Starved *Saccharomyces cerevisiae* Cells Have the Capacity to Support Internal Initiation of Translation*

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Internal initiation of translation, whereby ribosomes are directed to internal AUG codon independently of the 5′ end of the mRNA, has been observed rarely in higher eucaryotes and has not been demonstrated in living yeast. We report here that starved yeast cells are capable of initiating translation of a dicistronic message internally. The studied element that functions as an internal ribosome entry site (IRES) is hardly functional or not functional at all in logarithmically growing cells. Moreover, during the logarithmic growth phase, this element seems to inhibit translation reinitiation when placed as an intercistronic spacer or to inhibit translation when placed in the 5′-untranslated region of a monocistronic message. Inhibition of translation is likely due to the putative strong secondary structure of the IRES that interferes with the cap-dependent scanning process. When cells exit the logarithmic growth phase, or when artificially starved for carbon source, translation of the IRES-containing messages is substantially induced. Our findings imply that the capacity to translate internally is a characteristic of starved rather than vegetatively growing yeast cells.

The ribosome scanning model has been originally proposed by Kozak (1) to explain how the translation process is initiated. Numerous studies have corroborated the model whereby the initiation complex is assembled near or at the 5′ end of the mRNA, facilitated by the interaction of the cap structure with the eucaryotic initiation factor 4E, and starts scanning the mRNA until the first AUG is encountered (for recent review see Ref. 2). An alternative mode of selecting an initiation codon, whereby ribosomes are directed to an internal AUG by an internal ribosome entry sequence (IRES), has also been demonstrated. Well documented cases of internal initiation events are those of the uncapped picornaviral mRNAs (3). IRESes have also been found in the 5′-untranslated region (5′-UTR) of several cellular mRNAs (4–13). During evolution, IRESes have been utilized as targets for translation regulation during normal differentiation and development. For example, an IRES was shown to play a role in the translation of platelet-derived growth factor 2 mRNA that increases after megakaryocytic cells undergo terminal differentiation (10). IRESes have also been found to mediate the differential translation of Antennapedia and Ultrabithorax during *Drosophila melanogaster* development (6, 12).

Surprisingly, no IRES has been shown to function in yeasts, despite the observations that the yeast cell-free system is capable of recognizing plant viral IRESes (14) as well as natural yeast leader sequences (15). Attempts to promote internal initiation in living yeast cells by using IRESes of poliovirus (16) and encephalomyocarditis virus (17) have thus far failed. These studies were done with optimally growing cells. In their natural environment, however, yeast occasionally encounter starvation and enter into a distinct quiescent state called stationary phase (SP) (reviewed in Refs. 18 and 19). In the test tube, when yeast cells are cultured in rich media (e.g. YPD), nutrient consumption is gradual and entry into SP occurs in a stepwise manner. Thus, cells cultured in YPD display a characteristic growth pattern in which log phase is followed by a diauxic shift, a slow growth phase, and then by SP. During the diauxic shift, cell metabolism changes from mainly fermentation to aerobic metabolism (20), accompanied by morphological and biochemical changes (21–25). To preserve energy during SP, yeast cells shut off the transcription of most genes. Consequently, the global mRNA level is reduced about 35-fold (27, 28). Protein production during SP is reduced even more dramatically (200–300-fold) (29), suggesting that the availability of mRNAs is not the rate-limiting factor of protein synthesis. Instead, translation during SP is found to be inefficient or even actively repressed. Nevertheless, expression of a small repertoire of genes is not repressed in SP (18, 19, 27, 29). It is quite possible that the control of this dramatic strategic change in gene expression requires SP-specific mechanisms for ensuring the synthesis of the small repertoire of proteins that are essential for life during starvation.

Here we show that yeast cells are capable of recognizing an IRES element. The RNA element that functions as an IRES was found by serendipity and contains a sequence from the *Escherichia coli* *lacI* gene. Strikingly, the capacity to recognize this IRES is found in starved cells but not in growing cells. We therefore call this IRES stationary phase-induced IRES (SIRES).

**EXPERIMENTAL PROCEDURES**

Fortuitous Discovery of SIRES—By adapting a colony color test, we utilized the *ACT1p-UBI4-lacZ* reporter, described under "Results," and searched for mutants that failed to repress the reporter gene during SP. We thus identified a cell line whose reporter produced high levels of β-gal in SP. Later, we discovered that the defect was not in the cellular genome but, instead, in the plasmid that underwent a rearrangement event. A series of experiments have demonstrated that the RNA element, which was responsible for the translational induction in SP, contains the sequence shown in Fig. 1B.

Plasmid Constructions—Constructs are schematized in Fig. 1A. Construct 1 was described previously (26). Construct 2 was made by inserting SIRES at the HindIII site, located in-between *ACT1p* and *UBI4*, into construct 1. For construct 3, green fluorescent protein (GFP) was
amplified by PCR reaction, using OMC116 (5'-CCCAACGTTGGATCC-TAAAGATGTAATAGGAAG-3') and OMC117 (5'-GCGGTTTCTACTTTGATCCTCATCATTCCGCGATG-3') as the forward and reverse primers, respectively. HindIII sites are underlined and were used to introduce the PCR fragment into HindIII site of construct 1 (between ACT1p and UBI4). Two in-frame stop codons (marked by bold letters) are introduced immediately after the last codon of the GFP. XbaI was introduced downstream of the HindIII site and was used for inserting the intercistrionic spacers of constructs 4–6. For constructs 4–6, intercistronic spacers were amplified by PCR technology, using primers that contained the XbaI site at their 5' ends. The spacers were inserted in the single XbaI site of construct 3. All constructs were subject to sequencing analysis.

Detection of GFP—Cell extract was equilibrated with Laemmli sample buffer and kept at room temperature. Ten μg were subjected to SDS-PAGE analysis. Fluorescent bands were detected by exposing the gel to Image reader FLA2000 (Fujifilm) at 473 nm excitation and using the 520 nm filter.

RESULTS AND DISCUSSION

SIRES, an RNA Element That Mediates Translational Repression during the Logarithmic Growth Phase and Translation Enhancement during Post-logarithmic Phases—SIRES has been discovered by serendipity during a screen for SP-specific mutations (see “Experimental Procedures”). It contains a sequence from the E. coli lacI gene and a portion of a multiple cloning region. The sequence does not contain AUG or other codons that can function as translation start sites (Fig. 1B). It contains a high GC content (62%) and, by subjecting its sequence to a folding algorithm (foldRNA, GCG Sequence Analysis Package), was found to be capable of forming a highly structured molecule (ΔG = -43.8 kcal mol⁻¹). To study the effect of SIRES on the translation we used the UBI4-lacZ, encoding a ubiquitin-βgal fusion that contains isoleucine at the junction between the two protein sequences. Shortly after translation, the ubiquitin is cleaved off the fusion protein, and the resulting βgal is short lived both in dividing (30) and in stationary cells (39). This reporter thus enables the coupling between the level of the unstable βgal and the rate of its synthesis, even when this rate is decreased (39). Transcription of the reporter gene is governed by ACT1 promoter fragment, spanning positions +1 to -472 relative to the first ACT1 ATG codon and covering the previously described regulatory elements and transcription start sites of the gene (32) (construct 1 in Fig. 1A). SIRES was introduced downstream of the ACT1p and upstream of the UBI4 translation start codon in the ACT1p-UBI4-lacZ, as schematically shown in Figs. 1A and 2A, right panels (see also “Experimental Procedures”). The inclusion of SIRES had little effect on the level of the ACT1p-UBI4-lacZ transcript (Fig. 2B, compare left and right panels). In contrast, the presence of SIRES had a dramatic effect on the level of the translated protein, shown in Fig. 2C (compare left and right panels). During the logarithmic growth phase, the βgal level in cells carrying the SIRES-containing construct was two orders of magnitude lower than that in cells carrying the control construct (log lanes in Fig. 2C, compare the right and left panels). Thus, in logarithmically growing cells, the presence of SIRES in the 5'-UTR inhibits translation. This result is consistent with previous observations demonstrating that in yeast the inclusion in a 5'-UTR of an element that has the potential to form a secondary structure with a stability of ≥28 kcal mol⁻¹ inhibits translation by at least 98% (33). However, other explanations for the SIRES-mediated translational repression cannot be ruled out. For example SIRES may give the mRNA a competitive disadvantage.

The differential translation of the control mRNA and the SIRES-containing mRNA during log phase, when the translation of the former was higher than that of the latter, underwent a significant twist after the diauxic shift. Specifically, expres-
SIRES Functions as a Stationary Phase-induced IRES—Two observations raised the possibility that SIRES mediates cap-independent translation. First, SIRES is capable of forming a highly stable secondary structure, which is likely to impede the cap-dependent ribosome scanning process (see above). Second, SIRES-mediated translation is enhanced when the bulk of the translation machinery is compromised, raising the possibility that this is recognized by a different or modified machinery. To study the mode of action of SIRES, a series of dicistronic constructs were made (Fig. 1A). Dicistronic constructs have been effectively used in vitro to demonstrate the existence of IRES (3, 5, 6, 8, 13). As most ribosomes fail to continue through the intercistronic spacer, the translation of the second cistron is greatly reduced, unless preceded by an IRES. Using these constructs we attempted to answer the following questions. (i) Can SIRES promote translation of the second cistron by internal initiation? (ii) Is the translation of the second cistron influenced by that of the first cistron, or is the translation of the two cistrons controlled independently? (iii) Is the translation of the second cistron induced in SP like that of the SIRES-containing monocistronic mRNA?

The gene encoding the GFP was chosen as the first cistron in the dicistronic constructs, and the second cistron was UBI4-lacZ. (Fig. 1A). To enhance translational termination of the first cistron and to minimize reinitiation of the second one due to “leakiness” from the first cistron, we introduced an additional stop codon immediately downstream to the natural GFP stop codon. SIRES was placed as the intercistronic region, downstream to the stop codons. Three SIRES-less constructs were engineered to be used as controls. One control construct contained no intercistronic region (except for two restriction sites that accounted for “leakiness” from the first cistron, we introduced an additional stop codon). SIRES was placed as the intercistronic region, downstream to the stop codons. Three SIRES-less constructs were engineered to be used as controls. One control construct contained no intercistronic region (except for two restriction sites that accounted for “leakiness” from the first cistron into the second cistron), and this construct contained SIRES (Fig. 1A).

Fig. 2. Translation of SIRES-containing mRNA is repressed in log phase cells and induced in post-log phase cells. SUB62 cells (MFA1, lys2-801, leu2-3, 112, ura3-52, his3-200, trp1-t1 (amu)) (26, 36) carrying either the control fusion gene (left panels) or the SIRES-containing gene (right panels) were grown to SP. Samples containing equal numbers of cells were taken at the growth stages indicated at the top or bottom of each panel and analyzed as indicated. A, construct scheme. B, mRNA levels were determined by Northern blot hybridization as described previously (27, 28). Five μg of RNA were loaded per lane. To detect UBI4-lacZ mRNA, either UBI4 or ACT1 noncoding region (which was hybridized to the ACT1 sequence of the 5′-UTR) was used. Each of these probes detected the same transcript and gave similar results. Shown are the results using UBI4 as the probe. EtBr staining of the rRNAs (18 S and 25 S) is shown at the bottom to demonstrate equal loading. Amounts of UBI4-lacZ mRNA and rRNAs were quantitated by scanning the autoradiograms and the EtBr-stained bands, respectively, using ImageMaster 1D (Amersham Pharmacia Biotech). The obtained ratios of mRNA/rRNA are indicated. These values were normalized to that of the log sample in the left panel. Note that the rRNA content per cell is reduced by 2–3-fold after the transition from log to SP (26); accordingly, the observed decrease in the calculated ratios of mRNA/rRNA is an underestimate of the actual decrease in the amount of transcript on a per cell basis. Note also that both the mRNA and the rRNAs shown in the SG lane migrated faster than their counterparts in the other lanes, probably due to a salt effect in these samples. C, protein level was determined by Western analysis as described previously (37, 38). Anti βgal antibodies (Promega) were used at 1:2000 dilution. Twenty μg of protein was loaded per lane. Rpb4, whose level is little changed along the growth to SP (37), is shown to demonstrate equal loading. Enzymatic activity of Rpb4 was done as described (31, 39). Growth stages: Log, logarithmic phase; SG, slow growth phase; SP1, 1 day in SP; SP4, 4 days in SP.
in post-log phases (Fig. 3A, lanes 14–17). We suppose that the decrease of GFP translation in post-log phases is stronger than the observed decrease in its steady state level because GFP, like most proteins (29), is highly stable in SP. The different expression behavior of the two cistrons indicates that, after the diauxic shift, the translation of the two cistrons is independently controlled.

The expression pattern of the SIRES-containing construct was compared with that of the control constructs. In cells carrying the spacerless dicistronic mRNA no βgal was detected, indicating that following termination of the GFP translation no reinitiation occurred. It was demonstrated previously that reinitiation can occur in yeast and that the spacing between the sites of termination and initiation is important (2). We therefore examined expression of the second cistron in cells carrying the other control constructs. Indeed, the inclusion of either a 104- or a 282-nucleotide spacer in the dicistronic mRNA permitted little synthesis of βgal during log phase, which was detected only after overexposure (lanes 6 and 10 in Fig. 3B, lower panel). This βgal synthesis is most probably the result of translational reinitiation. In post-log phases, the level of the βgal expressed by these control dicistronic mRNAs declined (lanes 7–9 and 11–13 in Fig. 3B, lower panel). This is in contrast with the increase in the level of βgal expressed by the SIRES-containing mRNA (lanes 15–17 in Fig. 3B). In summary, the differences between the expression pattern of the control constructs and the expression pattern of the SIRES-containing construct indicates that the induced synthesis of the second cistron is SIRES-specific. We consider the possibility that the SIRES-mediated translation of the second cistron is due to reinitiation as unlikely because it is much higher than the translation of the second cistron of the control mRNAs. Furthermore, the observation that following the diauxic shift the translation of the two cistrons that are separated by SIRES is independently controlled (i.e. one is repressed and the other one is induced) strongly argues against the reinitiation possibility.

Interestingly, the level of βgal produced by the SIRES-containing mRNA during log phase was even lower than that produced by the mRNA containing the control spacers (compare lane 14 with lanes 6 and 10, in Fig. 3B, lower panel). This result is consistent with our suggestion that SIRES, which is not active as IRES in log phase, impedes the scanning-mediated reinitiation process.

We then examined whether the SIRES-containing construct gave rise to one transcript that encompasses both GFP and UBI4-lacZ genes. Transcripts were analyzed by Northern blot hybridization using either GFP or lacZ as probes. Fig. 4 shows that the SIRES-containing dicistronic gene produced a dicistronic transcript, which migrated slower than the monocistronic transcript (compare lane 1 with lanes 2–5) and hybridized with both probes. The dicistronic message was by far the most prominent RNA species that was detected by both probes. In addition to this band, a few minor bands were detected after overexposure. These RNA species could not have promoted translation of the second cistron by an end-initiated scanning process for the following reasons: (i) no RNA species was detected that hybridized only (or preferentially) with lacZ but not with the GFP probe, indicating that no transcript has a 5' end located downstream of the GFP open reading frame that might promote a cap-dependent translation of the second cistron; and (ii) the intensities of the minor bands were not increased after the shift from log to post-log phases. Because SIRES mediates an increase in the expression of the second cistron in post-log phases (see Fig. 3), this increase cannot be attributed to one of the minor bands. We conclude that the cells can support an induced translation of the second cistron during post-log phases only by internal initiation of translation (see also the note in the legend to Fig. 4).

Taken together, the results shown in Figs. 3 and 4 indicate that translation of the two cistrons that are separated by SIRES is controlled differently. Thus, following the shift from log to post-log phases, when the translation of the first cistron decreases, translation of the second cistron increases. We conclude that, after cells exit the log phase, SIRES promotes the initiation of the second cistron translation from an internal site in the mRNA. Little or no internal initiation can be detected in growing cells.

Previous experiments failed to identify IRESes in vegetatively growing yeast (see introduction). The lack of success of finding IRESes in dividing cells, taken together with our results that the capacity to recognize an IRES is found in starved cells, raises the possibility that putative IRESes are not recognized in optimally growing yeast cells but rather in starved, or otherwise stressed, cells. Cumulative data from several laboratories suggest that IRESes are best recognized when the main initiation pathway is compromised. The most remarkable examples are the observations that the activities of picornaviral IRESes (3) and BiP IRES (4, 5) are enhanced as a result of the inactivation of the cap-dependent mechanism. Similarly, when yeast encounter starvation, the overall translation declines by more than 2 orders of magnitude (29, 34). We propose that a capacity to recognize putative natural SIRESes has evolved in yeast to provide a means to escape the general loss of the cap-dependent translation capacity, and that SIRES is fortuitously recognized by this machinery. Thus, putative natural SIRESes are likely to render the translation of some mRNAs, encoding proteins that are important for surviving starvation, independent of the main initiation pathway. SIRES can be utilized in the future to isolate factors that are capable of identifying IRESes during starvation. Identifying these factors may also help with the identification of natural SIRESes.
UTR sequences as IRESes (15). However, natural IRESes have not been identified in living yeast cells. This discrepancy suggests that, for the identification of IRESes, the currently available in vitro systems might be irrelevant to the biological systems. Alternatively, it is possible that the capacity to recognize IRESes exists also in log phase yeast cells, and the in vitro system reliably detects this capacity. However, this capacity is repressed in dividing cells, either because the cap-recognizing machinery competes very efficiently with the IRES-recognizing machinery or because dividing cells express specific repressor(s), or because of both possibilities. According to this view, one or more of these repressing features is lost in the in vitro system.

Starvation-induced increase of translation has been previously demonstrated in the case of GCN4 (review in Ref. 35). In a series of studies, Hinnebusch and his co-workers (35) have shown that induction of GCN4 translation in response to amino acid or purine deprivation is mediated by four short open reading frames (uORFs) in the leader of its mRNA. The uORFs inhibit the GCN4 translation in nonstarved cells by restricting the progression of the scanning ribosomes through the leader. Upon starvation, the scanning ribosomes bypass the most inhibitory uORF, uORF4, and repression is partially relieved, leading to an increased translation of the GCN4 ORF (35). Apparently, the starvation-induced up-regulation of GCN4 differs from that mediated by SIRES. First, up-regulation of GCN4 translation results from a derepression mechanism that is imposed by the uORFs, whereas the SIRES-mediated translation seems to be governed by activation. Second, up-regulation of GCN4 mRNA translation is cap-dependent, whereas that mediated by SIRES is not. We propose that during starvation, when the general cap-dependent translation is repressed (29, 34), there are at least two types of mechanisms that mediate the translation of a small repertoire of mRNAs whose products are important for coping with starvation.

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