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The *ERD2* Gene Determines the Specificity of the Luminal ER Protein Retention System

Michael J. Lewis, Deborah J. Sweet, and Hugh R. B. Pelham
MRC Laboratory of Molecular Biology
Hills Road
Cambridge CB2 2QH
England

Summary

Luminal ER proteins carry a signal at their C terminus that prevents their secretion; in S. cerevisiae this signal is the tetrapeptide HDEL. Indirect evidence suggests that HDEL is recognized by a receptor that retrieves ER proteins from the secretory pathway and returns them to the ER, and a candidate for this receptor is the product of the *ERD2* gene (see accompanying paper). We show here that presumptive ER proteins from the budding yeast K. lactis can terminate either with HDEL or, in the case of BiP, with DDEL. S. cerevisiae does not efficiently recognize DDEL as a retention signal, but exchange of its *ERD2* gene for the corresponding gene from K. lactis allows equal recognition of DDEL and HDEL. Thus the specificity of the retention system is determined by the *ERD2* gene. We conclude that *ERD2* encodes the receptor that sorts luminal ER proteins.

Introduction

Proteins that reside in the lumen of the ER are characterized by a C-terminal signal that prevents their passage along the secretory pathway (for review see Pelham, 1989). The optimal signal in animal cells is the tetrapeptide KDEL, whereas in Saccharomyces cerevisiae it is HDEL. Proteins bearing this retention signal are able to leave the ER by vesicular transport but are specifically retrieved from the Golgi or a pre-Golgi "salvage" compartment. In yeast, the retrieval system is readily saturable; it is presumed to involve a membrane-bound receptor that cycles between the Golgi and the ER (Dean and Pelham, 1990).

In an attempt to identify components of this sorting system, and in particular the presumed HDEL receptor, we have isolated yeast mutants that fail to retain HDEL-tagged proteins in the ER (Pelham et al., 1988; Hardwick et al., 1990). These mutants define two genes, one of which (*ERD2*) is a good candidate for the receptor. In support of this, we have found that the capacity of the retention system is controlled by the level of *ERD2* expression: low levels of *ERD2* protein result in secretion of ER proteins, while high levels render the system resistant to saturation (Semenza et al., 1990).

The definitive property of a receptor is its ligand specificity. Thus one way to test whether *ERD2* encodes the receptor is to identify differences in the specificity of the retention system between species, and see whether they are determined by the *ERD2* gene. We report here that one of the major ER proteins of the budding yeast Kluyveromyces lactis, namely BiP, has at its C terminus the sequence DDEL, although at least one glycoprotein in this species carries the HDEL signal. S. cerevisiae does not efficiently retain fusion proteins that terminate with DDEL, but replacement of the *ERD2* gene with the equivalent one from K. lactis allows efficient retention of both DDEL and HDEL. This result argues very strongly that the *ERD2* protein is indeed the receptor responsible for retrieving ER proteins from the Golgi.

Results

K. lactis BiP Lacks HDEL

We have raised anti-peptide antisera that are specific for a C-terminal HDEL sequence. These antibodies recognize two different peptides whose only common sequence is HDEL (Hardwick et al., 1990), but will not bind to HDEL when it is present at an internal position, nor to KDEL. In a search for a suitable organism whose ER proteins have a signal different from those in S. cerevisiae, we used these antibodies to probe blots of total proteins from the yeasts Schizosaccharomyces pombe and Kluyveromyces lactis (Figure 1). K. lactis had at least one glycoprotein that was detected by the antiserum; from its mobility and abundance, it seems likely that this glycoprotein corresponds to the grp94 protein of animal cells. A smaller, unglycosylated protein was also detected (Figure 1). However, only an extremely faint signal was detected in the position expected for K. lactis BiP, implying that it carries a divergent retention signal. S. pombe contained no abundant proteins that reacted with the antiserum (Figure 1), and thus this species must also use a retention signal other than HDEL.

K. lactis and S. cerevisiae are related budding yeasts, and genes from one are usually efficiently transcribed and correctly spliced in the other (e.g., Salmeron and Johnston, 1986; Desher et al., 1989); in contrast, many S. pombe genes are nonfunctional in S. cerevisiae. K. lactis thus seemed a suitable organism with which to test the hypothesis that the *ERD2* gene encodes the HDEL receptor.

Cloning the K. lactis BiP Gene

To identify the retention signal on K. lactis BiP, we cloned the corresponding gene. Low stringency hybridization with parts of the S. cerevisiae BiP gene failed to distinguish the K. lactis BiP gene from other members of the hsp70 family. K. lactis DNA was therefore digested with EcoRI and electrophoresed on an agarose gel, and DNA from regions corresponding to individual cross-hybridizing bands was eluted, cloned, and sequenced. The third fragment sequenced showed clear homology with BiP and was used to isolate a full-length clone. From this, we obtained sequence corresponding to the C terminus of the protein (Figure 2).

Strikingly, the C-terminal tetrapeptide of K. lactis BiP is

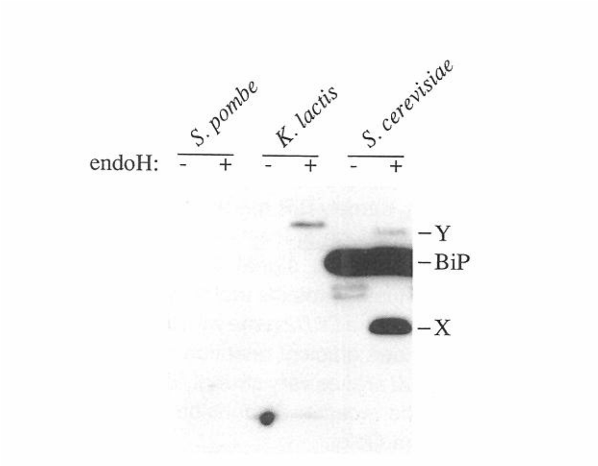

Figure 1. Divergence of the ER Retention Signal in Different Yeasts

Samples of total protein from the indicated yeasts were incubated with endoglycosidase H to remove high mannose oligosaccharides. Endoglycosidase H-treated and control samples were immunoblotted with anti-HDEL antibodies. X and Y refer to the glycoproteins previously identified in S. cerevisiae; Y probably corresponds to grp94 (Hardwick et al., 1990). Staining of the blot revealed similar amounts of protein in each lane.

DDEL. The presence of an aspartic acid in place of the histidine found in S. cerevisiae accounts for the failure of our anti-HDEL antibodies to recognize the protein.

Cloning of K. lactis *ERD2*

Probing Southern blots of K. lactis DNA at low stringency with the S. cerevisiae *ERD2* gene revealed a single cross-hybridizing sequence, which could be localized to a 1.25 kb EcoRI–HindIII fragment. This fragment was cloned and sequenced (Figure 3A). The gene contained a single in-

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...GEGFEDEDDD-DYF-DDEL    K. lactis
       ****** ***  ***
...DYDDEDEDDDGDYFEHDEL    S. cerevisiae
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Figure 2. C-Terminal Sequences of K. lactis and S. cerevisiae BiP Proteins

Asterisks indicate identical residues; two gaps have been introduced into the K. lactis sequence to maximize homology. The complete sequence of the K. lactis gene will be reported elsewhere.

tron in the same location as the intron in the S. cerevisiae gene, and the encoded proteins showed 59% identity, with many of the changes being conservative in nature. Furthermore, expression of the K. lactis gene in S. cerevisiae was sufficient to complement the growth-arrest phenotype of an *ERD2* deletion mutant. It thus seems clear that the K. lactis gene that we have isolated corresponds to the *ERD2* gene of S. cerevisiae.

A comparison of the two sequences is shown in Figure 3B. One difference is that the 15 amino acid duplication near the N terminus of S. cerevisiae *ERD2* is barely recognizable as a repeated sequence in K. lactis. Moreover, the D→N change at amino acid 51, which causes a strong *erd* phenotype in S. cerevisiae, is naturally present in the heterologous gene. At the C terminus, three hydrophobic stretches are very highly conserved, although the hydrophilic sequences flanking them are more divergent. These hydrophobic residues may form part of a conserved transmembrane structure.

Changing the Specificity of the Retention System

To determine the effects of *ERD2* on the specificity of the retention system, we expressed fusion proteins carrying at their C termini the potential retention sequences FEH-

A

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GAATTCTTCACTCTTCACCCCTCCTCTCTAACGAGACTCGGATAAACAGACTGTAACGAT
                                                         120
GACGACGACGACGATGATGATAATGATTATGATGATGATGATTGAATGTGCCATTTGACG
TTTTTGACCTGTAAACCCCTTTTGTAAATAGCCTCTGTCCTTTTTAAGTACTTATGCGGT
                                                         240
GTCTGTTAATACAATCTTTGTGGCTCACGTGATTTATTATTTTTCTCAATGTGGGCCAAA
ATTGCAATCTCTTAACTCTGAAAAATAAAACAAGGCAGTACTAATCGCTTCTTAAACACT
                                                         360
TGTTCTTTAAAGATTGAAATAAACTACCGACATTTGTCGTTCTAAGTTAAATTCAAATCA
AAACAGCTTTCAATTGAGTGTGTCACATTATGTTGAACGTTTTCAGAATAGCAGGTATGT
                                                         480
GAAAAGAACTGATTTTATATCATTGTTGGACGTCAACCTCGATCGGAACGTTATATCTTG
GTTCTGAATGATGATTTCCATCGGCTTATCTCTACTGGCAGCATCTTTACTAACATTGTT
                                                         600
ATTACCAACTATTTTGTAATCGAGTGTTTAGGTGATTTCTCTCATTTGGCTAGTATCATC
ATTTTGATACAATCGATCACAACATCTAACTCAGTTGATGGTATCTCATTGAAAACTCAA
                                                         720
CTGCTATACACCTTGGTCTTTATCACACGTTATTTGAACCTATTTACCAAATGGACCTCC
TTGTACAACTTCTTAATGAAAATTGTTTTCATTTCATCTTCGGTTTACGTCATTGTGTTA
                                                         840
ATGCGCCAACAAAAATTTAAAAACCCTGTCGCATATCAAGACATGATCACCAGAGATCAA
TTTAAAATCAAGTTTTTAATAGTACCATGCATTCTCCTAGGATTAATTTTCAATTATCGT
                                                         960
TTCAGTTTTATACAAATATGCTGGTCCTTCTCTCTATGGTTGGAAAGTGTTGCAATCCTT
CCTCAATTGTTTATGTTGACTAAAACAGGTAAAGCAAAACAATTGACATCTCATTATATT
                                                        1080
TTTGCATTGGGGTTATACCGTGCCTTGTACATTCCAAATTGGATATGGAGGTATTATACC
GAAGAAAGATTCGATAAATTATCAGTTTTCACCGGTGTGATTCAAACTTTAGTGTACTCT
                                                        1200
GATTTCTTCTACATCTATTATCAGAAGGTTATCAAACTCGGTGGAGATTTAGAATTACCA
CAATAGATGACAGGATTTGCATCTCCACTTGCAGTCAGTTCGAAGCTT
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B

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                                                             60
        +   -  +        +  +  +  -    +           +  -    +
                             _______________     ______________
S. M-NPFRILGDLSHLTSILILIHNIKTTRYIEGISFKTQTLYALVFITRYLDLLTFHWVSL
   * * *** ** *** ** ***  * *     *** *** ** ******** * *  * **
K. MLNVFRIAGDFSHLASIIILIQSITTSNSVDGISLKTQLLYTLVFITRYLNLFT-KWTSL
        +   -  +                 -    +           +       +

                                                            120
   _    +                 ++       -   +-  +  +            +++
S. YNALMKIFFIVSTAYIVVLLQGSKRTNTIAYNEMLMHDTFKIQHLLIGSALMSVFFHHKF
   ** **** ** *  *  **    *  *  **  *   * ***  *     *    *   *
K. YNFLMKIVFISSSVYVIVLMRQQKFKNPVAYQDMITRDQFKIKFLIVPCILLGLIFNYRF
        +              +  + +      -   +-  + +               +

                                                            180
      -         -            +  + +    +        +         +   -
S.•TFLELAWSFSVWLESVAILPQLYMLSKGGKTRSLTVHYIFAMGLYRALYIPNWIWRYSTE
    *    **** *********** ** * **   ** ***** *************** **
K. SFIQICWSFSLWLESVAILPQLFMLTKTGKAKQLTSHYIFALGLYRALYIPNWIWRYYTE
                -            +  + +    +        +         +   -

                                         221
   -++ -+              -       +  +  +  +  +
S. DKKLDKIAFFAGLLQTLLYSDFFYIYYTKVIR-GKGFKLPK
       **   * *  *** ********* ***  *    **
K. ER-FDKLSVFTGVIQTLVYSDFFYIYYQKVIKLGGDLELPQ
   -+  -+              -       +  +   - -
```

Figure 3. Sequence of K. lactis *ERD2*

(A) Nucleotide sequence of the gene. Double underlining indicates important features: the initiation codon; splice donor, branchpoint, and acceptor; and termination codon. The sequence illustrated is sufficient for full *ERD2* function.

(B) Alignment of the S. cerevisiae (upper) and K. lactis (lower) *ERD2* protein sequences. Asterisks indicate identical residues, and charged amino acids are indicated. Two spaces have been introduced into each sequence to maximize homology. Bars above the S. cerevisiae sequence indicate a 15 amino acid duplication.

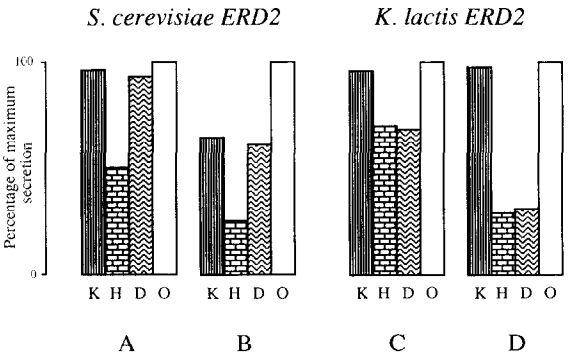

Figure 4. Effect of *ERD2* on the Secretion of Invertase Fusion Proteins Bearing Different C Termini

The percentage of total invertase activity that had been secreted was determined for fusion proteins terminating with SEEDLN (O), or with the same sequence followed by SEKDEL (K), FEHDEL (H), or YFDDEL (D).

(A) Wild-type S. cerevisiae strain with its normal chromosomal copy of *ERD2*.

(B–D) Isogenic strains that have the chromosomal *ERD2* gene deleted, and carry the following plasmids: a high copy number plasmid (PER220) with the S. cerevisiae *ERD2* gene (B), a low copy number plasmid (LE22) expressing K. lactis *ERD2* (C), and a high copy number plasmid (LE21) with the K. lactis *ERD2* gene (D).

Values (average of four or more determinations) have been normalized to the control construct (O) to aid comparison; this corrects for minor differences in overall secretion efficiency, which reflect the slightly different growth rates of the strains. About 30% of the invertase activity was secreted from the control strains.

DEL (corresponding to S. cerevisiae BiP), YFDDEL (K. lactis BiP), or SEKDEL (rat BiP). Control constructs lacking these sequences terminated with SEEDLN. Initial experiments used a fusion protein containing the enzyme invertase. Secretion of this protein can easily be monitored by assaying unlysed cells for invertase activity; intracellular enzyme is inaccessible to substrate under these conditions, but can be detected after cell lysis.

As shown in Figure 4A, the HDEL-tagged fusion protein was secreted less efficiently from a wild-type strain than were the KDEL or DDEL versions, neither of which showed significant retention relative to the control. Thus, neither KDEL nor DDEL is efficiently recognized by the S. cerevisiae receptor under these conditions.

A slightly different result was obtained with a strain that lacked the chromosomal copy of *ERD2* but instead carried the gene on a multicopy vector. Retention of the HDEL construct was improved, but in addition partial retention of both DDEL and KDEL was observed (Figure 4B). Thus when the capacity of the system is increased by overexpression of *ERD2*, its apparent specificity is broadened. Presumably, under normal conditions the receptor is saturated with endogenous HDEL-containing proteins, which effectively compete with the DDEL and KDEL sequences, but when receptor is present in excess, a weak affinity for the latter sequences is revealed. Nevertheless, with both high and low levels of *ERD2* expression, HDEL was the preferred substrate.

A different result was obtained when the plasmid-borne *ERD2* gene was replaced with K. lactis *ERD2*. Two different plasmids expressing the K. lactis gene were used; one was a low copy number vector with the *ERD2* coding sequences fused to the *TPI* promoter, while the other was a high copy number plasmid carrying the gene with its own promoter. Strains containing these plasmids showed different efficiencies of retention, presumably because they expressed *ERD2* at different levels, but in each case the specificity was the same: invertase fusion proteins bearing HDEL and DDEL were retained equally well, while the KDEL construct was secreted as efficiently as the control (Figures 4C and 4D).

As a further test, we examined the secretion of prepro-α factor fusion proteins in strains lacking the chromosomal *ERD2* gene and carrying either S. cerevisiae or K. lactis *ERD2* on a multicopy plasmid. Pro-α factor is secreted rapidly, and the fusion proteins can most easily be assayed by pulse-labeling and immunoprecipitation. Molecules that pass through the Golgi are proteolytically processed and thus escape detection, so the amount of labeled protein remaining after a short chase gives a direct indication of the efficiency of retention (Dean and Pelham, 1990; Semenza et al., 1990).

Figure 5 confirms the results obtained with the invertase constructs. With S. cerevisiae *ERD2* expressed at a high level, the pro-α factor–HDEL protein was retained best, but some retention of both the DDEL and KDEL versions was also observed. In contrast, when only K. lactis *ERD2* was present, HDEL- and DDEL-tagged proteins were retained with equal efficiency, whereas the KDEL-tagged and control proteins were rapidly secreted.

We conclude from these experiments that the *ERD2* gene controls the specificity of the ER retention system. S. cerevisiae *ERD2* allows strong recognition of HDEL and weak recognition of DDEL and KDEL; K. lactis *ERD2* allows recognition of HDEL and DDEL, but not KDEL. The dual specificity of K. lactis *ERD2* is consistent with the presence of both DDEL and HDEL on ER proteins of this organism.

Discussion

Our aim in these experiments was to test the hypothesis that the *ERD2* gene encodes the receptor that sorts luminal ER proteins. We first looked for a species of yeast with a receptor specificity different from that in S. cerevisiae, then isolated its *ERD2* gene, transferred it to S. cerevisiae, and tested for a change in the specificity of the retention system. The results indicate that the pattern of retention is indeed determined by the *ERD2* gene.

Divergence of the Retention Signal for Luminal ER Proteins

We were surprised to find that the BiP protein of K. lactis had the C-terminal sequence DDEL, because it implies considerable divergence of the retention signal between two related budding yeasts. All known examples of luminal ER proteins in S. cerevisiae have HDEL at their C terminus; these include BiP (Rose et al., 1989; Normington et al., 1989; Nicholson et al., 1990), protein disulfide isomerase (M. F. Tuite and R. Freedman, personal communica-

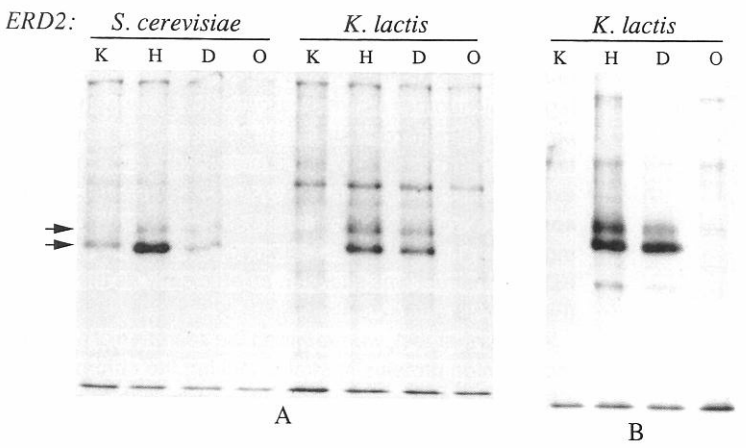

Figure 5. Effect of *ERD2* on Secretion of Pro-α Factor Fusion Proteins

Fusion proteins bearing the same termini as those in Figure 4 were assayed by pulse-labeling and immunoprecipitation.

(A) Strains carrying the chromosomal *erd2* deletion and high copy number plasmids expressing either S. cerevisiae or K. lactis *ERD2* were labeled for 10 min and chased for 15 min.

(B) Deletion strains carrying the low copy number plasmid expressing the K. lactis *ERD2* gene were labeled for 10 min and chased for 5 min.

Arrows indicate pro-α factor. The lower band is the ER form, and the upper band corresponds to protein that has cycled through an early Golgi compartment.

tion), the *KRE5* gene product (Meaden et al., 1990), and two other proteins identified only by their reactivity with anti-HDEL antibodies. Proteins from other species, including plants, nematodes, insects, and vertebrates, almost invariably have a positively charged amino acid 4 residues from the terminus (reviewed by Pelham, 1989), although Plasmodium falciparum BiP has serine (Kumar et al., 1988). The occurrence of an acidic residue at this position in K. lactis BiP is thus unprecedented.

Despite this striking difference, at least one glycoprotein in K. lactis has an HDEL sequence, as judged from its ability to bind anti-HDEL antibodies. The presence of two rather different sequences raises the question of how recognition of both of them is achieved.

ERD2 Encodes the Sorting Receptor

Previous experiments had identified the *ERD2* protein as a good candidate for the HDEL receptor, because its abundance controls the capacity of the retention system (Semenza et al., 1990). We have shown here that the signal specificity of the system is also determined by *ERD2*. With S. cerevisiae *ERD2*, HDEL is the preferred signal, whereas when the gene is replaced by the K. lactis homolog, HDEL and DDEL are retained with equal efficiency. This difference is independent of the level of expression of the *ERD2* genes and thus cannot be explained by a mere quantitative difference in the activity of the two *ERD2* proteins. Furthermore, the observed preferences are entirely consistent with the known sequences of ER proteins in the two species. We therefore conclude that *ERD2* indeed encodes the receptor.

In the case of K. lactis, this single receptor must be capable of binding both FEHDEL and YFDDEL, which suggests a surprising degree of flexibility in its interactions. The specificity of the S. cerevisiae receptor is also not absolute, because when it is expressed at high levels there is some recognition of both DDEL and KDEL sequences, neither of which has been found in this species. Presumably, this reflects weak binding of the receptor to DEL. The dual specificity of the K. lactis receptor cannot, however, be explained simply by binding to DEL, because SEKDEL is not recognized.

One possibility that we cannot rule out is that binding of YFDDEL to the K. lactis receptor is influenced by the two aromatic residues upstream of the terminal tetrapeptide, but there is no evidence that more than 4 amino acids are recognized in other species. We therefore prefer a model in which HDEL and DDEL each interact with the receptor in a highly specific manner.

The *ERD2* protein not only functions as a receptor, but is also required for normal vesicular traffic through the Golgi (Semenza et al., 1990). If ligand binding involves parts of the protein that are also required for viability, it will be difficult to identify the binding site by mutation. The results in this paper suggest that it may be possible to identify the sequences responsible for ligand specificity by creating chimeric *ERD2* genes with sequences from S. cerevisiae and K. lactis. It should also be possible to identify conserved functional motifs by isolating the receptor gene from more distantly related species. Very recently, an anti-idiotypic antibody approach has identified a 72 kd glycoprotein that is good candidate for the KDEL receptor in animal cells (Vaux et al., 1990). It will be interesting to see whether part of this protein shows similarity to the 26 kd *ERD2* product.

Experimental Procedures

Strains

Derivatives of S. cerevisiae strain SEY2102 (*MATα suc2-d9 ura3-52 leu2-3,-112 his4-519*) were used for Figure 4A. Other results were obtained with derivatives of the *erd2* deletion strain D209 (Semenza et al., 1990). K. lactis was kindly provided by Stephen Johnston (Duke University), and S. pombe by Andrew Newman.

Cloning

The K. lactis BiP and *ERD2* genes were detected by hybridization with probes containing the corresponding S. cerevisiae coding sequences, which we had isolated previously (Hardwick et al., 1990; Semenza et al., 1990). Hybridization was in 4× SSC, 10% formamide at 42°C, and filters were washed with 2× SSC at 50°C. Fragments migrating with the hybridizing bands were excised from an agarose gel and cloned into M13 vectors. The relevant clones were identified in these mini-libraries by plaque hybridization.

For high level expression, the EcoRI–HindIII fragment containing the K. lactis *ERD2* gene was inserted into the *LEU2*-containing 2μm vector ZUC13 to form plasmid LE21. Expression at a lower level was obtained by fusing the gene at the ScaI site (position 280) to the *TPI*

promoter and inserting it into a vector containing *TRP1*, *ARS1*, and *CEN3* to form plasmid LE22.

Isogenic strains carrying a deletion of the chromosomal *ERD2* gene and the K. lactis *ERD2* plasmids LE21 or LE22, or the S. cerevisiae *ERD2* plasmid PER220, were generated as described by Semenza et al. (1990).

Invertase and pro-α factor fusion constructs were identical to those used previously (Pelham et al., 1988; Dean and Pelham, 1990) except that additional constructs encoding proteins terminating with YFDDEL were prepared using synthetic oligonucleotides. All were inserted at the *URA3* locus. For the experiments shown in Figure 5, only strains containing a single inserted gene (identified by Southern blot analysis) were used. Invertase assays, pulse-labeling, and immunoprecipitation are described elsewhere (Pelham et al., 1988; Dean and Pelham, 1990). Note that the α factor constructs contain the epitope recognized by the monoclonal antibody 9E10, allowing their specific immunoprecipitation without interference from endogenous α factor.

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