean and maximum life spans were 23 and 37 generations, respectively, compared to a mean of 17 and a maximum of 24 generations for control cells.

LAG2 expression may be due to excessive Lag2 levels early in the life span. Indeed, when *LAG2* expression was induced later in the yeast life span at generation 12, when endogenous expression is low, an extension in the mean (36%; $P \leq 0.0001$) and maximum (54%) life span was observed (Fig. 5c), and there was no increase in the initial mortality compared to the control. Similar results were obtained in three experiments with three independent clones for Fig. 5(a) and (b), and two experiments with three independent clones for Fig. 5(c).

DISCUSSION

The senescence of an organism may be characterized as a progressive inability to maintain homeostasis and endure damage, stress and disease. Several theories have been proposed concerning the mechanisms involved in senescence. It is generally agreed that genetic determinants play an important role (Finch, 1990). The key is to identify the genes and their molecular mechanisms of action. One of these genes in yeast is *LAG2*, a gene preferentially expressed in young cells.

The *LAG2* gene is unique in that the nucleotide and protein sequence as yet has shown no similarity to any sequence previously entered in the databases. The protein appears to possess a transmembrane segment close to the C terminus. Potential phosphorylation sites were predicted at both ends of the Lag2 protein, particularly near the C terminus. The significance of these sites depends on the intracellular location of Lag2. In addition, several glycosylation sites were indicated throughout this protein. A potential mitochondrial transit peptide is predicted at the N terminus. Functional tests will be required, however, to establish the role of this sequence in import of Lag2 into the mitochondrion. Bcl-2 is an inner mitochondrial membrane protein involved in prevention of the apoptotic death of pro-B-lymphocytes (Hockenbery *et al.*, 1990). However, Lag2 does not share any sequence similarity to Bcl-2. An argument for possible mitochondrial function of Lag2 comes from the observation that cells with a *LAG2* deletion were unable to utilize non-fermentable carbon sources. However, it should be noted that the life span of spontaneous and induced petites (mutants defective in mitochondrial function) are not significantly different from parental strains (Muller & Wolf, 1978). Also, an accumulation of petites during yeast ageing has not been observed (Muller, 1971). An alternative, suggested by the sensitivity of the *LAG2* deletion strain to caffeine, is an interaction of *LAG2* with the Ras-cAMP pathway (Broach & Deschenes, 1990). Caffeine is a 3',5'-cAMP phosphodiesterase inhibitor. One possibility is that Lag2 is a plasma membrane protein that interacts with Ras.

Age-responsive promoter elements may control gene expression at various stages during the life span. Sequence analysis of the *LAG2* gene for promoter and possible regulatory elements revealed a TATA-box located 51 bp upstream from the single transcription start site. A CCAAT box was also predicted 122 bp upstream of the transcription start site. Other regulatory mechanisms,

including RNA processing and degradation, translation initiation and elongation, transcriptional elongation and interaction of Lag2 with other proteins may be involved. Post-translational modifications may also regulate the activity of this gene in the cell. This may involve targeting the PEST region or one of the predicted phosphorylation sites.

A null mutation in *LAG2* (*LAG2Δ::TRP1*) had no effect on vegetative growth of haploid cells. However, it decreased significantly the mean and maximum life span. Overexpression early in the life span, when endogenous levels of *LAG2* mRNA are high, resulted in an increase in initial mortality. However, surviving cells realized a 20% extension in the mean life span. Induction of *LAG2* later in the yeast life span, when endogenous levels of *LAG2* mRNA are lower, resulted in a 36% extension in the mean and a 54% extension in the maximum life span. These results suggest that the level and time of *LAG2* expression in the life span are important for longevity. Considering that the overexpression construct of *LAG2* lacked the first 87 amino acids of the gene, there remains a possibility that the lack of this region may impart an additional influence on the extension of the observed life span of the cells. The life span attenuation observed on deletion of *LAG2* and the extension seen upon overexpression, coupled to the fact that this gene is preferentially expressed in young cells, indicate that this is a longevity-assurance gene. One view of the role played by *LAG2* is that its level of expression determines the probability that the yeast cell will continue dividing later in the life span. Once expression drops below a threshold, another gene or gene hierarchy becomes limiting for longevity. Alternatively, once expression of *LAG2* declines, the yeast organism may simply coast, dependent on its life maintenance reserves. Longevity is polygenic in yeast (C. Pinswasdi & S. M. Jazwinski, unpublished). The question arises as to how many different gene-dependent processes impinge on longevity. The identification of a gene that determines longevity in yeast does not indicate that ageing is genetically programmed as a series of events each dependent on the completion of the previous one, starting with birth and ending with the death of the cell.

ACKNOWLEDGEMENTS

We thank Linda Riles for providing blots with the ordered array of yeast genomic contigs. This work was supported by grants from the National Institute on Aging of the National Institutes of Health (USPHS).

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Received 22 November 1995; revised 8 March 1996; accepted 1 April 1996.