Étude des éléments impliqués dans le transport et la régulation traductionnelle de l'ARNm *ASH1* chez la levure *Saccharomyces cerevisiae*.

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Département de Biochimie
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Mémoire présenté à la Faculté des études supérieures
En vue de l'obtention du grade de Maîtrise ès Science en Biochimie

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Présenté par :

Guillaume POIRIER

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Résumé

La localisation asymétrique d'ARNm dans le cytoplasme est un mécanisme utilisé par plusieurs types de cellules pour contrôler l'expression de protéines dans l'espace et dans le temps. Nous étudions l'ARNm *ASH1*, qui est localisé à l'extrémité du bourgeon de la levure *Saccharomyces cerevisiae* lors de l'anaphase, afin que la protéine Ash1 inhibe le changement de type sexuel dans la levure fille. Avec l'aide d'éléments de localisation situés en grande partie dans la région codante de l'ARNm *ASH1*, ce transcrit est transporté de la levure mère vers la levure fille en se déplaçant sur les filaments d'actines. La protéine She2, qui se lie à ces éléments de localisation, interagit aussi avec plusieurs autres ARNm dans le but d'assurer leur transport vers la cellule fille. Pour l'instant, aucun motif spécifique sur ces ARNm, permettant d'expliquer leur liaison par la protéine She2, n'a encore été identifié. La première partie de ma maîtrise consistait à déterminer le motif commun ou la séquence commune entre les quatre éléments de localisation de l'ARNm *ASH1* nécessaire pour la liaison avec la protéine She2.

Pour que la localisation de la protéine Ash1 soit efficace, la traduction de son ARNm doit être contrôlée pour permettre le transport vers le bourgeon avant que la synthèse de la protéine soit terminée. La formation du complexe de localisation lié aux structures secondaires de l'ARNm *ASH1* est l'un des mécanismes permettant ce contrôle traductionnel. De plus, la présence de protéines telles que Puf6 et Khd1 augmente l'efficacité de la régulation de la traduction de l'ARNm *ASH1* durant son transport. Chacune de ces protéines diminue l'efficacité de la traduction de l'ARNm *ASH1* d'un facteur de 2 à 3 fois. La protéine Khd1 se lie à la fois à eIF4G, une protéine impliquée dans l'initiation de la traduction, et à une séquence située dans les 900 premiers nucléotides de l'ARNm *ASH1* grâce à ses domaines KH. La seconde partie de ma maîtrise consistait à déterminer la région de l'ARNm *ASH1* responsable de la liaison avec la protéine Khd1. Nous montrons que la boucle terminale de l'élément de localisation E1 contient le motif pouvant lier cette protéine.

Mots clés: Khd1p, ARNm *ASH1*, Localisation, Traduction, She2p

Abstract

mRNA localization is a mechanism used by different cell types to control the expression of specific proteins in space and time. We are using the budding yeast *Saccharomyces cerevisiae* as a model system to study the molecular determinants behind this process. In yeast, the *ASH1* mRNA localizes at the bud tip of the future daughter cell, where the Ash1 protein inhibits mating type switching. The *ASH1* mRNA contains four localization elements, mainly within the coding sequence, which are used to transport this transcript along the actin cytoskeleton. The She2 protein recognizes these fours localization elements and it is known to bind more than 20 other mRNAs. A common motif or structure in these mRNAs should be recognized by She2p in order to transport them in the daughter yeast. For now, no common RNA motif has been identified to explain the specificity of She2p recognition. The first part of my studies consisted to identify the common motif in all four localization elements of the *ASH1* mRNA, which is recognized by She2p and to compare the finding with other mRNAs which are known to bind She2p.

In order to have an efficient localization of Ash1p, it is important to regulate the translation of the *ASH1* mRNA during its transport. Secondary structures of the localization elements of the *ASH1* mRNA, which binds the transport machinery, are known to regulate the translation of this mRNA. Proteins like Puf6 and Khd1 are also involved in translational regulation. Puf6p binds the 3'UTR of *ASH1* mRNA and reduces translation efficiency. Khd1p has the ability, with his KH domains, to bind proteins involved in translation initiation and also in a region within the first 900 nucleotides of the *ASH1* mRNA . The second part of my studies was to identify the *ASH1* mRNA sequence required for the interaction with Khd1p. We found that the terminal stem-loop of the localization element E1 is recognized by Khd1p.

Key words: Khd1p, *ASH1* mRNA, Localization, Translation, She2p

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Liste des abréviations

A	Adénine
ARN	Acide ribonucléique
ARNm	Acide ribonucléique messager
ARNt	Acide ribonucléique de transfert
ASH1	Asymetric Synthesis of HO
B-Gal	Béta-galactosidase
C	Cytosine
C-terminal	Carboxy terminal
G	Guanine
GTP	Guanine triphosphate
kDa	KiloDalton
L	Lysine
MAPK	Map kinase
^{7}mGTP	7-methyl guanine triphosphate
Poly(A)	Polyadénine
T	Thymine
U	Uracile
3'UTR	Région 3' non codante
5'UTR	Région 5' non codante
Y	Tyrosine

Liste des symboles

ϕ	Acide aminé hydrophobe
α	Alpha
β	Bêta
γ	Gamma
%	Pourcentage

Remerciements

Je tiens à remercier le Dr. Pascal Chartrand pour m'avoir offert la possibilité d'effectuer ma maîtrise dans son laboratoire.

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1. INTRODUCTION

1.1 La localisation asymétrique d'ARNm dans le cytoplasme

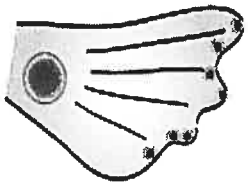
La localisation d'ARNm est un mécanisme important qui permet la ségrégation de la traduction de protéines à des endroits ciblés de la cellule. Ce mécanisme offre l'avantage de concentrer la protéine où elle est requise et permet aussi un contrôle local de la traduction de ces ARNm. Chez la levure, il a été estimé qu'environ 1% des ARNm exportés du noyau sont ciblés à des endroits spécifiques du cytoplasme : bourgeon, mitochondries, ... (**Marc *et al*, 2002**; **Shepard et al., 2003**).

1.1.1 Fonctions de la localisation d'ARNm

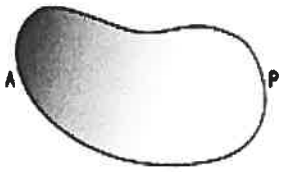
La localisation d'un ARNm à l'intérieur d'une cellule peut avoir différents buts (Figures 1 et 2). Il est possible grâce au phénomène de localisation d'ARNm d'augmenter la concentration d'une protéine particulière à un endroit spécifique de la cellule, par exemple la localisation de l'ARNm de la β-actine aux extrémités des fibroblastes afin de favoriser la motilité cellulaire (**Hill and Gunning, 1993**). Ce mécanisme permet aussi de créer un gradient de morphogènes à l'intérieur d'un ovocyte afin de favoriser le développement de l'embryon chez la Drosophile (**Salles *et al*, 1994**) ou le développement de lignées cellulaires spécialisés chez *Xenopus* (**Yisraeli *et al*, 1990**). Ce même mécanisme est observé chez la levure où la localisation de l'ARNm *ASH1* amène une inhibition du changement de type sexuel de la levure fille, créant ainsi une asymétrie entre les deux cellules issues de la mitose. Finalement, l'allongement du cône de croissance des neurones ainsi que la présence de canaux récepteurs situés dans les dendrites, nécessitent le ciblage d'ARNm (**Eberwine *et al*, 2001**). Des problèmes de développement dus à une mauvaise localisation des ARNm ou à leur traduction dérégulée pourraient mener à des maladies neurodégénératives chez l'humain, telles que le syndrome du chromosome X fragile (**Verker *et al*, 1991**).

Figure 1 : Exemples de mécanismes cellulaires utilisant la localisation d'ARNm (Kloc et al, 2002)

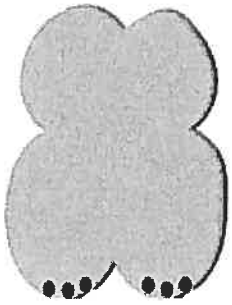
A. Haute concentration en protéine aux extrémités des fibroblastes



B. Gradient de morphogenèse de bicoïd chez *Drosophile*



C. Lignée cellulaire spécialisée chez *Xenopus*



D. Traduction localisée dans les neurones

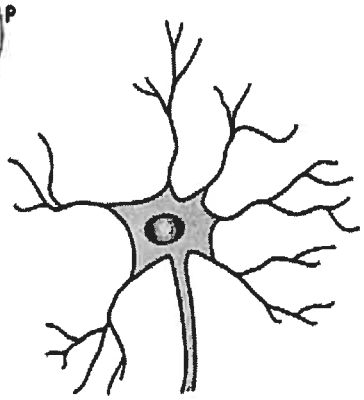
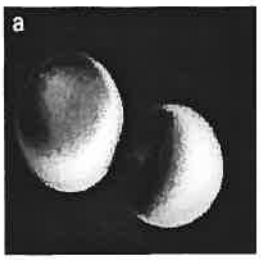
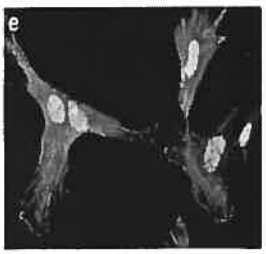


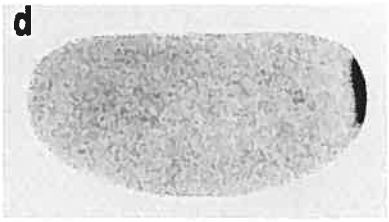
Figure 2 : ARNm localisés impliqués dans le développement et la croissance cellulaire (tiré de Kloc *et al*, 2002)



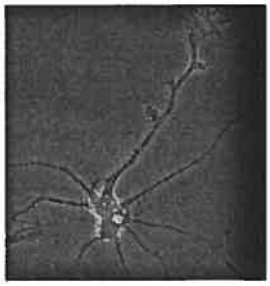
ARNm *Vg1* chez *Xenopus*



ARNm de la *ß-actine* des fibroblastes



ARNm *nanos* chez la *drosophile*



ARNm de la *ß-actine* des neurones

1.2 Mécanismes de contrôle traductionnel des ARNm localisés :

Le contrôle traductionnel des ARNm localisés est nécessaire afin de maintenir une distribution asymétrique des protéines synthétisées. Cette régulation peut se réaliser via plusieurs mécanismes. Il peut avoir lieu, entre autre, au niveau du 3'UTR de l'ARNm par la présence d'une structure secondaire, ou une séquence spécifique, qui permet le recrutement de protéines responsables de la régulation de la traduction (**Gu *et al*, 2004; Serano *et al*, 1995**). Plusieurs protéines s'associent au niveau du 5'UTR des ARNm dans le but d'initier la traduction en permettant le recrutement des sous-unités ribosomales sur les ARNm. D'autres vont inhiber l'association des protéines responsables de l'initiation de la traduction. Comme par exemple la traduction de l'ARNm de la ferritine qui est régulée par la rotéine responsable de la liaison avec le fer (IRE-BP) qui se lie à l'IRE situé au 5'UTR et empêche le recrutement des ribosomes sur l'ARNm lorsque sa traduction n'est plus requise (**Rouault *et al*, 2002**). Certains mécanismes de régulation de la traduction peuvent même survenir à l'intérieur de la séquence codante, comme c'est le cas de l'ARNm *ASH1* chez *Saccharomyces cerevisiae* (**Chartrand *et al*, 2002**). En résumé, le contrôle traductionnel requiert la présence de plusieurs protéines et chaque ARNm traduit requiert la présence de différents facteurs pour permettre l'activation ou l'inhibition de la traduction. Néanmoins, il y a des facteurs communs qui jouent un rôle dans l'initiation de la traduction des ARNm en permettant le recrutement et le bon fonctionnement des ribosomes.

1.2.1 Traduction

Plusieurs protéines ont pour fonction d'enclencher le processus d'initiation de la traduction des ARNm. Il y a d'abord les protéines responsables de la circularisation de l'ARNm pour permettre une traduction plus efficace (Figure 3A, **Sachs, 2000; Wakiyama *et al*, 2000**). La protéine PABP se lie à l'extrémité 3' de l'ARNm, au niveau de la queue de poly(A) ainsi qu'à la protéine eIF4G. Cette dernière a un rôle de protéine d'échafaudage qui permet le rapprochement de plusieurs autres protéines requises pour favoriser l'initiation de la traduction (**Sachs *et al*, 1997; Bernstein *et al*, 1989**). Il existe

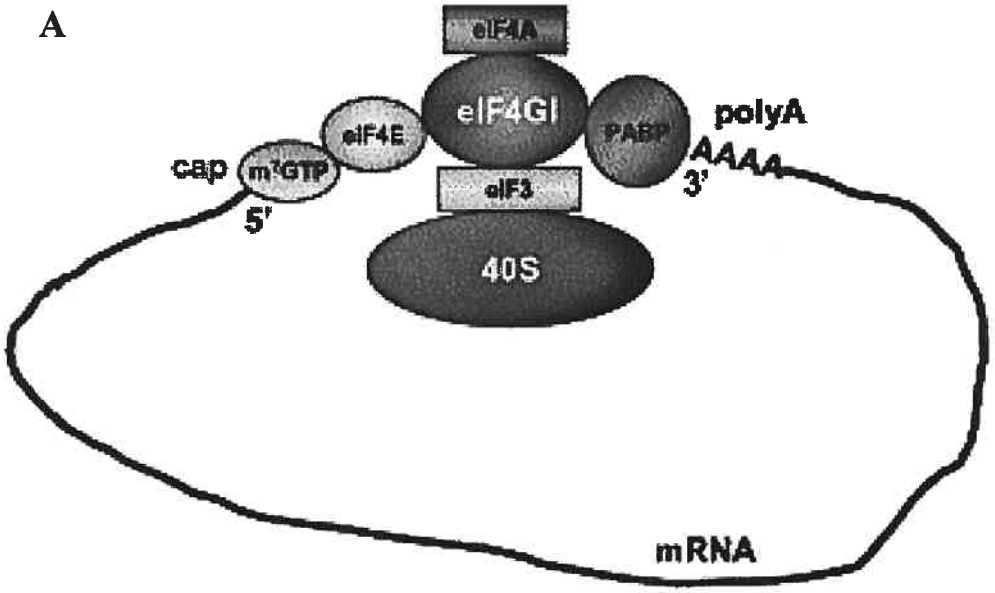
deux isoformes de la protéine eIF4G. La protéine eIF4G1 est l'isoforme la plus utilisée au niveau de la traduction et diminue l'efficacité de traduction lorsqu'elle est délété. La protéine eIF4G2 possède environ 50% d'homologie de séquence avec la protéine eIF4G1 et demeure moins utilisée par les cellules.

Du côté de l'extrémité 5' de l'ARNm se retrouve une coiffe ^{7}mGTP (**Shatkin *et al*, 1976**) reconnue par la protéine eIF4E, qui possède aussi la capacité de lier la protéine eIF4G (**Marcotrigiano *et al*, 1997**). Malgré le fait que ces trois protéines permettent la circularisation de l'ARNm, leur présence n'est pas suffisante pour optimiser la traduction. En effet, les structures secondaires dans la région 5' non codante de l'ARNm doivent être défaites pour permettre le balayage par la sous-unité 40S du ribosome et un meilleur recrutement au codon d'initiation. Les protéines eIF4A et eIF4B, associé à la protéine eIF4G, facilitent le balayage de la région 5' non-traduite par la sous-unité 40S du ribosome grâce à leur activité hélicase (**Pause and Sonenberg, 1992**). Le complexe formé entre les protéines eIF4E, eIF4G et eIF4A est nommé eIF4F. Ce complexe est suffisant pour permettre certaines traductions dont celle de la globine. (**Grifo *et al*, 1983**).

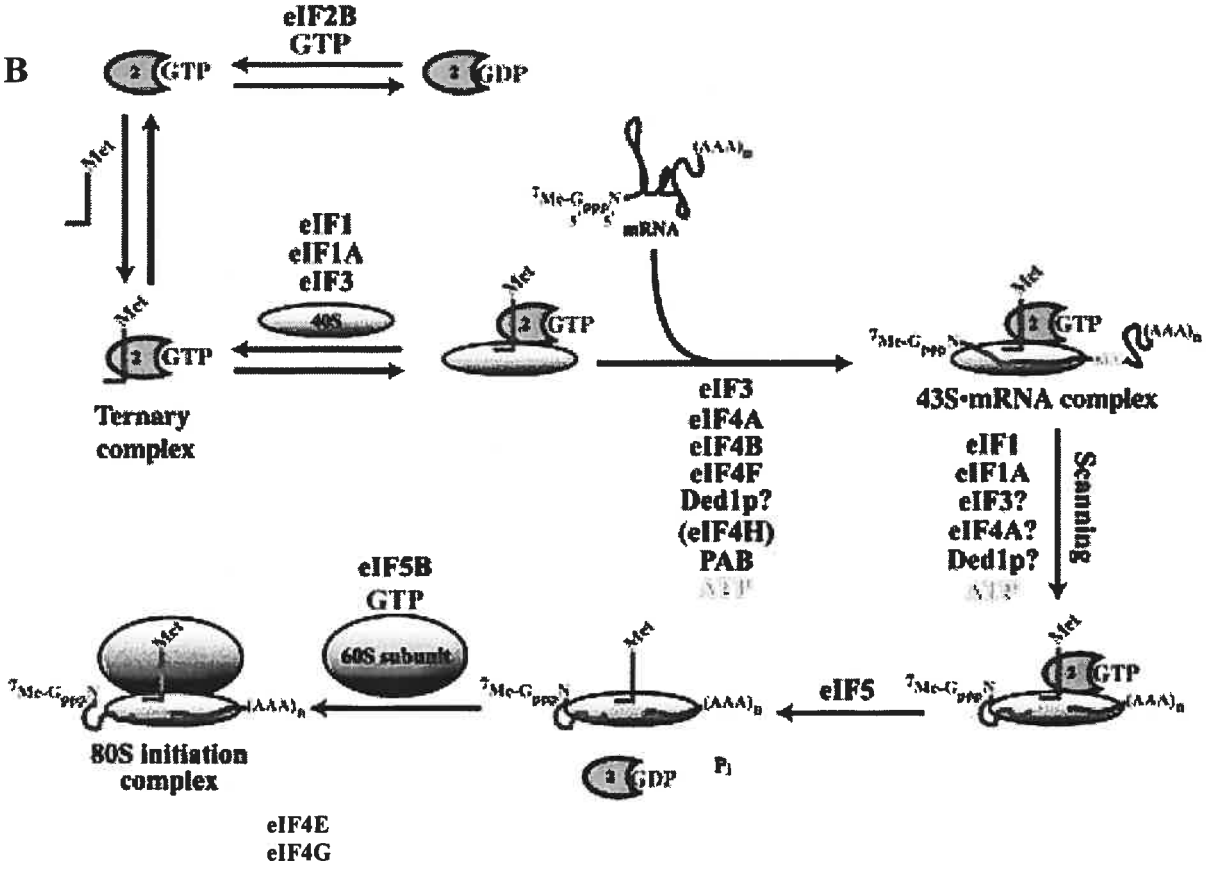
Une fois, le complexe eIF4F associé, la petite sous-unité ribosomale associée aux facteurs eIF3, eIF1A ainsi qu'à l'ARNt^{met} apporté par eIF2-GTP est en mesure de se lier à l'ARNm cible et de débuter le balayage du 5'UTR (Figure 3B, **Kapp *et al*, 2004; Maitra *et al* 1982**). Le facteur eIF3 a la fonction de recruter le complexe eIF2-GTP-ARNt^{met} sur la petite sous-unité et aussi d'empêcher le recrutement précoce de la sous-unité 60S du ribosome sur le petite sous-unité durant le balayage (**Garcia-Barrio *et al*, 1995; Maitra *et al*; 1982**). Une fois le codon d'initiation reconnu par la sous-unité 40S, la protéine eIF5 se lie à la sous-unité γ de la protéine eIF2 afin d'hydrolyser le GTP et ainsi séparer la protéine eIF2 de la sous-unité ribosomale et de conserver ARN^{met} sur le ribosome à l'aide de la protéine eIF1A (**Charkrabarti et Maitra, 1991; Chaudhuri *et al*, 1997**). La présence de la protéine eIF5 facilite aussi le recrutement de la sous-unité 60S, qui peut s'associer à la petite sous-unité 40S afin de permettre l'initiation de la traduction.

Figure 3 : Mécanisme d'initiation de la traduction d'un ARNm (A) : Association des protéines responsables de l'initiation de la traduction de l'ARNm (Déborah *et al*, 2003) (B) : Étapes menant au recrutement des ribosomes **sur un ARNm (Kapp *et al*, 2004)**

A


eIF4A
eIF4GI
eIF4E
PABP
polyA
AAAA
3'
cap
m7GTP
5'
eIF3
40S
mRNA

B


eIF2B
GTP
GTP
GDP
Met
eIF1
eIF1A
eIF3
40S
mRNA
Ternary complex
43S•mRNA complex
eIF3
eIF4A
eIF4B
eIF4F
Ded1p?
(eIF4H)
PAB
eIF1
eIF1A
eIF3?
eIF4A?
Ded1p?
Scanning
eIF5B
GTP
60S subunit
80S initiation complex
eIF5
GDP
Pi
eIF4E
eIF4G

1.2.2 Répression de la traduction

Vu sa complexité, la traduction d'un ARNm peut être régulée de différentes façons. Il est possible, par exemple, de réguler la formation de la queue de poly(A) qui est un élément essentiel à la traduction puisqu'elle permet à PABP de se lier à ce segment pour circulariser l'ARNm (**Salles *et al*, 1994; Borman *et al*, 2002**). La protéine ORB utilise cette voie pour réguler la traduction. En effet, la protéine ORB est située au pôle postérieur de l'embryon de la Drosophile dans le but d'allonger la queue de poly(A) de l'ARNm *Oskar*, requis précisément à cet endroit pour favoriser le développement de l'abdomen ainsi que la formation de cellules germinales chez la larve de Drosophile (**Chang *et al*, 1999**). De cette façon, la traduction d'*Oskar* n'est possible que dans des endroits ciblés. Afin d'éviter une traduction durant le transport de l'ARNm *Oskar* au niveau du pôle antérieur, la protéine YPS (Ypsilon-Schachtel) inhibe l'effet de la protéine ORB sur *Oskar*, ce qui cause une inhibition de la traduction (**Mansfield *et al*, 2002**). Avec ce système, ORB est assuré de demeurer au niveau du pôle postérieur. De plus, la protéine Bruno se lie au 3'UTR de l'ARNm *Oskar* afin d'inhiber sa traduction, mais sans modifier la longueur de la queue de poly(A) (**Lie *et al*,1999; Castagnetti *et al*, 2000**).

Un autre exemple chez la Drosophile est la protéine Nanos, qui forme un gradient de concentration à partir du pôle postérieur de l'embryon de la Drosophile vers le pôle antérieur. La fonction de la protéine Nanos est d'empêcher l'allongement de la queue de poly(A) de l'ARNm *Hunchback*, ce qui inhibe la traduction de cet ARNm où celle-ci n'est pas désirée (**Wreden *et al*, 1997**). Chez *Xenopus laevis*, l'élongation de la queue de poly(A) est contrôlée par la protéine (Cytoplasmic Polyadenylation-Element Binding protein) qui se lie à une séquence CPE (Cytoplasmic Polyadenylation Element) au niveau 3'UTR de l'ARNm ciblée (**Minshall *et al*, 1999**). Cette protéine a pour effet de laisser des ARNm « dormants » dans des endroits spécifiques de la cellule sans être dégradés afin de permettre une activation rapide de la traduction lors d'une stimulation, comme c'est le cas chez les neurones de l'*Aplysie* (**Wu *et al*, 1998**). L'activité de la protéine CPEB est régulée par la protéine Maskin, qui empêche la traduction des ARNm en se liant à la fois à la protéine CPEB ainsi qu'à la protéine eIF4E (Figure 4, **Stutz *et al*, 1998**). Par ces liaisons, la protéine Maskin compétitionne avec la protéine eIF4G pour la

liaison à eIF4E et empêche le recrutement des autres protéines responsables de l'initiation de la traduction. L'inhibition de l'initiation de la traduction en bloquant l'interaction entre eIF4E et eIF4G est utilisée par plusieurs facteurs de régulation traductionnelle. Comme par exemple, la protéine Cup inhibe complètement la traduction de l'ARNm *Oskar* localisé au niveau du pôle postérieur jusqu'au stade 8 du développement de la Drosophile (Figure 5) Le mode d'action de la protéine Cup est d'interagir avec la protéine eIF4E à l'aide de deux sites de reconnaissance, dont l'un des sites fait partie de la séquence Y-X-X-X-X-L-ϕ retrouvée chez eIF4G et eIF4EBP (**Mader *et al*, 1995; Nelson *et al*, 2004**). Une autre approche est de compétitionner avec eIF4E pour la liaison à la coiffe ^{7}mGTP. La protéine humaine homologue à eIF4E (heIF4EHP) se lie à la fois avec la protéine Bicoid à la coiffe ^{7}mGTP de l'ARNm *Caudal* afin d'être en mesure d'inhiber la traduction de cet ARNm en empêchant le recrutement des protéines initiatrices eIF4E et eIF4G (**Cho *et al*, 2005**).

Finalement, plusieurs virus qui utilisent le système de traduction de la cellule hôte pour leur prolifération, détruisent une partie de la protéine eIF4G afin d'empêcher celle-ci de recruter les protéines eIF4E ou PABP selon le type de virus (**Piron *et al*, 1998**). De cette façon, les virus favorisent la traduction d'ARNm contenant une structure IRES qui rend possible la traduction des ARNm non cappés, dont la grande majorité des ARNm viraux (**Nomoto *et al*, 1976**).

Figure 4 : Régulation de la traduction des ARNm par la liaison de CPEB sur le CPE situé dans le 3'UTR de l'ARNm ciblée (Groisman *et al*, 2001)

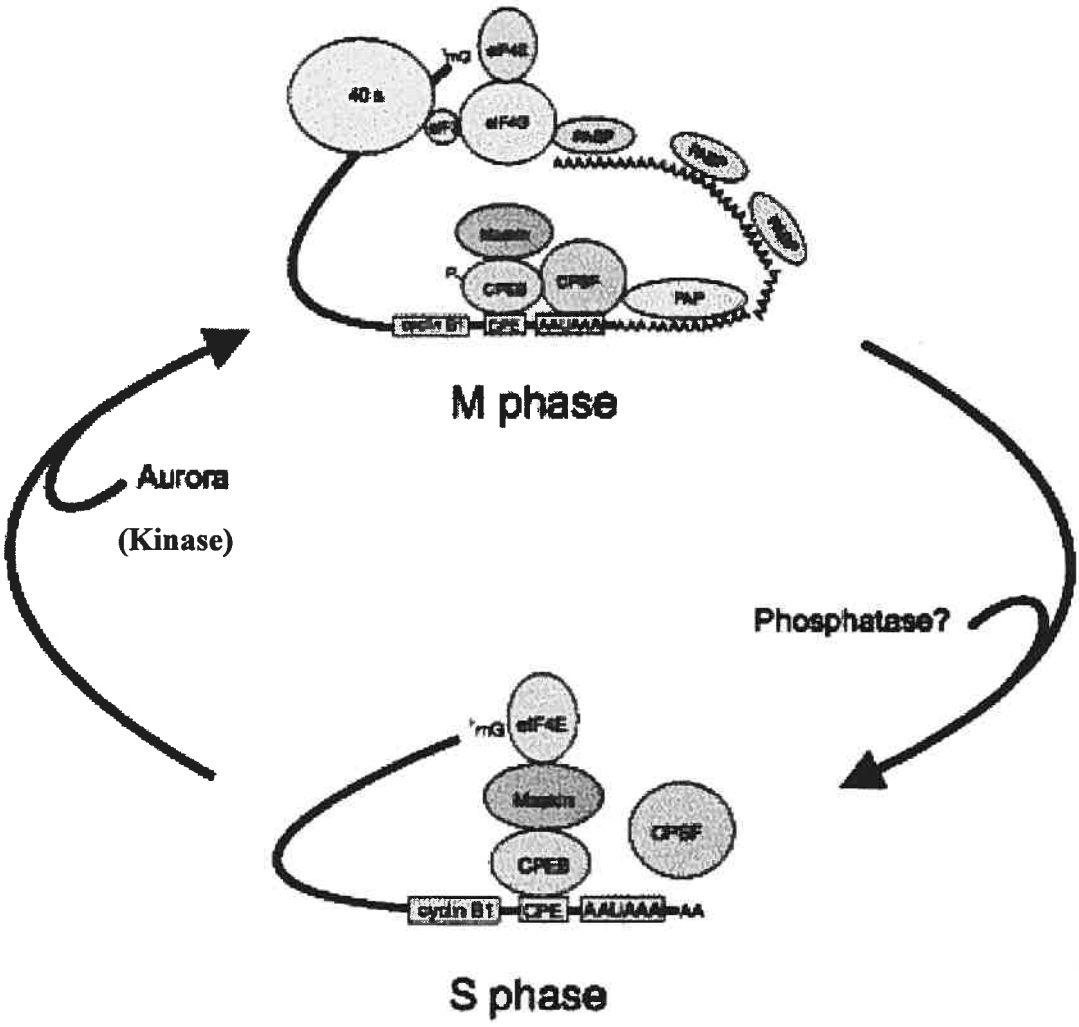
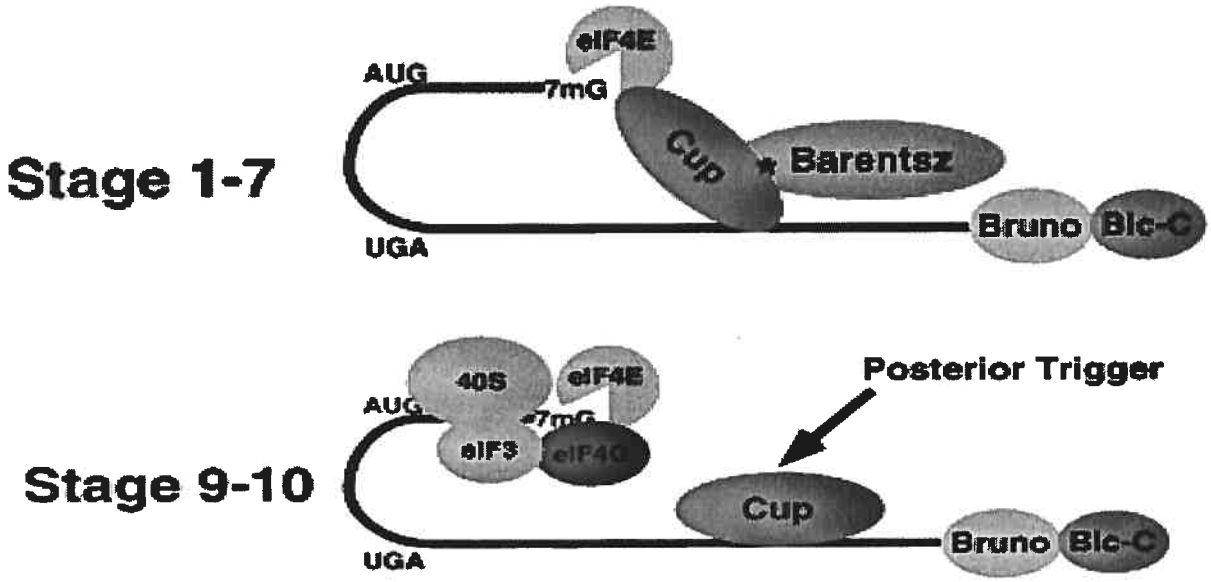

40s
eIF4E
eIF4G
PABP
CPEB
CPSF
PAP
cyclin B1
CPE
AAUAAA
M phase
Aurora
(Kinase)
Phosphatase?
Maskin
S phase

Figure 5 : Contrôle de la traduction de l'ARNm *Oskar* par la protéine Cup lors du


Stage 1-7
eIF4E
AUG
7mG
Cup
Barentsz
Bruno
Ble-C
UGA
Stage 9-10
40S
eIF4E
AUG
7mG
eIF3
eIF4G
Posterior Trigger
Cup
Bruno
Ble-C
UGA

1.3 ARNm localisés chez *Saccharomyces cerevisiae*

Dans notre laboratoire, nous utilisons la levure *Saccharomyces cerevisiae* comme système modèle pour étudier les mécanismes moléculaires requis avec de permettre les processus de transport et de régulation traductionnelle des ARNm. Grâce aux puissants outils génétiques, de biologie moléculaire et de biologie cellulaire développés pour cet organisme, plusieurs des facteurs impliqués dans ces processus ont déjà été identifiés. De plus, plusieurs ARNm chez *Saccharomyces cerevisiae* sont localisés au bourgeon des levures en mitose (Table I; **Sheppard *et al*, 2003**).

Tableau I : Exemples d'ARNm localisés au bourgeon chez *Saccharomyces cerevisiae*

ARNm	Fonction de la protéine	Référence
BRO1	Coordination de la déubiquitation dans les corps multivésiculaires	Odorizzi *et al*, 2003 Luthtala *et al*, 2004
CLB2	Cycline permettant la terminaison de la division Cellulaire	Hood *et al*, 2000
DNM1	Conservation de la morphologie des mitochondries	Bleazard *et al*, 1999
		Haut du formulaire Kovacech *et al*, 1996
EGT2	Impliquée dans la séparation des cellules en G1	Bas du formulaire
ERG2	Impliquée dans la synthèse de l'ergostérol	Arthington *et al*, 1991 Ashman *et al*, 1991
IST2	Protéine membranaire impliquée dans l'équilibre salin de la levure	Takizawa *et al*, 2000
MID2	Récepteur Membranaire	Philip *et al*, 2001
TPO1	Transporteur polyamine	Tomitori *et al*, 1999
WSC2	Récepteur Membranaire	Philip *et al*, 2001
KSS1	Impliquée dans la croissance des filaments	Madhani *et al*, 1997
LCB1	Synthèse des sphingolipides	Nagiec *et al*, 1994
MET4	Activateur de la traduction	Thomas *et al, 1992*

1.3.1 L'ARNm *IST2*

L'ARNm *IST2* code pour un transporteur membranaire et contrôle la concentration des ions dans le milieu intracellulaire (**Tomitori *et al*, 1999**). L'ARNm *IST2* est localisé au bourgeon de la levure à l'aide des mêmes protéines She qui localisent l'ARNm *ASH1* (**Takizawa *et al*, 2000**). Il a été démontré que l'absence du réticulum endoplasmique périnucléaire dans la levure fille n'est pas un obstacle au niveau de traduction de l'ARNm *IST2* localisé à cet endroit, ce qui suggère que l'ARNm *IST2* n'emploie pas le chemin standard de traduction via le réticulum endoplasmique et fait partie des ARNm qui sont localisés avant d'être traduits (**Jüschke *et al*, 2004; Prinz *et al*, 2000**). En effet, il a été suggéré que l'ARNm *IST2* n'est en mesure d'être traduit que lorsqu'il est bien localisé dans le milieu cytoplasmique de la levure fille. La protéine Ist2 synthétisée est par la suite retenue par l'anneau de septine situé dans le col de la levure en bourgeonnement afin de permettre sa rétention dans la membrane de la cellule fille (**Takizawa *et al*, 2000; Jüschke *et al*, 2004**).

1.4 L'ARNm *ASH1* comme modèle d'étude

Notre laboratoire étudie l'ARNm *ASH1*, qui est localisé à l'extrémité du bourgeon de la levure en mitose (Figure 6, **Long *et al*, 1997**). La fonction de la localisation de cet ARNm est d'empêcher le changement de type sexuel de la levure fille afin de faciliter la formation d'une levure diploïde par la fusion des levures mère et levures fille haploïdes de types sexuels opposés (**Bobola *et al*, 1996; Sil *et al*, 1996**). La protéine Ash1 une fois traduite, se déplace vers le noyau de la levure fille, où elle inhibe la transcription du gène codant pour l'endonucléase Ho, qui est le facteur responsable du changement de type sexuel (**Sil *et al*, 1996**). Pour remplir sa fonction, la protéine Ash1 inhibe le recrutement du complexe Swi/Snf au niveau du promoteur du gène *HO* empêchant ainsi sa transcription (Figure 7, **Cosma *et al*, 1999**).

La localisation des ARNm à des régions précises de la cellule nécessite la présence d'éléments de localisation dans leurs séquences (**Serano *et al*, 1995**). Dans le cas de l'ARNm *ASH1,* quatre éléments de localisation appelés E1, E2A E2B et E3, sont responsables de la localisation de cet ARNm au bourgeon des levures (Figure 8, **Chartrand *et al*, 1999**). La particularité de ces éléments de localisation est que les trois premiers (E1, E2A et E2B) se situent à l'intérieur de la région codante de l'ARNm, alors que le quatrième englobe la fin de la séquence codante, le codon stop, et le début du 3'UTR (**Gonzalez *et al*, 1999; Chartrand *et al*, 1999**). Ces quatre éléments de localisation se lient à la protéine She2 qui est un facteur essentiel pour la localisation de l'ARNm *ASH1.* Chacun des éléments adopte une structure secondaire en forme de tige-boucle (Figure 8). Par contre, aucune similarité de séquence parmi les quatre éléments de localisation n'a été établie à ce jour. Ainsi, les bases moléculaires de l'interaction entre la protéine She2 et les quatre éléments de localisation de l'ARNm ne sont toujours pas élucidées.

Figure 6 : Localisation de l'ARNm *ASH1* chez la levure *Saccharomyces cerevisiae* et distribution asymétrique de la protéine Ash1 (Long *et al*, 1997)

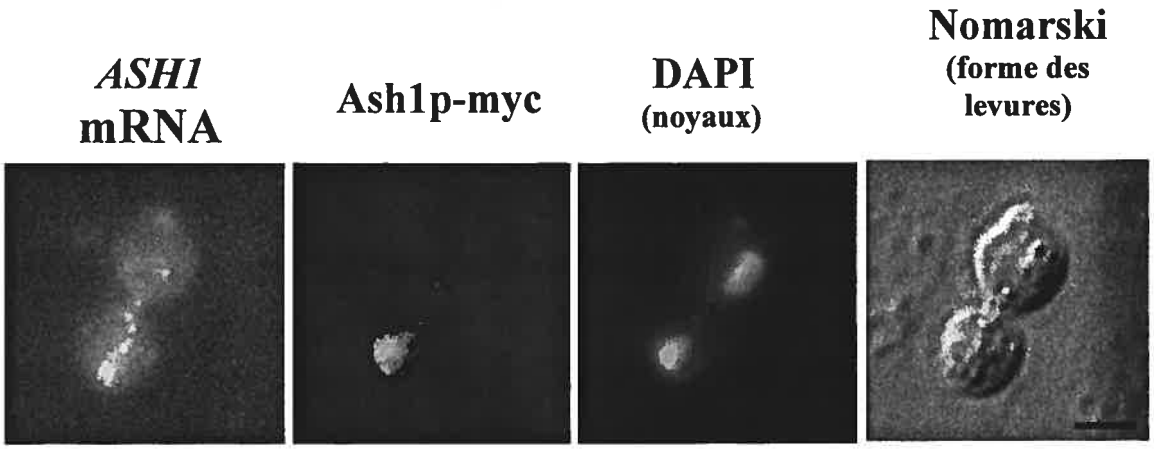

ASH1 mRNA
Ash1p-myc
DAPI (noyaux)
Nomarski (forme des levures)

Figure 7 : Inhibition du changement de type sexuel de la levure fille provoquée par la protéine Ash1

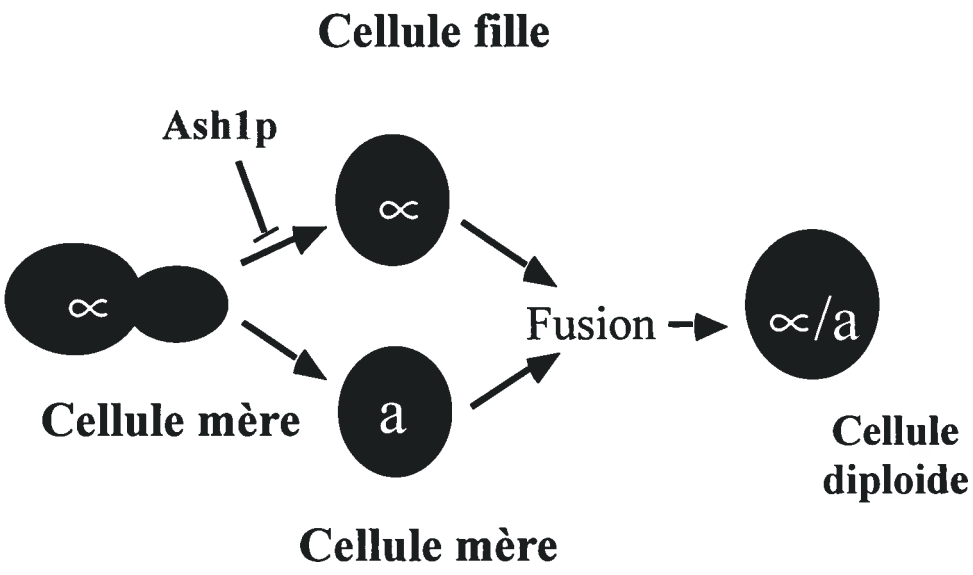
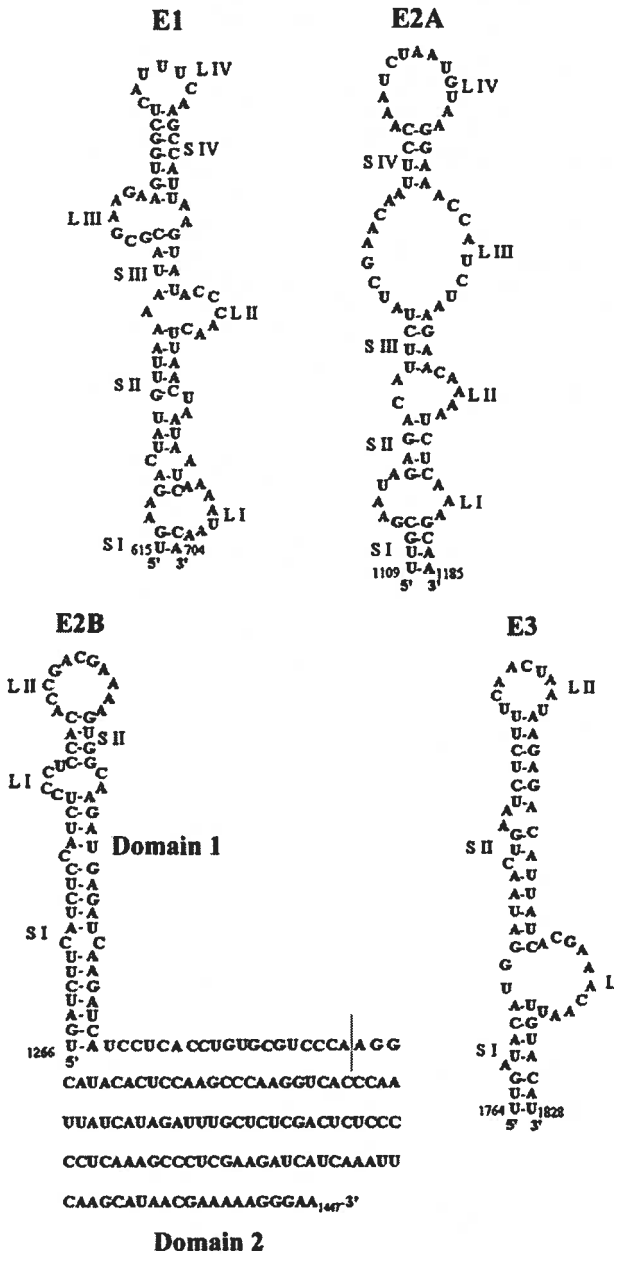

Cellule fille
Ash1p
∝
∝
Fusion
∝/a
Cellule mère
a
Cellule diploide
Cellule mère

Figure 8 : Structures secondaires des éléments de localisation E1, E2A, E2B, E3 de l'ARNm *ASH1* (Olivier *et al*, 2005)

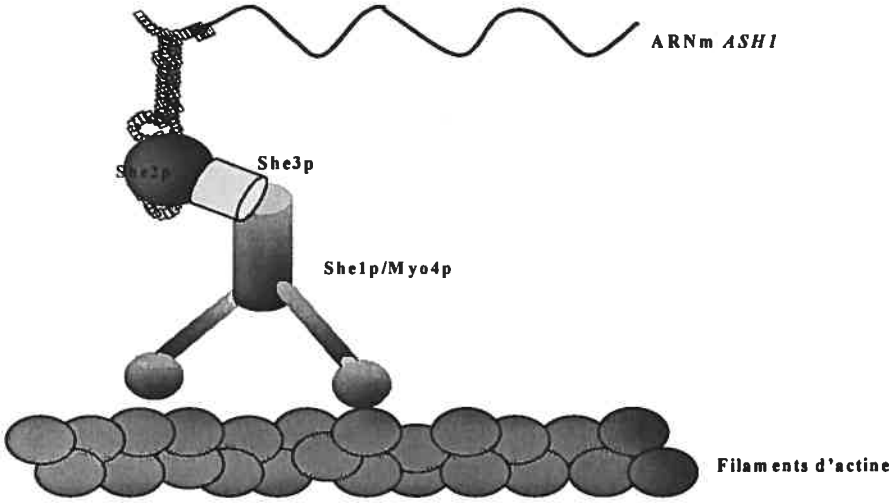

E1
E2A
E2B
E3
Domain 1
Domain 2
CAUACACUCCAAGCCCAAGGUCACCCAA
UUAUCAUAGAUUUGCUCUCGACUCUCCC
CCUCAAAGCCCUCGAAGAUCAUCAAAUU
CAAGCAUAACGAAAAAGGGAA

1.5 Les protéines She

La localisation de l'ARNm *ASH1* est possible grâce aux protéines du groupe She, signifiant « Swi5-dependent *HO* Expression » (**Jansen *et al*, 1996**), qui possèdent chacune des fonctions complémentaires. Sur les cinq protéines She, trois sont reconnues pour leur capacité à former un « locasome » (un complexe de localisation) pour permettre le transport de l'ARNm *ASH1* vers le bourgeon (Figure 9, **Chartrand *et al*, 2001**).La première protéine du locasome est la protéine She1 (ou Myo4) qui est une myosine de type V. Elle se déplace sur les filaments d'actine polarisés de la levure mère vers la levure fille (**Long *et al*, 1997; Takizawa *et al*, 2000, Münchow *et al*, 1999**). La protéine She3 fait le pont entre la protéine She1 par son extrémité N-terminale et la protéine She2 en la liant à l'aide de son extrémité C-terminale (**Böhl *et al*, 2000**). She1 et She3 jouent aussi d'autres rôles puisque sans ces deux protéines, la localisation de certaines organelles de la levure mère, comme par exemple le réticulum endoplasmique, vers la levure fille n'est plus possible (**Estrada *et al*, 2003**). La troisième protéine du complexe, She2, est une petite protéine de 28 kDa qui a la capacité de se lier aux quatre éléments de localisation de l'ARNm *ASH1* (**Long *et al*, 2000; Böhl *et al*, 2000**). Les autres protéines du groupe She jouent un rôle indirect dans la localisation de l'ARNm *ASH1* puisqu'elles ne sont pas incluses dans le locasome. En effet, la protéine She4, qui possède la capacité de se lier aux myosines de classes I et V (soit Myo2 et Myo4 respectivement), semble effectuer un rôle en tant que chaperone pour le repliement de ces myosines (**Toi *et al*, 2003; Wesche *et al*, 2003**). Finalement, la protéine She5 possède un rôle au niveau de l'organisation et la polarisation du cytosquelette d'actine (**Beach *et al*, 1999, Miller *et al*, 1999**). Malgré que les fonctions des protéines She4 et She5 demandent des études plus approfondies, nous savons que la délétion de l'une ou l'autre de ces protéines entraîne des effets néfastes pour la localisation de l'ARNm *ASH1* et la polarité de l'actine (**Miller *et al*, 1999; Toi *et al*, 2003; Chartrand *et al*, 2001**).

Un modèle général a été proposé par Chartrand *et al* (2001) au niveau du mécanisme de la localisation de l'ARNm *ASH1* par le locasome (Figure 10). L'ARNm *ASH1* nouvellement transcrit est reconnu par la protéine She2 qui se lie aux quatre éléments de localisation. Par contre, il n'a pas encore été démontré que la protéine She2 se lie à l'ARNm *ASH1* dans le noyau ou dans le cytoplasme puisqu'elle est présente dans ces deux compartiments. La protéine She3 s'associe par la suite à la protéine She2 afin d'accomplir sa fonction de pont entre les protéines She1 et She2. En dernier lieu, la protéine She1 se lie à la protéine She3 et complète le locasome. L'ARNm *ASH1* est transporté le long des filaments d'actines vers l'extrémité de la levure fille. Une fois rendu à la membrane, l'ARNm est ancré à la paroi cellulaire et la conformation du locasome est modifiée (**Gonzalez *et al*, 1999; Gonsalvez *et al*, 2004**). La protéine Ash1 est par la suite synthétisée afin de se rendre dans le noyau de la levure fille pour inhiber l'expression du gène HO (**Bobola *et al*, 1996; Sil *et al*, 1996**).

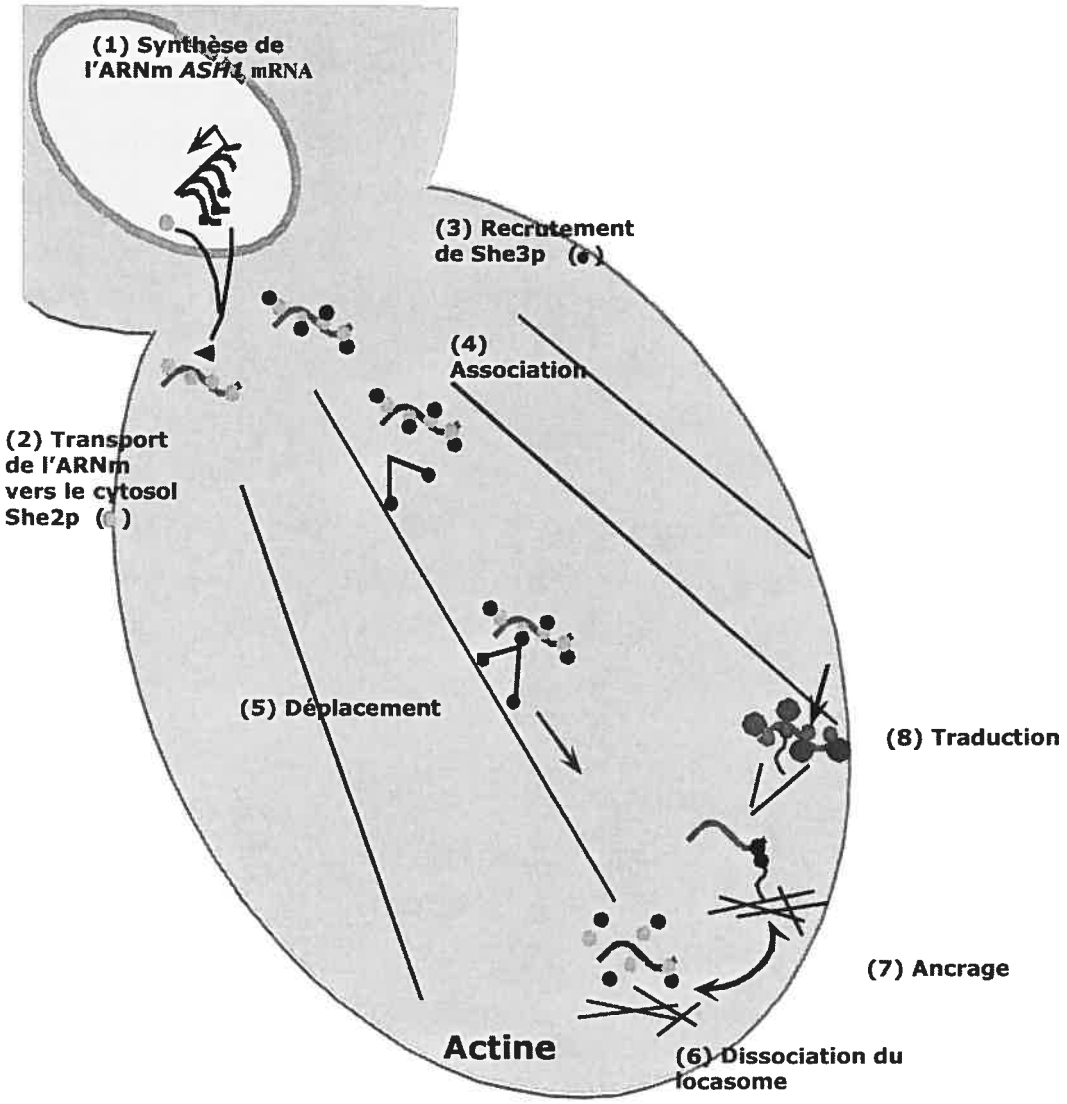
Figure 9 : Schéma de l'association des protéines She1-3 à l'ARNm *ASH1* (Chartrand *et al*, 2001)


ARNm *ASH1*
She3p
She1p/Myo4p
Filaments d'actine

1.5.1 Propriétés de She2p

L'interaction de la protéine She2 avec des éléments de localisation qui ne possèdent aucune similarité de séquence soulève des questions à propos de ses propriétés de liaison à l'ARN. Sa structure tridimensionnelle a été récemment résolue et montre que She2p est composé de 5 hélices α et forme un homodimère. (**Niessing *et al*, 2004**). Son mécanisme d'interaction avec les ARNm est encore discuté. Selon des études récentes, il semblerait que la protéine She2 se dimérise afin de pouvoir se lier avec l'ARNm *ASH1* (**Gonsalvez *et al.*, 2003**). En effet, des études de mutagenèse ont montré que plusieurs acides aminés basiques (Arg et Lys) sont essentiels à son interaction avec l'ARN (**Gonsalvez *et al.*, 2003**). La structure tridimensionnelle des protéines She2 démontre qu'une fois liée, ces acides aminés sont regroupés et forment un nouveau motif qui permet l'interaction des protéines She2 à l'ARN (**Niessing *et al.*, 2004**). Étant donné que la protéine She2 possède la capacité de se lier à plus d'une vingtaine d'ARNm différents (**Sheppard *et al*, 2003**), il serait possible de croire que l'ensemble de ces ARNm ciblés possèdent un motif commun qui serait reconnu par cette protéine. Pour notre premier projet, nous avons concentré nos recherches sur les quatre éléments de localisation de l'ARNm *ASH1* en espérant trouver un motif commun entre chacun d'eux et rechercher ce motif chez d'autres ARNm pouvant être liés par la protéine She2.

Figure 10 : Mécanisme de transport et de traduction de l'ARNm *ASH1* à l'extrémité du bourgeon (Chartrand *et al*, 2001)


(1) Synthèse de l'ARNm *ASH1* mRNA
(3) Recrutement de She3p
(4) Association
(2) Transport de l'ARNm vers le cytosol She2p
(5) Déplacement
(8) Traduction
(7) Ancrage
Actine
(6) Dissociation du locasome

1.6 Traduction et localisation de l'ARNm *ASH1*

Le transport de l'ARNm *ASH1* au niveau du bourgeon n'est pas le seul mécanisme requis pour permettre une bonne distribution asymétrique de la protéine Ash1. En effet, en dépit du fait que la localisation de l'ARNm à l'extrémité du bourgeon s'effectue en 2 minutes (**Bertrand *et al*, 1998**), il serait toujours possible que l'ARNm *ASH1* soit traduit durant son transport si la traduction n'était pas régulée. Il a été montré que le déplacement des structures secondaires des éléments de localisation vers la région 3' UTR de l'ARNm *ASH1* ne modifie pas le taux d'efficacité de localisation de l'ARNm vers la membrane de la levure fille en bourgeonnement (**Chartrand *et al.*, 2002**). Par contre, cette modification a un impact au niveau de la distribution de la protéine Ash1. En effet, le transfert des éléments de localisation vers la région 3'UTR de l'ARNm *ASH1* réduit de façon importante la distribution asymétrique de la protéine Ash1. Ces résultats ont montré que la présence des éléments de localisation peut remplir deux rôles à la fois. Tout d'abord, par sa liaison avec le complexe protéique She1, 2 et 3, l'ARNm *ASH1* est assuré d'être transporté vers le bourgeon. Par la suite, leur présence dans la région codante diminuerait l'efficacité de traduction durant le transport et augmenterait le taux de distribution asymétrique de la protéine Ash1 (**Chartrand *et al*, 2002**).

1.6.1 Contrôle traductionnel de l'ARNm *ASH1*

Le ralentissement de la traduction par les structures secondaires des éléments de localisation n'est pas le seul facteur pouvant réduire la synthèse de la protéine Ash1. D'autres protéines comme Puf6, Scp160 ainsi que Khd1 participeraient possiblement à la régulation de la traduction, et indirectement, de la localisation de la protéine Ash1 (**Long *et al*, 2001; Irie *et al*, 2002; Gu *et al*, 2004; Lang *et al* 2000**). La protéine Puf6, nouvellement découverte, lie l'élément E3 dans le 3'UTR de l'ARNm *ASH1* et est localisée au bourgeon de la levure au même endroit que cet ARNm. La délétion du gène *PUF6* entraîne une diminution de l'efficacité de localisation de l'ARNm *ASH1* de l'ordre de 35% (passant de 87% à 53% de distribution asymétrique) ainsi qu'une augmentation de la traduction de ce transcrit (**Gu *et al*, 2004**). La perte d'efficacité de localisation de la protéine Ash1 est due à une absence de la protéine Puf6 ainsi que par le transfert des

éléments de localisation vers la région 3'UTR de l'ARNm. Par contre, l'ajout d'une structure secondaire dans la région 5'UTR de l'ARNm *ASH1* compense la perte d'efficacité obsersée par le déplacement des éléments de localisation vers le 3'UTR. Ce type de structure est connu pour réduire le niveau de traduction d'un ARNm (**Vega Laso *et al.*, 1993**), ce qui suggère encore une fois que le protéine Puf6 et les structures des éléments de localisation possèdent un rôle au niveau du contrôle de la traduction (**Gu *et al*, 2004; Chartrand *et al*, 2002**). La protéine Scp160 a aussi un effet sur la localisation de l'ARNm *ASH1*, avec une efficacité de localisation passant de 87% à 23 % dans une souche *scp160*, mais son rôle au niveau de l'ARNm *ASH1* semble être plutôt général que spécifique à un seul ARNm. En effet, la protéine Scp160 possède une fonction importante au niveau de l'association des polyribosomes sur plusieurs ARNm (**Li *et al*, 2004**). Elle peut aussi se lier entre autre aux ARN ribosomaux et former de larges complexes ribonucléoprotéique (**Weber *et al*, 1997; Lang *et al*, 2000; Mendelsohn *et al*, 2003**)

1.6.2 Protéines à domaines KH

Le contrôle de la traduction de l'ARNm *ASH1* nécessite la présence d'une protéine à domaines KH appelé Khd1p (**Irie *et al*, 2002**). Les protéines à domaines KH ont la capacité de se lier à l'ARNm simple brin par leur configuration β_1-α_1-α_2-β_2-β_3-α_3 sans toutefois avoir une séquence particulière qui pourrait être identifiée comme protéine à domaines KH (**Siomi *et al*, 1993; Paziewska *et al*, 2004**). Les ARN pouvant interagir avec les protéines à domaines KH semblent par contre contenir une séquence consensus UCAY qui peut varier légèrement entre différentes protéines, mais l'insertion de guanine dans la séquence consensus réduit de façon importante la force d'interaction entre la protéine et l'ARNm ciblé (Tableau II, **Paziewska *et al*, 2004; Musunuru *et al*, 2004**). Chaque protéine à domaines KH possède des fonctions particulières, il est donc difficile de comparer les fonctions de ces protéines par une autre façon que leur capacité d'interaction à l'ARNm simple brin. La protéine hnRNPK par exemple peut se lier au poly(rC) à l'aide de son troisième domaine KH et est impliquée au niveau de la stabilisation et la régulation de plusieurs type d'ARNm (**Dejgaard et Leffers 1996;**

Kiledjian *et al*, 1995; Ostarek *et al*, 1997). La protéine Nova, qui possède trois domaines KH, serait impliquée au niveau de la régulation de l'épissage des ARNm localisés dans le cerveau (**Jensen *et al*, 2000; Ule *et al*, 2003**). La protéine ZBP1 (zipcode- binding protein) localise l'ARNm de la β-actine et permet une bonne motilité de la cellule (**Farina *et al*, 2003**). La protéine KSRP peut, grâce à son domaine KH, accélérer le processus de dégradation de l'ARNm en recrutant la machinerie de dégradation au niveau de la séquence riche en AU (AU rich element; **Gherzi *et al*, 2004**).

Notre protéine d'intérêt, Khd1, est une protéine à trois domaines KH qui aurait un rôle au niveau de l'ancrage et de la traduction de l'ARNm *ASH1*. Les recherches de Irie et al., publiées en 2002, constituent le seul article à ce jour qui traite de cette protéine. Selon eux, la protéine Khd1 interagit avec l'ARNm *ASH1* dans un endroit situé dans les 900 premiers nucléotides, dont l'élément de localisation E1 fait partie intégrante. Tout comme la protéine Puf6, une surexpression de la protéine Khd1 entraîne une importante diminution de la synthèse de la protéine Ash1, ce qui suggère que les protéines Puf6 et Khd1 jouent un rôle au niveau de la régulation de la traduction de l'ARNm *ASH1* (**Irie *et al*, 2002; Gu *et al*, 2004**). De plus, des études de purification de complexes protéiques à large échelle, effectuées par Gavin *et al* (**2002**), ont permis d'établir que la protéine Khd1 se retrouve associée *in vivo* avec un groupe de protéines impliquées dans l'initiation de la traduction : eIF4E, eIF4G1, eIF4G2, CBP80, ainsi que la sous-unité bêta de eIF2.

Tableau II : Comparaison des séquences d'ARN reconnues par diverses protéines à domaines KH

Protéine	Séquence	Référence
hnRNPK	UCCCU/UCAUC	Paziewska *et al. 2004*
ZBP1	YCACCC	Farina *et al. 2003*
Nova	UCAY	Jensen *et al.* 2000 Musunuru et al. 2004
Era	GAUCA	Manjuli *et al.* 2005

1.7 Contexte et objectifs

L'ARNm *ASH1* chez *Saccharomyces cerevisiae* est l'un des 22 ARNm liés par la protéine She2 et transporté vers le bourgeon de la levure. A cet endroit, la protéine Ash1 est traduite pour ensuite se localiser dans le noyau de la levure fille dans le but d'inhiber la transcription du gène de l'endonucléase HO. Ce gène a pour fonction de cliver l'ADN double brin pour permettre un remodelage de l'ADN causant ainsi le changement de type sexuel (**Oshima et Takano, 1971**). Son inhibition permet d'obtenir deux levures de types sexuels différents à proximité pour permettre la formation rapide d'une levure diploïde en cas de millieu non propice à la croissance. Pour réaliser sa fonction adéquatement, la traduction de l'ARNm *ASH1* doit être régulée durant son déplacement. La protéine She2 doit se lier à ces ARNm à l'aide d'une structure secondaire ou d'une séquence commune. Étant donné que l'ARNm *ASH1* possède plusieurs éléments de localisation présents, il serait plus facile d'utiliser cet ARNm pour trouver un motif commun de liaison à la protéine She2. Mon premier objectif fut d'identifier le motif d'ARN présent dans les éléments de localisation de l'ARNm *ASH1* qui est reconnu par She2p.

La protéine Khd1 quant à elle révèle d'intéressantes propriétés au niveau du contrôle de la traduction durant le transport de l'ARNm *ASH1*. L'article publié par Irie *et al* en 2002 semble suggérer que la protéine Khd1 serait liée à cet ARNm dans une région où un élément de localisation serait présent, l'élément E1. De plus, le fait que la protéine Khd1 se retrouve associée àdes protéines responsables de l'initiation de la traduction suggère un rôle dans le mécanisme de régulation traductionnelle. Mon objectif dans ce projet fut de déterminer à quel endroit se lie la protéine Khd1 sur l'ARNm *ASH1* et de déterminer son effet sur la traduction de ce transcrit. Combinés à des études sur la liaison

de la protéine Khd1 aux protéines initiatrices de la traduction, nos résultats proposent un nouveau mécanisme de régulation traductionnelle.

1.8 Hypothèses de travail

1) Malgré que les éléments de localisation de l'ARNm *ASH1* possèdent des séquences complètement différentes, ils forment toutefois des structures en tige-boucle. Il serait envisageable que la protéine She2 reconnaisse quelques nucléotides spécifiques exposé à des endroits précis du à la conformation aux structures tridimensionnelles des éléments de localisation. Pour vérifier cette hypothèse, un schéma des structures secondaires des quatre éléments de localisation doit être validé pour déterminer les nucléotides ne formant pas de tige. Ces nucléotides seraient fort possiblement situés dans les boucles internes des structures en tige-boucle des éléments de localisation.

2) Un autre facteur impliqué dans la régulation de l'ARNm *ASH1* est la protéine Khd1 (**Irie *et al*, 2002**). Étant donné qu'il a été démontré que la protéine Khd1 se lie à l'ARNm *ASH1* au niveau des 900 premières de bases, il serait préférable de trouver des structures secondaires connues comprises dans cette région. L'élément de localisation E1 se retrouve à l'intérieur de cette séquence cible et est connu pour son importance au niveau de la régulation de la traduction. Notre hypothèse est donc que la protéine Khd1 reconnaisse l'ARNm *ASH1* en se liant directement l'élément de localisation E1, mais à un endroit qui n'entre pas en compétition avec la protéine She2. De plus, Khd1 interagirait avec une des protéines responsables de l'initiation de la traduction. Ces interactions amèneraient une diminution spécifique de la traduction de cet ARNm.

2. Contributions

2.1 Olivier *et al.* MCB 2005

Durant ma maîtrise, j'ai participé à l'article d'Olivier *et al* publié dans « Molecular and Cellular Biology » en juin 2005. Cet article explore le motif d'ARN reconnu par la protéine She2 qui se retrouve dans les éléments de localisation de l'ARNm *ASH1*. Nous avons aussi identifié ce motif chez deux autres ARNm localisés au bourgeon de la levure.

- J'ai contribué à la Figure 1 de l'article, qui montre que la protéine She2 interagit de façon indépendante avec chacun des quatre éléments de localisation de l'ARNm *ASH1* dans un essai de triple-hybride chez la levure.

- J'ai contribué à la Figure Supplémentaire 1, qui montre que le signal d'interaction dans le triple-hybride est plus efficace en présence du domaine C-terminal de She3 en fusion avec le domaine d'activation de Gal4 plutôt que l'utilisation d'une protéine de fusion Gal4-She2.

- J'ai participé à la Figure 4, où les clones positifs issus du criblage de banques de mutants des éléments de localisation issus de l'essai de triple hybride ont été séquencés. J'ai travaillé sur l'élément de localisation E2B-D1. Cette expérience a permis de comparer les nucléotides essentiels entre chacun des éléments de localisation afin d'identifier ceux qui sont conservés entre les éléments, ce qui donnerait un indice pour découvrir les nucléotides requis pour l'interaction avec la protéine She2.

- J'ai participé à la Figure 6, qui montre que la taille de la tige entre les deux cytosines essentielles pour l'interaction avec la protéine She2 est aussi très importante pour cette interaction. Pour ce faire, il a fallu augmenter le nombre de nucléotides

présents dans la tige entre les deux cytosines essentielles. J'ai participé à cette figure en mutant les tiges des éléments de localisation E1, E2A et E3.

2.2 Paquin *et al*

Ma seconde participation à un article a été dans celui de Paquin *et al* qui est sur le point d'être soumis. Cet article propose un nouveau modèle de régulation de la traduction de l'ARNm *ASH1* à l'aide de la protéine Khd1 qui se lie à la fois à l'élément de localisation E1 et à l'extrémité C-terminal de la protéine eIF4G.

- J'ai contribué à la Figure 1 de l'article, qui montre que la protéine Khd1 diminue l'efficacité de régulation de la traduction de l'ARNm *ASH1* en se liant à l'élément de localisation E1.

- J'ai produit la Figure Supplémentaire 1, qui montre qu'une certaine quantité de protéines Khd1 est associée aux ribosomes.

- J'ai participé à la Figure 2, qui montre que la protéine Khd1 se lie à l'extrémité de l'élément E1 de façon directe. L'analyse des essais luciférases que j'ai effectués à démontré que l'interaction entre l'ARNm *ASH1* et la protéine Khd1 est perdue lorsque l'élément E1 est muté. J'ai aussi produit le clone E1-M6 utilisé pour les essais de retardement sur gel qui ont montré que l'interaction entre Khd1 et l'élément E1 est directe.

- J'ai produit la Figure 3, qui montre que la protéine Khd1 diminue la quantité de ribosomes présents sur l'ARNm *ASH1*.

3. Identification of a conserved RNA motif essential for She2p recognition and mRNA localization to the yeast

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ABSTRACT

In *Saccharomyces cerevisiae*, over twenty mRNAs localize to the bud tip of daughter cells, playing roles in processes as different as mating-type switching and plasma membrane targeting. The localization of these transcripts depends on interactions between cis-acting localization element(s) or "zipcodes", and the RNA-binding protein She2p. While previous studies identified four different localization elements in the bud-localized *ASH1* mRNA, the main determinants for She2p recognition are still unknown. To investigate the RNA-binding specificity of She2p, we isolated She2p-binding RNAs by *in vivo* selection from libraries of partially randomized *ASH1* localization elements. The RNAs isolated contained a similar loop-stem-loop structure with a highly conserved CGA triplet in one loop and a single conserved cytosine in the other loop. Mutating these conserved nucleotides or the stem separating them resulted in the loss of She2p binding and in the delocalization of a reporter mRNA. Using this information, we identified the same RNA motif in two other known bud-localized transcripts, suggesting that this motif is conserved among bud-localized mRNAs. These results show that mRNAs with zipcodes lacking primary sequence similarity can rely on a few conserved nucleotides properly oriented in their three-dimensional structure in order to be recognized by the same localization machinery.

INTRODUCTION

The cytoplasmic transport and localization of mRNAs is a mechanism used by various eukaryotic polarized cells to control the spatio-temporal expression of specific proteins. This process has been observed and widely studied in *Drosophila* and *Xenopus* embryos (2, 19, 24), neurons (22), fibroblasts (31) and yeast (26, 41). The mRNAs sorted by this mechanism contain in their sequences specific localization elements or "zipcodes" which are recognized by the cell localization machinery (23). These localization elements are usually found in the 3'untranslated region (3'UTR) of the mRNAs, but some have been found in the 5'UTR (43) or within the coding sequence of transcripts (7, 14, 36).

The budding yeast *Saccharomyces cerevisiae* has emerged as a model system for understanding the molecular basis behind cytoplasmic mRNA transport and localization (9). In this organism, more than twenty transcripts have been shown to be transported and localized to the bud tip of yeast cells via the same pathway (38, 40). The core components of the yeast mRNA localization machinery have been identified and were shown to form a complex called the "locasome", that includes She2p, She3p and Myo4p (3, 4, 25, 29, 42). The current model for the assembly of this localization complex suggests that the RNA-binding protein She2p recognizes the mRNAs to be localized and recruits the type V myosin Myo4p via their common interaction with the adapter protein She3p (4, 25). This ribo-nucleoprotein (RNP) complex is then transported along the actin cytoskeleton and anchored at the bud tip where translation occurs. Of these mRNAs, the yeast *ASH1* mRNA was the first identified and the most studied (26, 41). The localization of the *ASH1* mRNA to the distal tip of daughter cells during anaphase is responsible for the asymmetric sorting of Ash1p to the daughter cell nucleus (8, 26, 41) where it represses mating-type switching (17, 39). The *ASH1* mRNA contains four localization elements which are essential for the proper localization of this transcript (7, 8, 14). Of these localization elements, three (E1, E2A and E2B) are located within the coding region of the *ASH1* mRNA whereas the remaining element (E3) includes the

termination codon and is located primarily within the *ASH1* 3'-UTR. These localization elements were predicted to form RNA secondary structures containing stem-loops (7, 8, 14). Each single element is sufficient to localize a reporter mRNA at the bud of yeast cells (7, 14) and can promote the formation of the locasome complex (7). Other studies have also shown that the four elements interact independently and specifically with the RNA-binding protein She2p (4, 25). Deletion of the *SHE2* gene lead to the delocalization of the *ASH1* mRNA (26, 41) and *SHE2* is essential for the localization function of each one of the four *ASH1* localization elements (25). The X-ray structure of She2p, which has been recently published, shows that this protein forms a symmetric homodimer and contains two RNA-binding domains rich in basic residues (30). Niessing et al. also found that one RNA molecule binds per She2p homodimer and suggested that the RNA molecule would bind to both RNA-binding domains (30). To date, no consensus sequence and/or structure have been identified among the four elements which may act as an RNA recognition motif for the She2 protein. Since the transport and localization of mRNAs at the bud tip of yeast cells has been shown to be She2p dependent (38, 40), the identification of the specific RNA motif recognized by She2p is crucial for understanding the specificity of this pathway.

Here we report the identification of a three-dimensional motif conserved within the four *ASH1* mRNA localization elements and in two other bud-localized transcripts. Using an *in vivo* screen of partially randomized *ASH1* RNA localization elements, we have identified the nucleotides essential for She2p recognition. From these screens, a motif constituted of a loop-stem-loop structure with a highly conserved cytosine in one loop and a CGA triplet in the other loop was found to be present in the *ASH1* localization elements. Mutation of the conserved CGA triplet and the single cytosine in this motif resulted in the delocalization of a reporter mRNA and in the loss of binding to She2p. A computer search and analysis of this motif revealed a conserved three-dimensional fold in the *ASH1* localization elements, especially in the distance separating the two cytosines. Elongation of the stem between the two cytosines showed that the distance separating these two nucleotides and/or their spatial orientation were important for the recognition of the localization elements by the yeast mRNA localization machinery. Using this information, we identified the same motif in two other bud-localized mRNAs, *IST2* and

YMR171c, confirming the importance of this motif for the proper sorting of yeast bud-localized mRNAs. Altogether, these data show that the recognition of different mRNAs by a localization machinery can rely on a few conserved nucleotides properly oriented in the three-dimensional structure of these mRNAs.

RESULTS

A yeast three-hybrid assay to study the interaction between She2p and the *ASH1* mRNA localization elements.

Extensive mutagenesis had previously allowed the mapping of the four *ASH1* mRNA localization elements and had shown that each element adopts a specific stem-loop structure (Figure 1A) (7, 8, 14). In order to identify the motif(s) bound by She2p in each localization element RNA, we first set up a reliable system to study the interaction between She2p and the four *ASH1* localization elements *in vivo* using the yeast three-hybrid assay. In this assay, the formation of an RNA-protein complex leads to the activation of reporter genes (*lacZ* and *HIS3*), therefore large libraries of mutations on the RNA can be screened and selected for maintenance of the RNA-protein interaction (35). A fusion of She2p with the *GAL4* activation domain has already been shown to interact with the E3 localization element in the yeast three-hybrid assay in a previous study (13). However, we could only obtain a weak but specific interaction between this fusion protein and the four *ASH1* mRNA localization elements (data not shown; see Supplementary Material section). In order to increase the sensitivity of our assay, we used a variation of this three-hybrid assay, developed by Long et al. (25), where the C-terminal domain of She3p was fused to the *GAL4* activation domain (She3-Cterm-AD). The C-terminal domain of She3p has been shown previously to bind She2p directly *in vitro* and *in vivo* (4, 25). The idea behind this system is that the She3-Cterm-AD protein recruits the endogenous She2 protein, whose interaction with a hybrid RNA containing a

localization element RNA fused to two MS2 binding sites leads to the recruitment of the MS2-LexA DNA binding domain fusion protein. The formation of this "four hybrid" complex results in the activation of the reporter genes. This approach has been successfully used previously to characterize the interaction between She2p and the localization element E3 (25). As expected, using the C-terminal domain of She3p fused to the *GAL4* activation domain in the three-hybrid assay, we observed a strong interaction with all four *ASH1* mRNA localization elements (Figure 1B). Interestingly, we found that only the domain 1 of the localization element E2B (E2B-D1) interacted with She2p, whereas the domain 2 (E2B-D2) did not (Figure 1B). Since both domains of E2B are important for its localization function (7), the domain 2 may be involved in another aspect of the localization process. These interactions were dependent on the endogenous She2p since the same assay in a *she2* strain lead to the complete loss of β-galactosidase expression, as reported previously (25) (Figure 1B). Overall, these results are in agreement with previous data that showed that She2p interacts with the four *ASH1* localization elements *in vivo* (4, 25).

To confirm that She2p binds directly to the four localization elements, we used an electrophoretic mobility shift assay (EMSA) to test the binding of purified recombinant GST-She2 protein to the RNA localization elements *in vitro*. Previous *in vitro* studies had mostly focused on the binding of GST-She2p to the element E3 RNA using either GST-She2p pull-down (4), EMSA (4) or cross-linking (13, 25), but little had been done to confirm a direct interaction between She2p and the other three elements. Since this information is crucial for our investigation, we decided to complement these studies and focus only on the binding of GST-She2p to the localization elements E1, E2A and E2B-D1 RNAs.

As shown in Figure 2, these three RNA localization elements could form a complex with recombinant GST-She2p *in vitro*. While the E2B-D1 RNA with GST-She2p leads to the formation of only one main shifted complex, a second shift was observed for the element E2A and, at a lower yield, with the element E1 (Figure 2A and B). Since She2p acts as a dimer (30), the different shifts observed may correspond to the formation of RNA-protein complexes with a 1:1 or 2:1 ratio of the RNA zipcode with the

GST-She2p dimer. GST-She2p bound these RNAs with low affinity (in the micromolar range), but the complexes formed were saturable at high concentration of protein (Figure 2A). These complexes were specific for She2p since GST alone did not bind the localization elements (Figure 2C and data not shown). Moreover, the IRE (Iron Response Element) RNA, which also folds in a stem-loop structure (1), interacted only weakly with GST-She2p (Figure 2C), showing that the complex was specific for the *ASH1* localization elements. In order to confirm that the observed shifts were caused by the formation of a protein-RNA complex, we performed competition assays with a 5x, 50x and 500x excess of unlabelled wild-type localization element RNA. As shown in Figure 2B, these competitions lead to the loss of the RNA shift, indicating that this complex was formed by a GST-She2p:RNA species. Unlike what had been reported recently (13), we did not observe the formation of a base-paired RNA duplex between the labelled and the unlabelled RNA. We also found that an excess of mutated E2B-D1 localization element RNA competed the formation of the complex more poorly than the wild-type RNA (Figure 2B). However, mutations in other elements competed as efficiently as the wild-type RNAs (data not shown). GST-She2p was still able to bind to the mutated RNAs, possibly because of the low affinity of GST-She2p for RNA and the high concentration of protein used in these assays. Therefore we could not use this assay to detect mutations that disrupt the formation of this protein:RNA complex.

Identification of the She2p-binding domain in each *ASH1* localization element.

In order to identify which domain(s) of the localization elements were essential for binding She2p, we cloned several mutants of each *ASH1* mRNA localization element in the pIIIA/MS2-2 vector and tested their interaction with She2p in the yeast three-hybrid assay. Since the same localization element mutants had been previously tested for their mRNA localization function (7, 8), we looked for a correlation between the loss of She2p binding and the loss of mRNA localization function for each localization element. A similar study had already been done on the E3 element and showed that the internal loop and the upper stem of this RNA (see Figure 1A) were both essential for She2p binding and for the localization function of this element (25). We therefore used mutants of the localization elements E1, E2A and E2B-D1 that were previously found to have lost

their localization function (7, 8) and tested them for She2p binding. As shown in Figure 3, disruption of specific stems and loops in each localization element resulted in the complete loss of β-galactosidase expression and growth on -his selection medium. To test for the importance of the stems in the binding of She2p to these RNAs, we restored the predicted stems using compensatory mutations. As expected, these compensatory mutations allowed the binding of She2p to near wild-type levels and restored the localization function of the elements, suggesting that the stems, but not their sequences, are essential for She2p recognition (Figure 3). Altogether, these results showed a direct correlation between She2p binding and the localization function of these elements. Moreover, this analysis supported the predicted secondary structures for each localization element previously established using FISH (7, 8, 14).

Overall, in each localization element RNA, the specific region or domain previously found to be important for its localization function were also essential for She2p binding. These domains included two or three stems, and two asymmetric internal loops: stems II, III, IV and internal loops II and III for E1; stems II, III and internal loops II and III for E2A; stems I, II and loops I and II for E2B-D1 (see Figure 1A). Only in the E2B-D1 element was a terminal loop shown to be important for She2p binding. An exception is E3, where only the upper stem (stem II) and the internal loop (loop I) were found to be part of the She2p binding domain (25).

A set of specific nucleotides defines a conserved She2p-binding motif in each *ASH1* mRNA localization element.

Since the domain recognized by She2p in each localization element was constituted of asymmetric loops, we wanted to determine which nucleotides in these loops might be conserved between the four localization elements and might be part of a She2p-binding RNA motif. Using PCR and degenerated primers, we produced libraries for the four *ASH1* localization elements in which the sequences of the internal loops were partially randomized, with 40 to 50% of mutation at each position depending on the

localization element (see Figure 4A, underlined nucleotides). We did not mutate the nucleotides in the stems of these RNAs since it was the base pairs and not the sequences of these stems that were found to be determinant for She2p binding (see Figure 3). These libraries of partially randomized localization element RNAs were used in our yeast three-hybrid screen in order to identify the localization element "variants" that still interacted with She2p; which lead to the growth of yeast colonies on a –ura-leu-his medium with 3-AT. For each library, 40 or so positive clones were isolated, their pIIIA/MS2-2 plasmid (which expressed the localization element RNA) was purified and sequenced. In order to confirm the interaction of these localization element variants with She2p and eliminate the false positives, they were re-transformed into the yeast strain and tested for their β-galactosidase expression in the three-hybrid system. All the variants isolated, sequenced and tested for their β-galactosidase activity are listed in Tables III to VII of the Supplementary Material section. Overall, most of the variants had a β-galactosidase activity similar or superior to the wild-type localization element.

For each localization element RNA, we selected 5-6 variants in order to study their localization capabilities. These variants were chosen for the high number of mutations in their sequences and/or their high β-galactosidase expression in the three-hybrid assay (Table 1). Each variant was inserted at the 3'end of the *lacZ* reporter gene and the localization of this reporter mRNA was determined by FISH. As shown in Figure 4B and Table 1, all the variants of the four *ASH1* localization elements were able to localize the *lacZ* reporter mRNA like the wild-type localization elements. The localization element variants isolated from our three-hybrid assay were thus fully functional localization elements. An analysis of the sequences obtained revealed a certain number of highly conserved nucleotides (i.e present in >95% of the variants isolated) in each localization element RNA. These nucleotides are highlighted in the localization element structures in Figure 4A. Whereas the nucleotides in some loops were highly conserved between variants, like loop I of the E2A element, other loops, like the internal loop of E3 had only one conserved nucleotide, a C (Figure 4A). Looking at the nucleotides common between the four localization elements, we found a CGA sequence highly conserved in 3 of the 4 elements: E1, E2A and E2B (solid boxes in Figure 4A). Interestingly, while the E3 element also has a CGA sequence in its loop I

(marked by a solid box in Figure 4A), the only highly conserved nucleotide is the C of the CGA sequence. Another nucleotide highly conserved between these elements is a single C which is always present in a loop separated from the CGA sequence by a four base-pair stem (doted boxes in Figure 4A). We also observed a C about five base-pairs above the loop I of the E3 element (marked by an asterisk in Figure 4A), suggesting that these cytosines may be involved in She2p binding.

Mutation of the highly conserved nucleotides in the RNA motif resulted in loss of both She2p binding and localization function. Analysis of the sequences obtained by *in vivo* selection revealed a possible consensus She2p-binding RNA motif which contains two highly conserved determinants: a CGA triplet and a single cytosine, both in a terminal or an internal loop, and separated by a four base-pair stem. These two determinants were conserved in >95% of the sequences obtained for the E1, E2A and E2B localization elements (see Tables III to VII in the Supplementary Material section). Moreover, the four base-pairs stem separating these two determinants was essential for She2p binding in each of the three localization element (see Figure 3). In order to explore the importance of the CGA triplet and the single cytosine for She2p binding, we created point mutations by replacing the single cytosine and the cytosine of the CGA triplet by uracil or adenine, and tested the binding of the She2 protein to these mutants using the three-hybrid assay. As shown in Figure 5A, mutation of the two cytosines in the E1, E2A and E2B localization elements resulted in loss of the interaction between She2p and the element RNAs, suggesting that these nucleotides are essential for She2p binding. In the case of the E3 localization element, only one highly conserved cytosine was identified in the *in vivo* selection experiment. Once mutated (mutant E3-M15), the interaction with She2p was lost (see Figure 5A). Another cytosine is located in the five base-pairs stem above the internal loop of E3. This cytosine is bulged out of the stem (Figure 5A). This pattern is reminiscent of the two cytosines separated by a four base-pairs stem observed in the localization elements E1, E2A and E2B. Whereas the upper stem of the E3 element had been previously mutated, this cytosine was always maintained in all the mutants (7), so its importance in the function of this localization element was not tested. We replaced this cytosine by a uracil (mutant E3-M16) and tested the interaction of this mutated element E3 RNA with She2p in the three-hybrid

assay. Interestingly, this mutation resulted in the loss of interaction with She2p (Figure 5A).

In order to verify that these mutations affected the localization function of the elements, we inserted all the mutants at the 3'end of the *lacZ* reporter gene and the localization of this reporter mRNA was determined by FISH. As shown in Figure 5B and C, mutation of these cytosines in the four *ASH1* localization elements resulted in the delocalization of the reporter mRNA, suggesting that these nucleotides are essential for the proper recognition of the localization elements E1, E2A, E2B and E3 by the localization machinery. To confirm that the observed defect in localization was caused by a decreased interaction between the localization element and She2p *in vivo*, we immunoprecipitated a myc-tagged She2p and tested for the presence of the *lacZ* mRNA reporter by RT-PCR. As shown in Figure 5D, we detected the presence of the *lacZ* mRNA fused to the wild-type element E1 in the precipitate of She2p-myc. However, when the *lacZ* mRNA contained E1 with a mutation in the conserved cytosines, this transcript was not detected in the immunoprecipitates, suggesting that these mutations impaired the interaction between the localization element and She2p *in vivo*.

To further confirm the results from the *in vivo* selection experiments, we also mutated the guanosine and adenosine moieties of the CGA triplet in two localization elements, E1 and E2A, and compared them to mutants in poorly conserved regions of the asymmetric loops of these localization elements. As shown in Table 2, mutagenesis of the three nucleotides of the CGA triplet and the single cytosine all resulted in the same phenotype: lost of She2p binding and delocalization of the reporter mRNA. Meanwhile, mutations in the poorly conserved nucleotides of the asymmetric loops resulted in functional localization elements.

The She2p-binding domains in the four *ASH1* localization elements adopt a similar three-dimensional fold. Out of these analyses emerged a common RNA motif, in the *ASH1* localization elements, which is recognized by the RNA-binding protein She2p. This motif consists of two loops separated by a short stem of four base-pairs, with a conserved cytosine in one loop and a conserved CGA triplet in the other loop, both on opposite strands of the RNA loop-stem-loop structure. However, we observed some

variations in this RNA motif among the localization elements. For instance, in the E3 element, the GA of the CGA triplet is not essential, the stem has five base-pairs instead of four, and one of the loop is replaced by a single bulged cytosine. Also, the number of nucleotides that separate the two cytosines from the stem varies among the elements: from zero (for E2A, E2B-D1 and E3), one (both cytosines of E1, and E3) or two (for E2A and E2B-D1) nucleotides (see Figure 5A). A rule of distance emerged from the inspection of the structures of the She2p-binding motifs: there are always 6 nucleotides between the two cytosines, suggesting that it is the distance between the two cytosines, and not the length of the stem that separate them, that is important for She2p binding. We therefore described the localization elements with the following descriptor: (number of nucleotides that separate the 5' cytosine from the stem: length of the stem: number of nucleotides that separate the stem from the 3' cytosine). For the element E3, it gives 0:5:1; for E1, 1:4:1; for E2A and E2B, 2:4:0 (Figure 6A). The sum of these three numbers is always 6. We therefore predict from these results that other She2p-binding motifs may exist with the 1:5:0 and 0:4:2 descriptors. Even though there are variations in the secondary structures of these RNAs, they may adopt a similar three-dimensional structure which is recognized by She2p.

In order to explore this possibility, we used an automated program called MC-SEARCH (P.G and F. M, unpublished results) that searches for RNA structure patterns in the tertiary structures of the PDB database. Using secondary structure descriptors of the four *ASH1* localization element She2p-binding motifs (see Figure 6A), this program identified the RNA three-dimensional structures in the PDB database that fit these descriptors. Some 925 structures were thus identified: 123 for E1, 85 for E2A/E2B-D1 and 717 for E3 (this motif is less constrained than the others, mainly because of the absence of the CGA triplet). For each structure that fit the descriptors, the distance between the 3' phosphates of the two conserved cytosines was measured. Interestingly, this distance was highly similar among all the structures, with an average distance of 28.3±0.9Å for the structures that fit the E1 descriptor, 28.0±1.0Å for the structures that fit the E2A/E2B-D1 descriptor and 28.2±0.7Å for the structures that fit the E3 descriptor. Even when the structures of different descriptors were superimposed, we observed an excellent conservation of the distance between the two cytosines (see Figure 6B). These

results suggest that the variations in the secondary structures of the localization element RNAs can still lead to similar three-dimensional folds.

In order to test this model, we increased the length of the stem separating the two cytosines by adding four additional base-pairs (around 1/3 of a helix turn) in each *ASH1* localization element RNA and measuring the impact of this modification on their interaction with She2p with the three-hybrid assay. As shown in Figure 6C, increasing the distance between the two cytosines lead to the complete loss of the interaction between the localization elements and She2p, suggesting that the distance between these two cytosines and/or their spatial orientation are important for the recognition of this motif by She2p.

Identification of the She2p-binding RNA motif in other bud-localized mRNAs.

To further confirm the relevance of the She2p-binding RNA motif for the sorting of bud-localized mRNAs, we searched for this motif in the sequences of the other known bud-localized transcripts in yeast (38). To do so, we opted for a computer-based approach, with the RNAMotif software (11) that searches for RNA primary and secondary structure patterns in a sequence database using descriptors based on the main determinants identified in the She2p-binding RNA motif (see Supplementary Material section). With this approach, we identified four potential candidates, cloned these sequences and tested them in our yeast three-hybrid assay. Of the four candidate RNA motifs, two of them were found to interact with She2p: one in the *IST2* mRNA (position 2694-2785) and one in the *YMR171c* mRNA (position 1540-1645) (Figure 7B). Both RNAs could be folded in the loop-stem-loop structure with the conserved C and CGA determinants separated by a distance of six nucleotides (see Figure 7A). Mutation of the conserved cytosines in the putative localization element from the *YMR171c* mRNA resulted in loss of interaction with She2p in the three-hybrid assay, further supporting our prediction (Figure 7C). Interestingly, both cytosines in the *YMR171c* localization element are part of a CGA triplet. Finally, to confirm the function of these putative localization elements, they were inserted at the 3'end of the *lacZ* reporter gene and the localization of this reporter mRNA was determined by FISH. As shown in Figure 7D,

the RNA motifs from the *IST2* and *YMR171c* mRNAs were able to localize a reporter mRNA to the bud tip, suggesting that both act as functional localization elements. Mutation of the conserved cytosines in the *YMR171c* localization element resulted in the loss of localization of the *lacZ* reporter mRNA. Altogether, our results show that the RNA motif identified in this study contains all the necessary determinants that provide the specificity for the recognition of bud-localized transcripts by the yeast mRNA localization machinery.

DISCUSSION

In this study, we have identified a conserved three-dimensional RNA motif, present in the four *ASH1* localization elements and in two other bud-localized transcripts, that is essential for She2p recognition and for the localization function of these elements. An *in vivo* selection approach, using partially randomized libraries of the localization element RNAs coupled with a modified yeast three-hybrid assay, lead to the identification of the nucleotides essential for She2p binding in each element. While the interactions detected by the modified three-hybrid assay are indirect (since we used the C-terminal domain of She3p as the bait), we have also shown that more direct assays (like using She2p as the bait or using RNA band-shift assays) did not provide comparable readout and sensibility to measure the effect of mutations in the *ASH1* localization elements on their interaction with She2p. This approach has been vindicated by the consistent results obtained from the screens, where a motif consisting of a CGA triplet and a single conserved cytosine in two loops separated by a short stem was found to be the only conserved feature among the localization elements. Mutation of this motif resulted in a decreased interaction with She2p and loss of the localization function of the elements. Interestingly, a distance of ~28Å between the two cytosines must be maintained in order to preserve the interaction with She2p, suggesting that three-dimensional features in these RNAs are essential for She2p recognition. However, our experimental results cannot determine if it is the distance between the cytosines and/or the spatial orientation of these residues that are important, since any insertion or deletion within the helix that separate the cytosines will affect both.

Our results suggest that the RNA motif identified contains the main specificity determinants of the She2p binding domain. These determinants are highly conserved in three of the four *ASH1* localization elements (E1, E2A and E2B). For the element E3, several features of the She2p binding RNA motif are conserved: the two essential cytosines on opposite strands, separated by a stem and at a distance of 28Å from each other. However, variations from the "classic" motif are present in this element: one of the loop is replaced by a single bulged cytosine and the GA of the CGA triplet is not essential. In this case, a particularity of this localization element; a single cytosine bulging from a stem instead of being incorporated in a loop, may result in a conformation where She2p binding becomes less dependent on the GA of the CGA triplet. Interestingly, among the variants isolated for the element E1, we found three variants with mutations in the GA of the CGA triplet (mutants 8, 26 and 43). These variants can be folded like the element E3 (with a stem of 5 to 6 base-pairs between the two conserved cytosines; data not shown), suggesting that the GA of the CGA triplet may be less important for She2p binding in some structures.

While these determinants are essential for She2p recognition, other elements around this RNA motif have been found to be important. For instance, the stems around the internal loops of the elements E1, E2A and E2B-D1 were also shown to be important for She2p binding, possibly by participating in the proper folding of these RNAs. From the randomized libraries, we also identified highly conserved nucleotides in each individual element that were not present in the other three elements (see Figure 4A). The nucleotides surrounding the highly conserved CGA triplet, present in all four localization elements, provide such example (see the elements E1 versus E2B-D1). These nucleotides may be involved in non-canonical base-pairs and may improve the accessibility of the single cytosine or of the CGA triplet for She2p binding in the particular context of a given RNA structure. Overall, our results suggest that the She2p-binding motif requires a set of conserved specificity determinants around which several nucleotide sequence combinations are tolerated only if they maintain the proper folding of this motif.

Recently, more than twenty new mRNAs have been found to be specifically localized at the bud tip of yeast cells during mitosis (38). The localization of these

mRNAs depends on Myo4p, She3p and She2p, suggesting that the localization pathway of the *ASH1* mRNA is not restricted to this transcript. No specific zipcodes have yet been characterized in any of these transcripts, but since they were identified by co-immunoprecipitation with She2p, it is highly probable that they all contain She2p-binding domains similar to the one found in the *ASH1* zipcodes. Indeed, using the She2p binding RNA motif in a computer search, we have been able to identify functional localization elements in two of these transcripts: *IST2* and *YMR171c*. Ist2p is a membrane protein targeted to the plasma membrane via a new trafficking pathway that requires the localization of its mRNA to the daughter cell cortex (21, 40). The function of *YMR171c* is still unknown. The identification of the same RNA motif in the localization elements of the bud-localized *ASH1*, *IST2* and *YMR171c* mRNAs strongly support our conclusion that this motif contains the main determinants required for their recognition by She2p.

These results raise questions about how She2p may recognize these determinants. The recent publication of the three-dimensional structure of She2p revealed that this protein acts as a homodimer and that a series of basic residues essential for the interaction between this protein and the *ASH1*mRNA localization elements are clustered in a specific region that folds as a basic helical hairpin (30). Interestingly, this region of the protein, from R44 to K57, covers a distance of around 27Å in length (data not shown), which is very close to the conserved 28Å distance between the CGA triplet and the single cytosine of the She2p-binding motif. Therefore, we can speculate that this RNA motif may dock into the basic helical hairpin, where amino acid residues at the extremities of this RNA binding motif (R43, R44, K57, K60) may interact with the conserved CGA triplet and the single cytosine of the RNA motif, while basic residues in the middle (R52 and R63) may interact with the phosphate backbone of the helix that separate the two loops. While the presence of two RNA-binding domain per homodimer would suggest that two RNA molecules could bind the homodimer (2:1 ratio), Niessing et al. have shown instead that a She2p homodimer binds only one RNA molecule (1:1 ratio) (30). They proposed that the RNA molecule arches over the upper, uncharged surface of the She2p homodimer and binds simultaneously to the RNA-binding domain of both monomers. However, our electrophoretic mobility shift assays (EMSA) suggest the presence of both 1:1 and 2:1 ratio of RNA zipcode vs She2p homodimer. The ratios observed vary with the

localization element (E2B-D1 has only one shifted species while E2A has two shifted species of equivalent intensities), and may depend on the size of the RNA zipcode used in the EMSA (the E2B-D1 RNA has 75 nucleotides while the E2A RNA has 103 nucleotides).

The low level of sequence conservation and the dependence on secondary and tertiary structure of their target RNA is a common feature found in RNA-binding proteins involved in the transport of several different mRNAs within the same cell. For instance, in *Drosophila*, the *K10*, *bcd* and *hairy* mRNAs are localized during oogenesis and in blastoderm embryos by the Egalitarian (Egl) and Bicaudal-D (BicD) localization machinery (5). The localization of these transcripts depends on zipcodes containing short stem-loop structures without sequence similarities (6, 27, 37), suggesting that structural features in these RNAs are recognized by the Egl-BicD localization pathway (6). Another example is Staufen, an RNA-binding protein containing double-stranded RNA-binding domains (dsRBD), which interacts with the localized mRNAs *bicoid*, *oskar* and *prospero* in *Drosophila*, and *BC1* and *CamKIIα* mRNAs in neurons (28). Although Staufen interacts with specific mRNAs *in vivo*, it binds non-specifically to double-stranded RNA *in vitro* (18). Our study show that mRNAs with zipcodes lacking primary sequence similarity can rely on a few conserved nucleotides properly oriented in their three-dimensional structure in order to be recognized by the same localization machinery.

MATERIAL AND METHODS

Growth media and yeast strains

Yeast cells were grown either in synthetic growth media lacking the nutrients indicated or in rich media (32). Yeast strains used in this study are listed in Table VIII of the Supplementary Material Section. Transformation was performed according to the protocol of Gietz and Schiestl (34). A yeast gene disruption cassette was created by PCR amplification of the *loxP-KAN-loxP* construct in plasmid pUG6 and primers specific for

the gene of interest (15). Specific disruption was confirmed by PCR analysis of genomic DNA.

Plasmid constructions

All the mutants in the localization elements were generated by PCR using the "splicing through overlap" extension strategy (see (7, 8). Mutants in the localization elements E1, E2A and E2B used in the yeast three-hybrid assay were amplified by PCR from YEPlac195-*lacZ-ADHII* based plasmids containing the mutated *ASH1* localization elements (7, 8) and cloned in the SmaI site of plasmid pIIIA/MS2-2. These mutations are listed in the Table I of the Supplementary Material section. Point mutations of the cytosines and elongation of the stem in each localization element were generated by PCR using as template plasmids pXR113 (YEPlac195-*lacZ*-E1), pXR137 (YEPlac195-*lacZ*-E2A), pXR156 (YEPlac195-*lacZ*-E2B) and pXR63 (YEPlac195-*lacZ*-E3) (7) and cloned in the SmaI site of pIIIA/MS2-2. All mutations were confirmed by DNA sequencing. These mutations are listed in the Table I of the Supplementary Material section. For the localization assays, mutant localization elements were amplified by PCR from their pIIIA/MS2-2 based plasmids and cloned at the SmaI site of YEPlac195-*lacZ-ADHII* or YCPlac33-*lacZ-ADHII* plasmids. For *in vitro* transcription, localization elements E1 (70 nt), E2A (92 nt) and E2B-D1 (60 nt) were cloned in pGEM-4Z between the HindIII and EcoR1 sites.

Yeast three-hybrid assay

The strain YBZ1 was used in the three-hybrid assay (16). This strain was transformed with the appropriate plasmids and grown in synthetic media lacking uracil and leucine. Dilution assays were made on plates lacking uracil, leucine and histidine, with or without 3-amino-1,2,4-triazole (3-AT). The β-galactosidase expression level was measured quantitatively using O-nitrophenyl-β-D-galactopyranoside (ONPG) according to the protocol provided by Clontech. The reported β-galactosidase expression levels represent the average of at least three independent experiments. Proper expression of the RNA-MS2 fusions was confirmed by Northern blot (data not shown).

In vivo selection

Partially degenerated libraries of the localization elements E1, E2A, E2B-D1 and E3 were produced by PCR. The oligonucleotides used to produce the libraries are described in the Table II of the Supplementary Material section. The pXR113, pXR137, pXR156, and pXR63 plasmids were used as templates. The libraries were cloned in the SmaI site of the pIIIA/MS2-2 plasmid. The randomization of the sequences was verified by sequencing several clones for each library. The partially degenerated libraries were transformed in YBZ1 yeasts and selected for the interaction with She2p by a three-hybrid assay. The yeasts were grown on a -his -ura -leu X-Gal indicator media (33) containing 0,04 mg/ml of 5-bromo-4-chloro-3-indolyl-ß-D-galactoside (X-gal). The *in vivo* selection for the E1 and E2A libraries were realized in the presence of 0.1 mM and 0.25 mM 3-AT respectively, whereas the selection for the E2B-D1 and E3 libraries were realized in the presence of 1 mM 3-AT. The sequences positive for the interaction with She2p were finally identified by automated DNA sequencing.

Electrophoretic mobility shift assay

To produce *in vitro* transcribed RNA, plasmids pPC1 (pGEM-E1), pPC4 (pGEM-E2A) and pPC9 (pGEM-E2B-D1) were linearized with EcoR1 and transcribed with T7 RNA polymerase in the presence of [α-^{32}P]CTP (20). The transcripts were purified on 6% denaturing polyacrylamide gels, then extracted, and desalted on G25 Sephadex spin columns (Roche). For unlabelled RNAs, the transcripts were purified, after treatment with DNAse1, by phenol/chloroform extraction, ethanol precipitation and desalting on G25 Sephadex spin columns. GST-She2p was expressed and purified according to the protocol published in (4). For electrophoretic mobility shift assays, the ^{32}P-labelled RNA was denatured by heating at 85°C for 2 minutes in the binding buffer and allowed to fold at room temperature for 10 minutes. A total of 10 000 CPM of labelled RNA (~ 1 ng) were added to the binding buffer (10 mM Hepes pH. 7.4, 150 mM KCl, 1 mM DTT, 5 mM $MgCl_2$, 4 % glycerol, 15 U RNAse Inhibitor (Pharmacia)) prior to the addition of various concentration of recombinant GST-She2p, up to a final volume of 20 µl. The

reactions were incubated at 4°C for 30 minutes, then 2 µl of 10 mg/ml heparin was added and incubated for 10 more minutes at 4°C to prevent non-specific interactions. The samples were separated on a 4% non-denaturing gel at 120V for 4hrs at 4°C, dried and exposed overnight with Kodak films. For competition experiments, unlabelled RNAs were added prior to the addition of GST-She2p.

Immunoprecipitation and RT-PCR

Yeast strain K699 *she2* transformed with the plasmids YCP22-She2-myc and YEPlac195-*lacZ-E1*, YEPlac195-*lacZ-E1-M15* or YEPlac195-*lacZ-E1-M16* were grown overnight in a selective media lacking uracil and tryptophan. The hybrid RNA expression was induced by the addition of 3% galactose to the selective media. Cells were harvested by centrifugation and resuspended at 100 OD_{600}/ml in the extraction buffer (25mM HEPES-KOH pH 7.5, 150mM KCl, 2mM $MgCl_2$, 0.1% IGEPAL CA-630, 1mM DTT; 87,5µg/ml PMSF; 0,5µg/ml pepstatin; 0,5µg/ml leupeptin; 0,5µg/ml aprotinin; and 23U/ml of RNAguard). The yeast cells were broken with glass beads and the supernatant was used for immunoprecipitation and western blot. For the immunoprecipitation, an anti-Myc antibody (9E10) was added to 500µl of supernatant and incubated at 4°C with agitation for 1h. 40µl of protein A-sepharose beads was then added and the incubation at 4°C was continued for 2 hours. The beads were washed four times for 3 min at 4°C with a wash buffer (25mM HEPES-KOH pH 7.5, 150mM KCl, 2mM $MgCl_2$). The RNA was eluted from the beads in 200µl of 50mM Tris-HCl pH 8.0, 100mM NaCl, 10mM EDTA and 1% SDS 10 min at 65°C, followed by a phenol/chloroform extraction and ethanol precipitation. For the reverse transcription, 2µl of mRNA was incubated at 70°C for 5 min in presence of 0,5µg of pd(N)6 and quickly chilled on ice. The reverse transcription reaction was performed according to indications in a 1X buffer (50mM Tris-HCl pH8,3; 50mM KCl; 4mM $MgCl_2$; 10mM DTT) containing 10mM dNTP and 20U of RNAguard, with 200U Reverse transcriptase for 1 hour at 42°C. The cDNAs were then amplified by PCR using primers in the *lacZ* sequence.

In situ hybridization and imaging

Yeasts cells were processed for fluorescent in-situ hybridization (FISH) according to the protocols described in (10). For in-situ hybridization, yeast spheroplasts were hybridized with a pool of Cy3-conjugated *lacZ* DNA oligonucleotide probes (7). When quantitative data were obtained, 50 budding yeast cells were counted per experiment

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Pattern search in the PDB database (MC-SEARCH) and RNA motif search (RNAMotif)

Using MC-SEARCH (P.G. and F.M., unpublished data), we scanned all known tertiary structures in the PDB database for occurrences of the three variants of the motif. MC-SEARCH is a computer program, derived from the automated RNA annotation program MC-ANNOTATE (12), that searches RNA structure files for regions that match a user-defined pattern. The search patterns were described only in terms of primary and secondary structure elements: the type of essential nucleotides and the presence of Watson-Crick base pairs in the stems. No constraint was added concerning potential interactions of non helical nucleotides with other parts of the patterns or of the scanned structures.

To identify She2p-binding motifs in other bud-localized mRNAs, we used RNAMotif, a RNA secondary structure search algorithm (11). RNAMotif needs 2 input files: the sequence database and the descriptor of the searched motif. The database was a file with the 22 bud-localized mRNAs sequences (coding sequences, plus 250 bp in 5' and 3') in FASTA format. The descriptor defines the criteria required to generate a match: stem, loop sequences and length. The GU base-pairs are authorized. For

example, the descriptor looking for a 5 base-pairs family stem-loop (Motif Stem 5 in Figure 2 of Supplementary Material section):

Parms: wc+=gu; Descriptor: h5(tag='1', len=4), ss (minlen=1,maxlen=4), h5(tag='2', len=5), ss (minlen=7,maxlen=38, seq='^**c**ga'), h3(tag='2'), ss (minlen=3,maxlen=10, seq='^[atcg]**c**'), h3(tag='1').

Where "h5" and "h3" mean stem, "ss" means single strand. The authorized length of the stem and loop are mentioned by "minlen" and "maxlen". The required nucleotides are mentioned by "seq". The output file contains all the sequences and their position that match the searched motif. Once obtained, the sequences identified were further filtered with the MFOLD software (http://www.bioinfo.rpi.edu/applications/mfold/old/rna/form1.cgi) in order to confirm the predicted secondary structure. Only the RNA sequences that correctly fold like the She2p binding motif RNA were finally selected.

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FIGURES

Figure 1

The four *ASH1* localization elements interact with She2p in a yeast three-hybrid assay. (A) Predicted secondary structures of the *ASH1* localization elements E1, E2A, E2B and E3. Secondary structures are described with latin numbered stems (S) and loops (L). For the element E2B, two separate domains have been previously identified (8), but only the secondary structure of the Domain 1 has been defined. (B) The yeast strain YBZ1 expresses a dimerized bacteriophage MS2 RNA-binding protein fused to a single LexA DNA-binding domain (MS2-LexA), with either wild-type (larger case) or knockout (lowercase) *SHE2* gene. These strains were transformed with a plasmid expressing the GAL4 activation domain fused with the C-terminal domain of She3p (She3-Cterm-AD), along with a plasmid expressing an hybrid RNA containing the indicated RNA fused to two MS2 binding sites. The formation of a She2p-hybrid RNA complex allows the recruitment of the She3-Cterm-AD and MS2-LexA proteins and the activation of the reporter genes. Controls: empty vector (pIIIA/MS2-2) and the Iron Response Element RNA (IRE).

Figure 2

Recombinant GST-She2p binds to the *ASH1* localization element RNAs *in vitro*. (A) Electrophoretic mobility shift assays (EMSA) of GST-She2p with the localization elements RNA E1, E2A and E2B-D1. Increasing concentration of recombinant GST-

She2p (0, 0.5, 0.9, 1.8, 3.7 and 7.4 μM) was added to ^{32}P-labelled localization element RNA. The free RNA is indicated by an asterisk. (B) Competition of the RNA-protein complex with unlabelled localization element RNA E1, E2A and E2B-D1. A constant quantity of GST-She2p (3.7 μM for E1 and E2B-D1, 0.9 μM for E2A) was combined to an increasing concentration of unlabelled RNA (5x, 50x and 500x). The mutant M16 of the element E2B-D1 was used as a competitor (E2D-D1 MUT). (C) Controls using GST or the IRE RNA. For GST, increasing concentration of recombinant GST (0, 0.5, 0.9, 1.8, 3.7 and 7.4 μM) was added to the ^{32}P-labelled localization element E2B-D1 RNA. For the IRE RNA, increasing concentration of recombinant GST-She2p (0, 0.5, 0.9, 1.8, 3.7 and 7.4 μM) was added to the ^{32}P-labelled IRE RNA.

Figure 3

Identification of the She2p-binding domain in the localization elements E1, E2A and E2B-D1. Mutated RNAs were fused to the MS2 binding RNA in the pIIIA/MS2-2 plasmid and transformed in yeasts YBZ1 containing the plasmid pGAD-She3-Ct. The regions mutated are indicated by brackets (for stems) or in gray letters (for loops). The strength of the interaction of the RNA mutants with the She3p-Ct/She2p complex in the yeast three-hybrid assay is reported as *lacZ* expression level (Miller units) or growth on plate lacking histidine. Values for the percentage of localization of the mutants are from references (7, 8).

Figure 4

In vivo selection of partially randomized *ASH1* localization element RNAs. (A) Diagram of the mutagenesis performed on the She2p-binding domains of the four *ASH1* localization elements. Partially randomized positions are underlined. Nucleotides conserved in >95% of the clones isolated are indicated in larger case and italicized. The conserved CGA sequence is boxed with large dotted lines, whereas the conserved cytosine in E1, E2A and E2B-D1 is boxed with small dotted lines. A cytosine at an analogous position on the element E3 is indicated by an asterisk. (B) Fluorescent in situ hybridization on the *lacZ* mRNA fused to *ASH1* localization elements variants isolated

from the three-hybrid screen. Numbers correspond to clones listed in the Table 1. DAPI, DNA staining; DIC, Nomarski.

Figure 5

Two conserved cytosines in the four *ASH1* localization elements define a She2p-binding motif. (A) Mutagenesis of each highly conserved cytosine to uracil or adenine in the *ASH1* localization element results in the loss of interaction with She2p in the yeast three-hybrid assay. Mutated cytosines are boxed. *LacZ* expression is in Miller units. (B) Fluorescent in situ hybridization on the *lacZ* mRNA fused to *ASH1* localization elements mutated on the conserved cytosines. The presence of these mutations in the localization elements lead to the delocalization of the *lacZ-E1, lacZ-E2A, lacZ-E2B* and *lacZ-E3* reporter mRNAs. DAPI, nuclear DNA; DIC, Nomarski. (C) Measurement of the percentage of budding yeast cells with bud-localized *lacZ* mRNA fused to wild-type or mutated localization elements. (D) Co-immunoprecipitation of She2p-myc and *lacZ-E1* mRNA was disrupted by mutation of the conserved cytosines in the element E1. RT-PCR detection of the *lacZ* mRNA fused to wild-type localization element E1 (lane 1) or mutants M15 (lane 2) and M16 (lane 3) of the element E1 were performed on either total yeast extract (total) or on the pellet of the immunoprecipitated myc-tagged She2p (IP+RT). A yeast strain expressing the *lacZ-E1* mRNA and She2p without a myc tag (lane 4) or RT-PCR reactions on the immunoprecipitates without reverse transcriptase (IP-RT) were used as controls.

Figure 6

The distance between the two conserved cytosines is important for She2p recognition. (A) Descriptors used in the program MC-SEARCH. (B) Overlap of three

structures, one from each descriptor, that were identified by the program. The cytosines are colored according to their descriptor motif in (A). The distance indicated correspond to the distance between the 3' phosphates of the two cytosines. For E3 (yellow), the motif comes from the 23S rRNA, starting at position 295 (PDP# 1JZY). For E1 (green), the motif comes from an hairpin similar to the P5Abc region of group I intron, starting at position 4 (PDB# 1EOR). For E2A/B (red), the motif comes from the 23S rRNA, starting at the position 1463 (PDB# 1M1K). The root mean square deviation (RMSD) in their common region (the stem structure) is 1.9Å. (C) Increasing the length of the stem separating the two cytosines in each localization element lead to a decreased interaction with She2p in the yeast three-hybrid assay. The conserved cytosines are labeled in gray. *LacZ* expression is in Miller units.

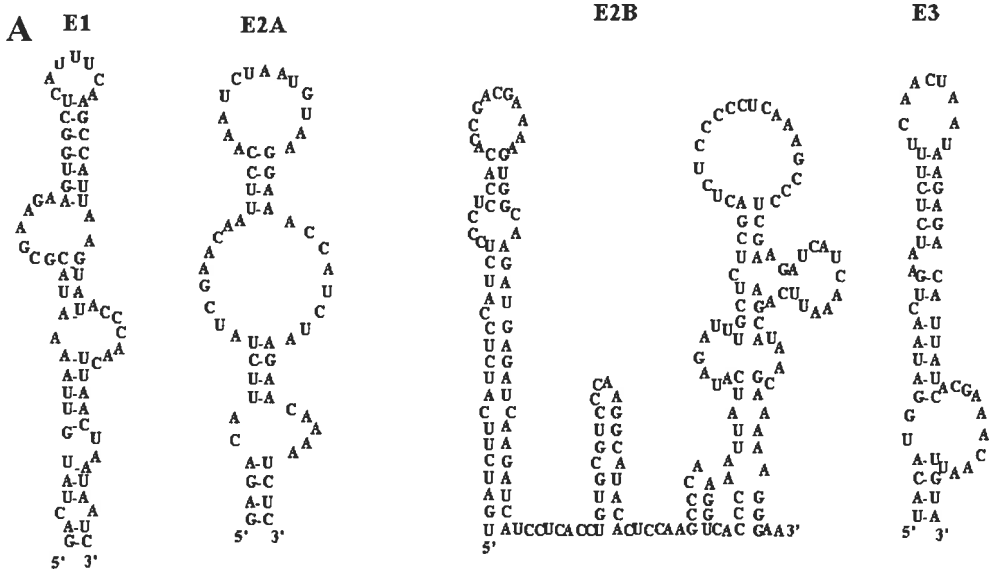
Figure 7

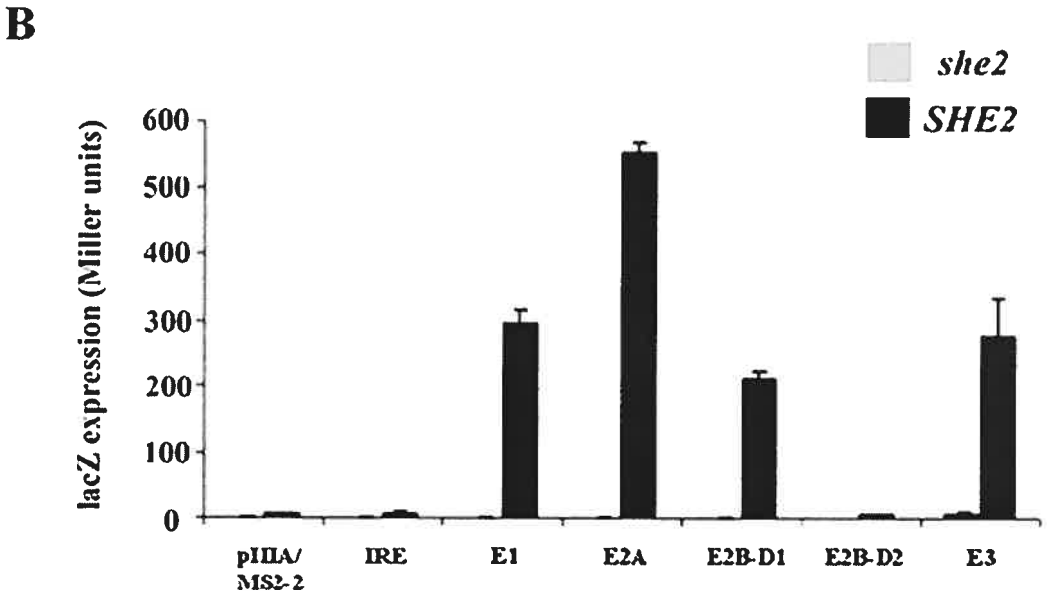
Identification of a She2p-binding motif in the bud-localized *IST2* and *YMR171C* mRNAs. A) Predicted secondary structures of the *IST2* and *YMR171C* mRNAs localization elements. The conserved CGA sequence is boxed with plain lines, whereas the conserved cytosine is boxed with dotted lines. The nucleotides are numbered starting from the adenosine of the start codon as +1. B) Three-hybrid assays using yeast strain YBZ1 with either wild-type or knockout *SHE2* gene, transformed with a plasmid expressing the GAL4 activation domain fused with the C-terminal domain of She3p, along with a plasmid expressing one of the indicated MS2 fusion RNA. Controls: empty vector (pIIIA/MS2-2) and the Iron Response Element RNA (IRE). C) Mutagenesis of each highly conserved cytosine in the *YMR171c* mRNA localization element results in the loss of interaction with She2p in the yeast three-hybrid assay. D) Fluorescent in situ hybridization on the *lacZ* mRNA fused to the *IST2* and wild-type, or mutated, *YMR171C*

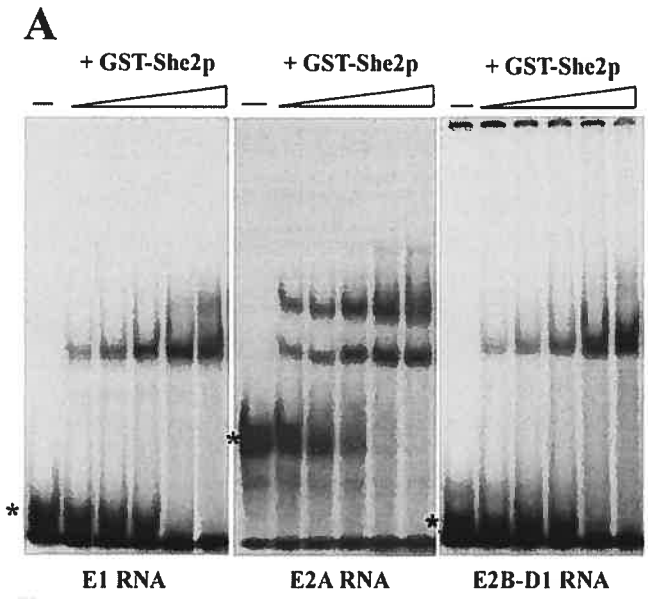
mRNAs localization elements. The percentage of budding yeast cells with bud-localized *lacZ* mRNA is indicated. DAPI, nuclear DNA; DIC, Nomarski.

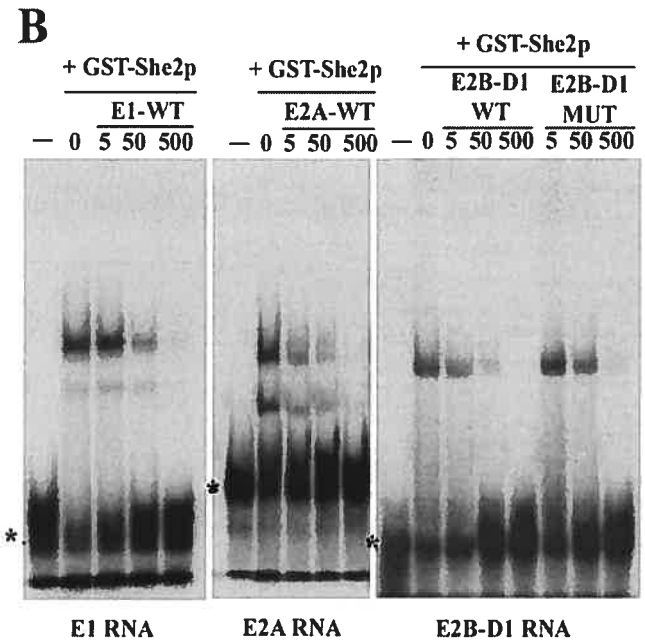
TABLE 1: Analysis of the functionality of localization elements variants isolated from the three-hybrid screens: interaction with She2p and localization function.

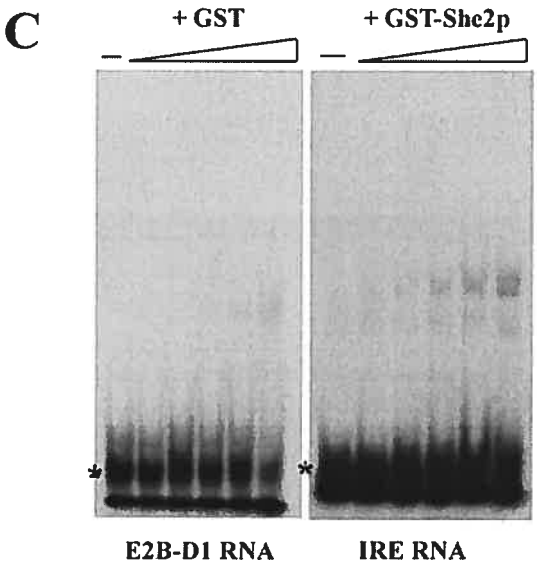
Localization element		Sequence[a]	LacZ[b]	Localization[c] (%)
E1	WT	GACTATGTTAAAATACGCGAAGAAGTGGCTCATTTCAAGCCATTAAGTATACCCAACTTAACTAATAATC	1	75
	8	GACTATGTTAAGATACACGCACAAGTGGCTCATTTCAAGCCATTAGGTATCCGGAAATTAACTAATAATC	4,2	66
	12	GACTATGTTAAGATACGCGAAAAAGTGGCTCATTTCAAGCCATTAAGTATACCCAACTTAACTAATAATC	10,1	70,5
	26	GACTATGTTAAGATACGCAAAAGAGTGGCTCATTTCAAGCCATTAAGTATACCCAACTTAACTAATAATC	3,4	76
	33	GACTATGTTAACATACACGA TTAAGTGGCTCATTTCAAGCCATTACGTATACTCGACTTAACTAATAATC	9,5	63,5
	43	GACTATGTTAACATACGCTAAGGAGTGGCTCATTTCAAGCCATTATGTATTGCTAAATTAACTAATAATC	3,0	65
E2A	WT	TTGCGAATAGAGACATTCTATCGAACAATTCCAAATCTAATGTAAGGAAACC ATCTAAGAACAAAATCTC	1	72
	10	TTGCGAATAGAGACATTCTATCGAACAATTCCAAATCTAATGTAAGGAATTT GGG TAAGAACAAAATCTC	10,1	69
	12	TTGCGAATAGAGACATTCTAACGAAGGATTCCAAATCTAATGTAAGGAATCGTTG GCAGAACAAAATCTC	9,2	74
	34	TTGCGAATAGAGACATTCTACCGAAGAATTCCAAATCTAATGTAAGGAAACC ATC TAAGAACAAAATCTC	7,0	73,5
	48	TTGCGAATAGAGACATTCTATCGAACAATTCCAAATCTAATGTAAGGAACGG AATCAAGAACAAAATCTC	8,6	72
	61	TTGCGAATAGAGACATTCTATCGAAAACTTCCAAATCTAATGTAAGGAAGTGTGC GTAGAACAAAATCTC	1,5	79
	73	TTGCGAATAGAGACATTCTAACGAACACTTCCAAATCTAATGTAAGGAAGTT AGC ATAGAACAAAATCTC	7,0	75
E2B/D1	WT	TGCATCTTCATCTCCATCTCCCTCCACACCGACGAAAAGTGGCAAGATGAGATCAAGATCATCCTCACCTG	1	73
	5	TGCATCTTCATCTCCATCTACATCCACACCGACGAACAGTGGCAAGATGAGATCAAGATCATCCTCACCTG	1,1	68
	12	TGCATCTTCATCTCCATCTCCCTCCACACCGACGTTACGTGGCAAGATGAGATCAAGATCATCCTCACCTG	1,4	65
	69	TGCATCTTCATCTCCATCTACCTCCACATCGACGAAAAGTGGCAAGATGAGATCAAGATCATCCTCACCTG	1,2	71
	87	TGCATCTTCATCTCCATCTCCTTCCACACCGACGAAAAGTGGCGAGATGAGATCAAGATCATCCTCACCTG	0,7	87
	106	TGCATCTTCATCTCCATCTCACTCCACCCCGACGTAAAGTGGCAAGATGAGATCAAGATCATCCTCACCTG-	0,4	69
E3	WT	AGAGAATTGATACATGGATAACTGAATCTCTTTCAACTAATAAGAGACATTATCACG AAA CAA TTGTACA	1	67
	3	AGAGAATTGATACAAGGATAACTGAATCTCTTTCAACTAATAAGAGACATTATCTCG TAT ATG TTGTACA	2,9	69
	20	AGAGAATTGATACACCGATAACTGAATCTCTTTCAACTAATAAGAGACATTATCACG GCG CTT ATGTACA	1,6	87
	30	AGAGAATTGATACATGGATAACTGAATCTCTTTCAACTAATAAGAGACATTATCTCA TCA AAT ATGTACA	1,2	68
	38	AGAGAATTGATACATTGATAACTGAATCTCTTTCAACTAATAAGAGACATTATCACG AAA CAA TTGTACA	3,4	74
	42	AGAGAATTGATACAAGGATAACTGAATCTCTTTCAACTAATAAGAGACATTATCCCG ACA TAC TTGTACA	6,1	64

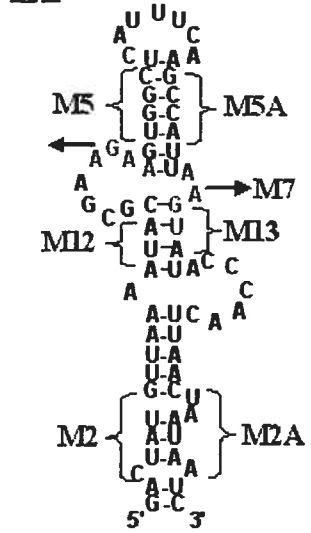
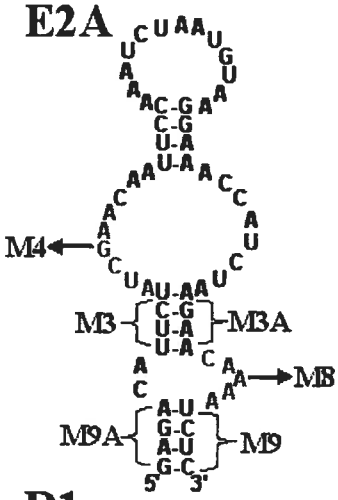
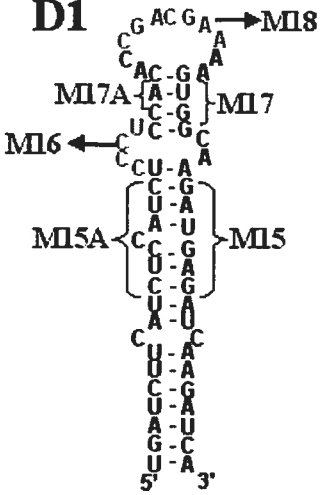

A
E1
E2A
E2B
E3
5' 3'
5' 3'
5'
5' 3'

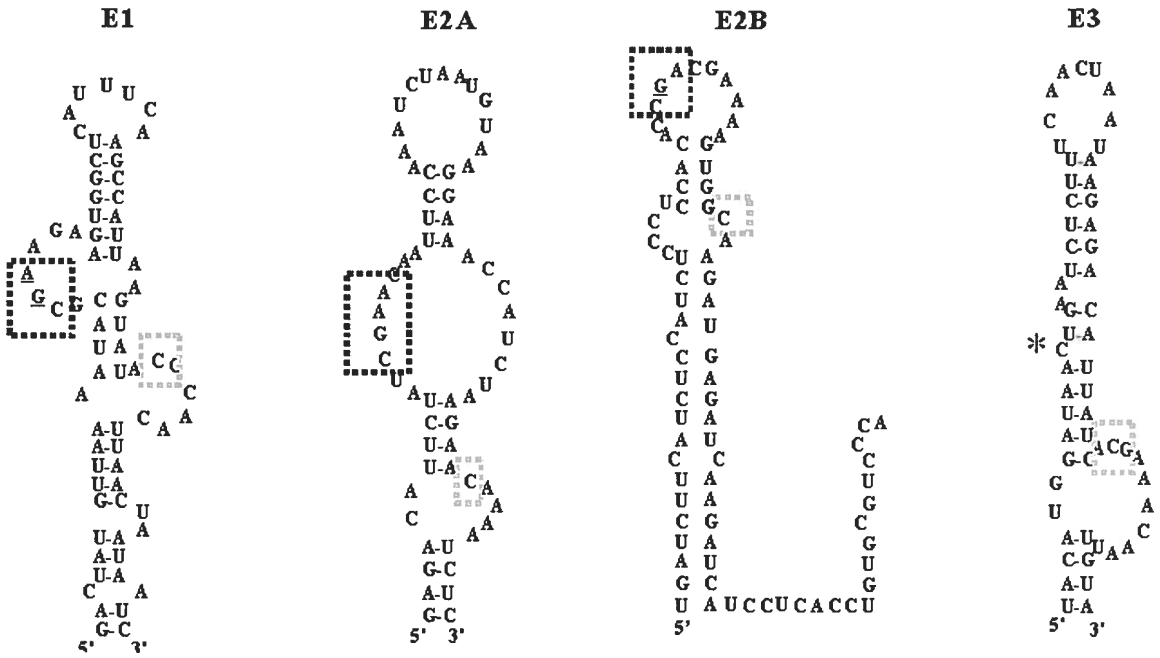

B
she2
SHE2
lacZ expression (Miller units)
600
500
400
300
200
100
0
pIIIA/
MS2-2
IRE
E1
E2A
E2B-D1
E2B-D2
E3


A
+ GST-She2p
+ GST-She2p
+ GST-She2p
E1 RNA
E2A RNA
E2B-D1 RNA

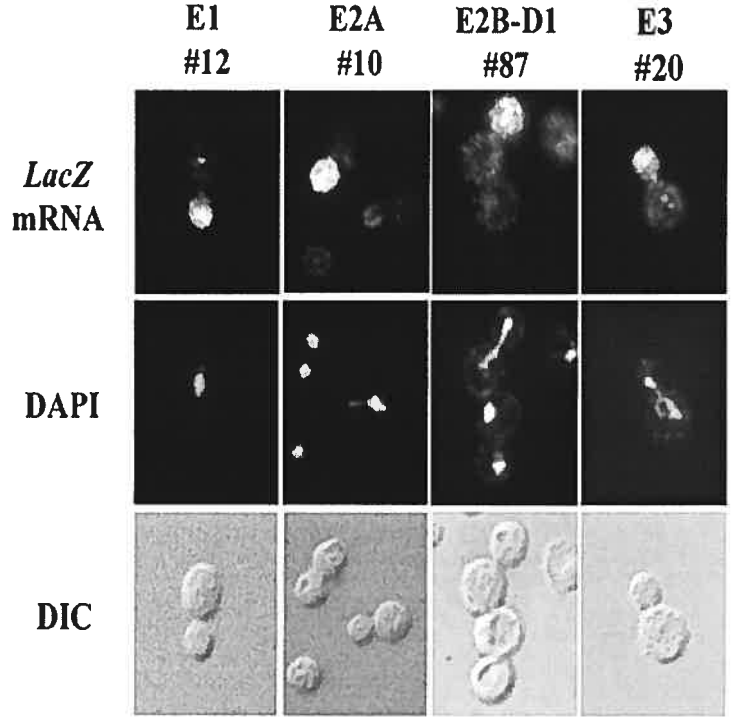

B
+ GST-She2p
E1-WT
— 0 5 50 500
+ GST-She2p
E2A-WT
— 0 5 50 500
+ GST-She2p
E2B-D1 WT
E2B-D1 MUT
— 0 5 50 500 5 50 500
E1 RNA
E2A RNA
E2B-D1 RNA

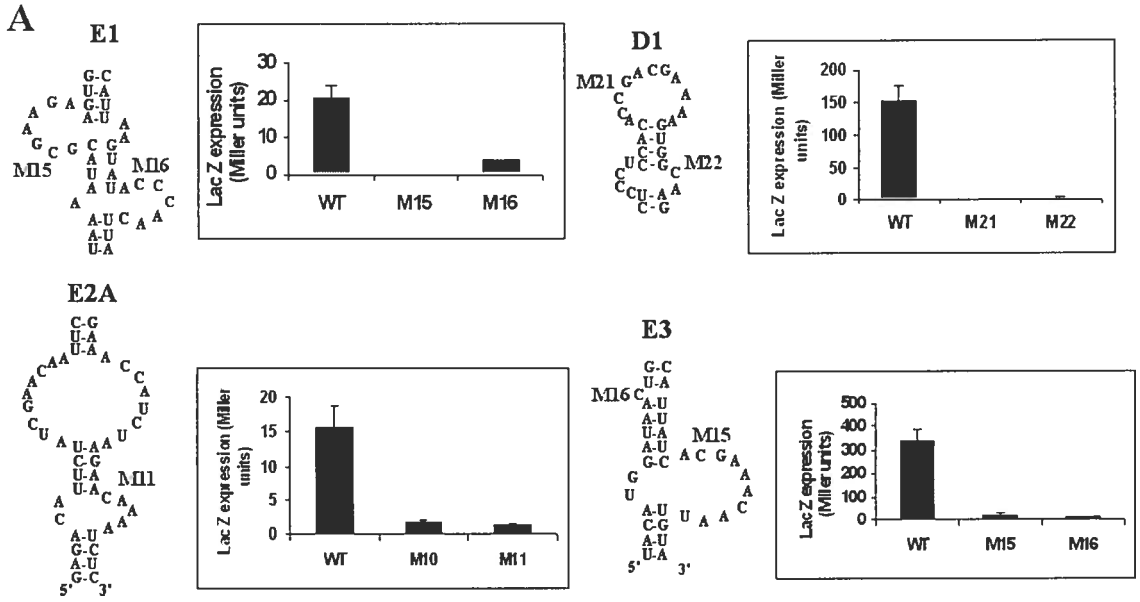

C
+ GST
+ GST-She2p
E2B-D1 RNA
IRE RNA

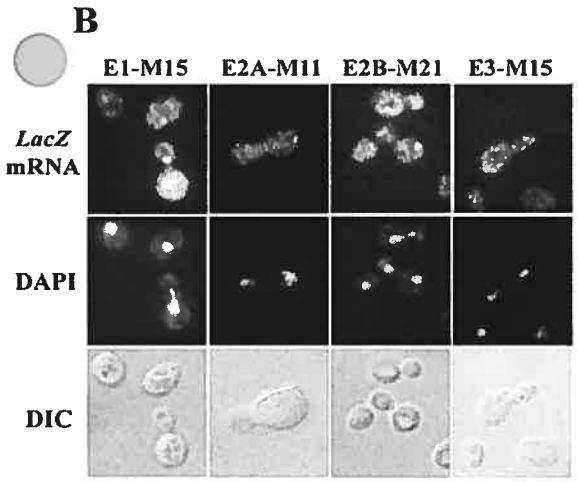
Localization element	Mutant	LacZ expression		Growth on -His medium	Frequency of localization (%) (taken from references (8,9))
		Miller Units	Normalized value		
E1 	WT	22,4 ± 2,2	1	++++	100
	M2	0,4 ± 0,5	0	-	23
	M2+2A	229,9 ± 23,2	10,3	+++++	77
	M5	1,1 ± 1,2	0	-	0
	M5+5A	26,0 ± 7,1	1,2	+++++	97
	M7	1,5 ± 1,2	0,1	-	2
	M10	2,4 ± 1,2	0,1	-	13
	M12	0,9 ± 1,1	0	-	ND
	M13	0,8 ± 0,6	0	-	ND
	M12/13	156,9 ± 12,6	7,0	+++++	ND
E2A 	WT	25,1 ± 5,4	1	+++++	100
	M3	2,3 ± 2,2	0,1	-	10
	M3+3A	44,1 ± 4,4	1,8	++	80
	M4	1,5 ± 1,7	0,1	-	10
	M8	0,9 ± 0,8	0	-	7
	M9	2,5 ± 2,0	0,1	-	7
	M9+9A	20,3 ± 5,4	0,8	+++	30
D1 	D1	168,2 ± 6,2	1	+++++	100
	D1-M15	16,4 ± 9,2	0,1	+	50
	D1-M15+15A	142,6 ± 21,0	0,8	+++++	ND
	D1-M16	15,7 ± 1,3	0,1	++	20
	D1-M17	2,1 ± 0,6	0	-	20
	D1-M17+17A	125,6 ± 5,7	0,7	++++	30
	D1-M18	3,9 ± 0,9	0	-	ND

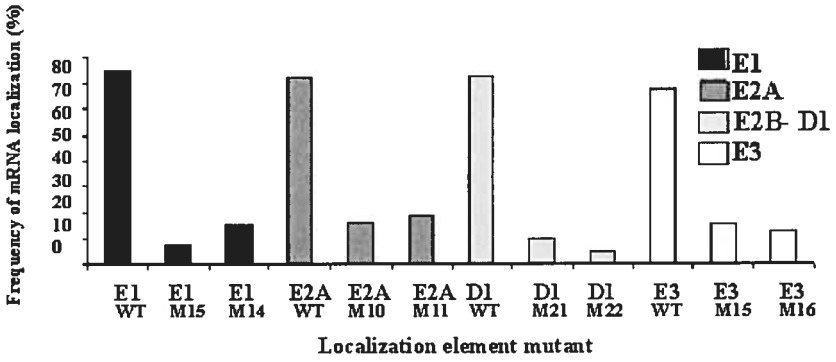


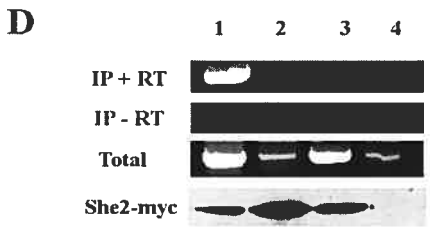
B

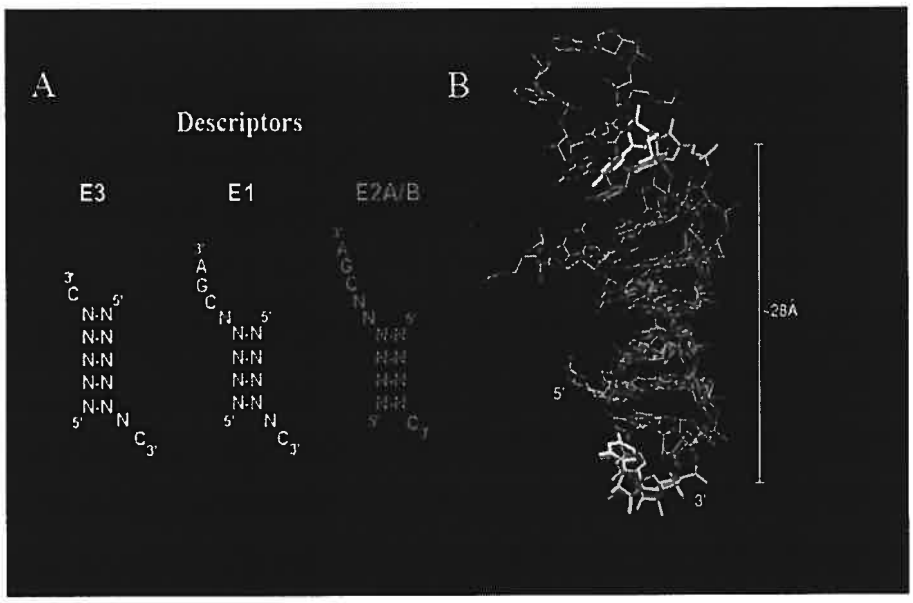


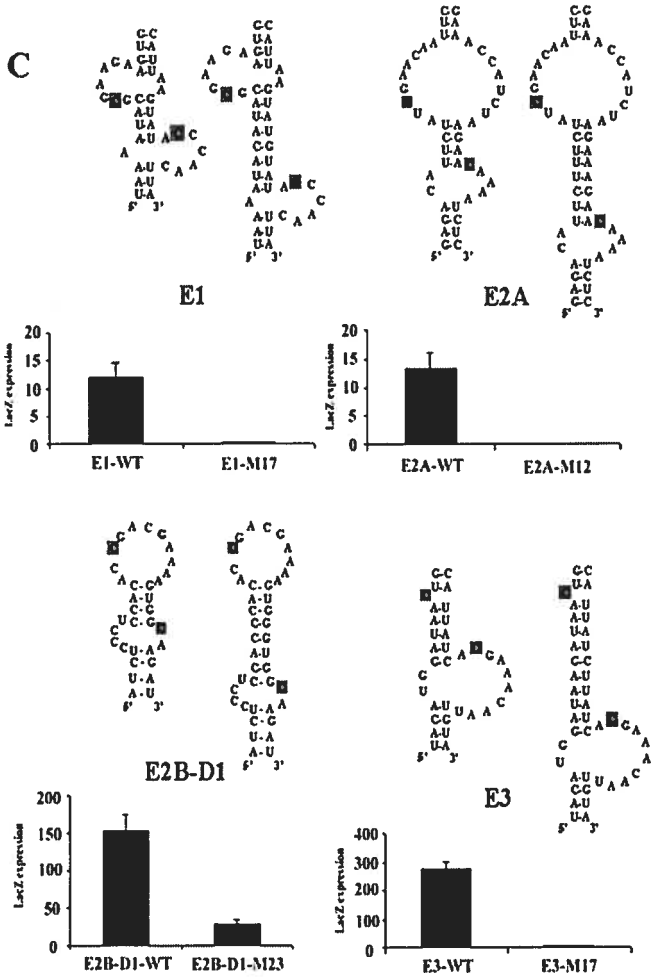

A
E1
M15
M16
Lac Z expression (Miller units)
WT
M15
M16
D1
M21
M22
E2A
M11
M10
E3
M16
M15

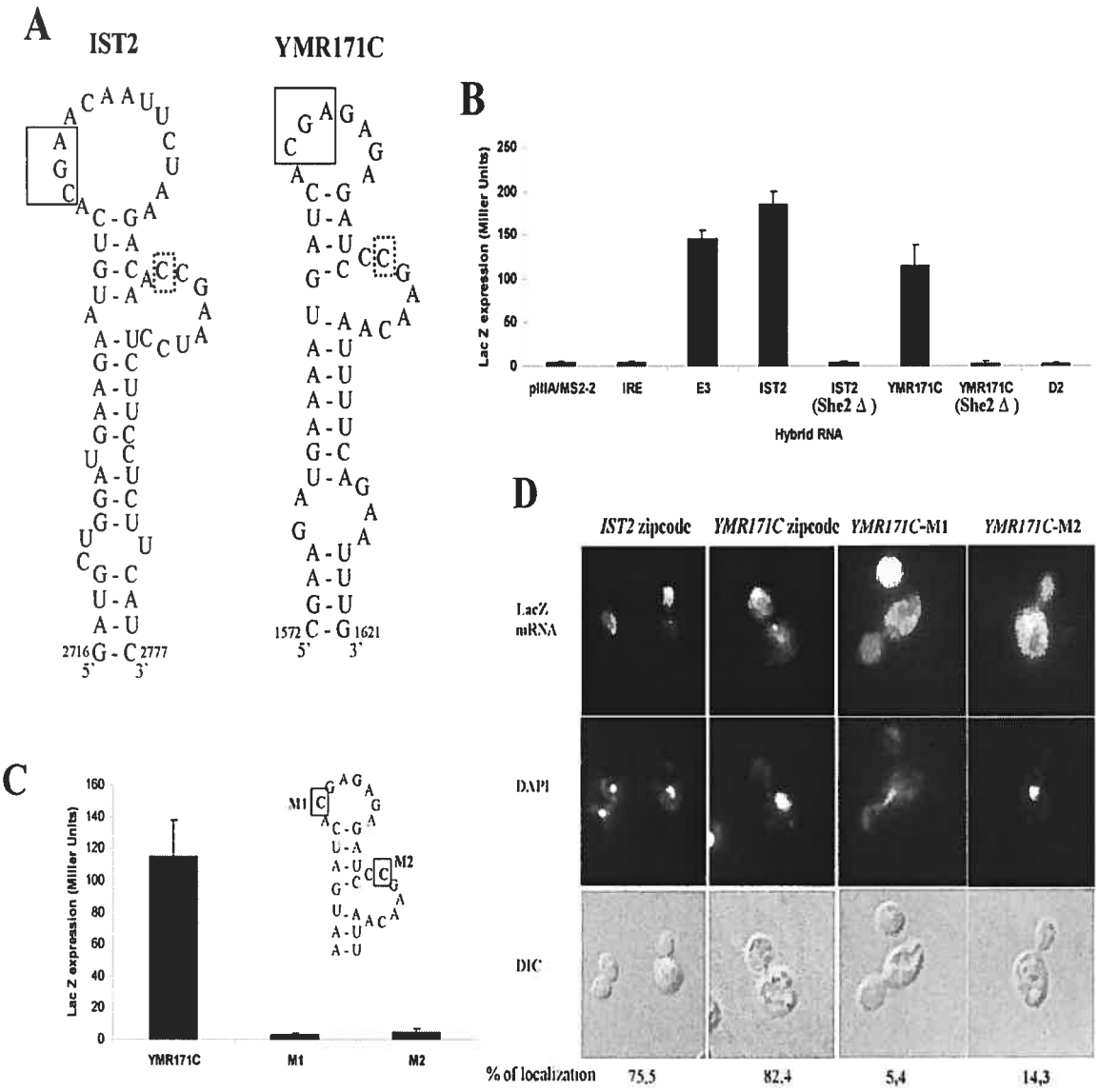

B
E1-M15
E2A-M11
E2B-M21
E3-M15
LacZ mRNA
DAPI
DIC


C
Frequency of mRNA localization (%)
E1
E2A
E2B- D1
E3
E1 WT
E1 M15
E1 M14
E2A WT
E2A M10
E2A M11
D1 WT
D1 M21
D1 M22
E3 WT
E3 M15
E3 M16
Localization element mutant


D
1
2
3
4
IP + RT
IP - RT
Total
She2-myc


A
Descriptors
E3
E1
E2A/B
B
~28Å
5'
3'


C
E1
E2A
E1-WT
E1-M17
E2A-WT
E2A-M12
E2B-D1
E3
E2B-D1-WT
E2B-D1-M23
E3-WT
E3-M17


A
IST2
YMR171C
2716 G - C 2777
5' 3'
1572 C - G 1621
5' 3'
B
Lac Z expression (Miller Units)
250
200
150
100
50
0
pIIIA/MS2-2
IRE
E3
IST2
IST2 (She2 Δ)
YMR171C
YMR171C (She2 Δ)
D2
Hybrid RNA
C
Lac Z expression (Miller Units)
160
140
120
100
80
60
40
20
0
YMR171C
M1
M2
D
IST2 zipcode
YMR171C zipcode
YMR171C-M1
YMR171C-M2
LacZ mRNA
DAPI
DIC
% of localization
75,5
82,4
5,4
14,3

SUPPLEMENTARY MATERIAL SECTION

FIGURES AND TABLES

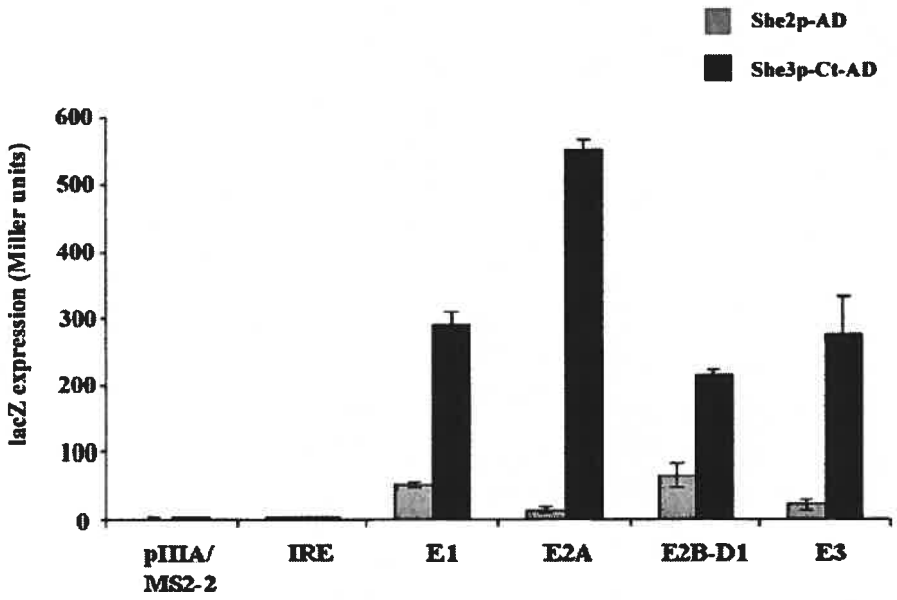


Figure 1.

The four *ASH1* localization elements interact with She2p in a yeast three-hybrid assay. Yeast strain YBZ1 transformed with a plasmid expressing the GAL4 activation domain fused with She2p (She2-AD) or the C-terminal domain of She3p (She3-Ct-AD), along with a plasmid expressing one of the indicated MS2 fusion RNA. Controls: empty vector (pIIIA/MS2-2) and the Iron Response Element RNA (IRE).

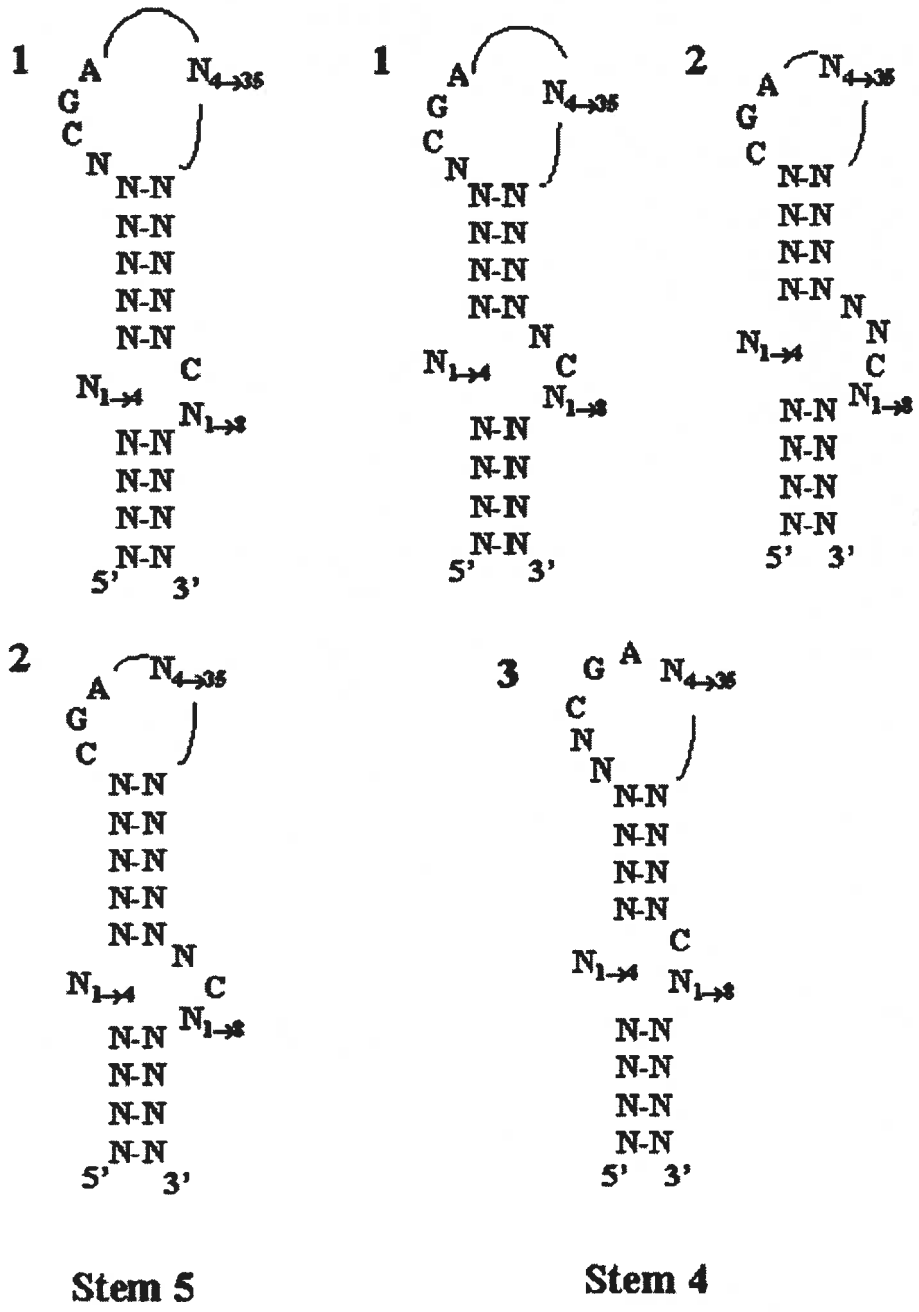


Figure 2.

Descriptors used in the RNAMotif search. Two groups of descriptors have been tested: one with a five base-pairs stem between the two loops (Stem 5), and one with a four base-

pairs stem between the two loops (Stem 4). Within each group, we classified the descriptors according to the following rule: (number of nucleotides that separate the 5'cytosine from the stem) + (length of the stem) + (number of nucleotides that separate the stem from the 3' cytosine) = 6 (see Figure 6A). For the Stem 5 group, we used the 1:5:0 ratio (number 1) and the 0:5:1 ratio (number 2). For the Stem 4 groups, we used the 1:4:1 ratio (number 1); the 0:4:2 ratio (number 2) and the 2:4:0 ratio (number 3). In order to connect both strands of the stem, a linker between 4 and 35 nucleotides was inserted.

TABLE I: List of the mutations used in this study

Mutant	Mutation [a]
E1-M2	$A_{626}CTATG_{631} \rightarrow GAATTC$
E1-M2+2A	$A_{626}CTATG_{631} \rightarrow GAATTC$; $C_{686}TAATAAT_{693} \rightarrow GAATTC$
E1-M5	$G_{648}TGGCT_{654} \rightarrow GAATTC$
E1-M5+5A	$G_{648}TGGCT_{654} \rightarrow GAATTC$; $A_{662}GCCAT_{667} \rightarrow GAATTC$
E1-M7	$A_{669}AGTAT_{674} \rightarrow GAATTC$
E1-M10	$A_{645}GAA_{648} \rightarrow GCGC$
E1-M12	$A_{631}UAC \rightarrow TCCT$
E1-M13	$G_{672}UAU \rightarrow AGGA$
E1-M12+13	$A_{631}UAC \rightarrow TCCT$; $G_{672}UAU \rightarrow AGGA$
E1-M15	$C_{636} \rightarrow A$
E1-M16	$C_{677} \rightarrow A$
E1-M17	$A_{631}UAC \rightarrow AUACAUAC$; $G_{672}UAU \rightarrow GUAUGUAU$
E1-M18	$G_{637} \rightarrow C$
E1-M19	$A_{638} \rightarrow G$
E1-M20	$C_{678}C_{679} \rightarrow AG$
E2A-M3	$T_{1124}TCT_{1127} \rightarrow AAGA$
E2A-M3+3A	$T_{1124}TCT_{1127} \rightarrow AAGA$; $A_{1166}GAA_{1169} \rightarrow TCTT$
E2A-M4	$A_{1128}TCGAA_{1133} \rightarrow GAATTC$
E2A-M7	$A_{1158}CCATC_{1163} \rightarrow CTGCAG$
E2A-M8	$C_{1170}AAAA_{1174} \rightarrow GTTTT$

E2A-M9	$C_{1176}TC_{1178}\rightarrow$ GAG
E2A-M9+9A	$C_{1176}TC_{1178}\rightarrow$ GAG;
	$G_{1118}AG_{1120}\rightarrow$ CTC
E2A-M10	$C_{1130} \rightarrow$ U
E2A-M11	$C_{1170} \rightarrow$ A
E2A-M12	$U_{1124}UCU \rightarrow$ UUCUUUCU;
	$A_{1166}GAA \rightarrow$ AGAAAGAA
E2A-M14	$G_{1131} \rightarrow$ C
E2A-M15	$A_{1132} \rightarrow$ C
E2B-D1-M15	$T_{1271}CTCCATCT_{1278} \rightarrow$ GAATCTCGA
E2B-D1-M15+15A	$T_{1271}CTCCATCT_{1278} \rightarrow$ GAATCTCGA;
	$A_{1304}GATGAGA_{1311} \rightarrow$ TCGAATTC
E2B-D1-M16	$C_{1279}CCT_{1282} \rightarrow$ GGGA
E2B-D1-M17	$G_{1298}TGG_{1301} \rightarrow$ CACC
E2B-D1-M17+17A	$G_{1298}TGG_{1301} \rightarrow$ CACC;
	$C_{1283}CAC_{1286} \rightarrow$ GGTG
E2B-D1-M18	$C_{1289}GACGAA_{1295} \rightarrow$ GAATTCT
E2B-D1-M21	$C_{1295} \rightarrow$ A
E2B-D1-M22	$C_{1308} \rightarrow$ U
E2B-D1-M23	$C_{1289}CAC \rightarrow$ CCACCCAC;
	$G_{1304}UGG \rightarrow$ GUGGGUGG
E3-M15	$C_{1813} \rightarrow$ U
E3-M16	$C_{1779} \rightarrow$ U
E3-M17	$G_{1774}AUAA \rightarrow$ GAUAAGAUAA;
	$U_{1773}UAUC \rightarrow$ UUAUCUUAUC
YMR171c-M1	$C_{1590} \rightarrow$ A
YMR171c-M2	$C_{1602} \rightarrow$ A

[a] The nucleotides are numbered starting from the adenosine of the start codon as +1.

TABLE II: Oligonucleotides used for the generation of the partially randomized libraries

E1-A:	5'AATGGCTTGAAATGAGCCACTNNNTCGCGTATTTTAACA3'
E1-B:	5'AGTGGCTCATTTCAAGCCATTNNNNNNNNNCAACTTAACTAA TAA3'
E1-C:	5'AATGGCTTGAAATGAGCCACTNNNNNNNGTATNTTAACATA GTCTTGA3'
E1-D:	5'AGTGGCTCATTTCAAGCCATTNNGTATNNNNNNNTTAACTAA TAATCAAAATAA3'
E2A-A:	5'AGATGGTTTCCTTACATTAGATTTGGAANNNNNNNNNAGAA NNTCTCTATTCGCAAGCAA3'
E2A-B:	5'TCCAAATCTAATGTAAGGAAACCATCTNAGAANNNNNTC TCAAAGCAAGCATCA3'
E2A-C:	5'TTCCTTACATTAGATTTGGAANNNNNNNNNAGAATGTCTCT ATTCGC3'
E2A-D:	5'TTCCAAATCTAATGTAAGGAANNNNNNNNAGAACAAAATC TCAAAG3'
E2B-D1-B:	5'AGGTGAGGATGATCTTGATCTCATCTNNCCACNNNNNNNNN NNGTGGNNNNAGATGGAGATGAAG3'
E2B-D1-C:	5'GATGAGATCAAGATCATCCTCACCTGTG3'
E3-A:	5'GTCTCTTATTAGTTGAAAGAGATTCAGTTATCNNTGTATC AATTCTCTACTG3'
E3-B:	5'ACTGAATCTCTTTCAACTAATAAGAGACATTATCNNNNN NNNNNTGTACATTTCTCTCCTTGTCTG3'

Each N position was degenerated at the level of 50% for E1, E2A-A and –B, and E3, or 60% for E2A-C and –D, and E2B-D1

TABLE III. Sequences of variants from a partially degenerated library of the localization element E1 that interacted with She2p in a yeast three-hybrid screen.

Clone	Sequence	LacZ expression	
		Miller units	N[a]
WT	5'-GACTATGTTAAAATACGCGAAGAAGTGGCTCATTTCAAGCCATTAAGTATACCCAACTTAACTAATAATC-3'	**12,2**	**1,0**
2	5'-GACTATGTTAAAATACGCGACGAAGTGGCTCATTTCAAGCCATTTAGTATACGAAACTTAACTAATAATC-3'	77,1	6,3
6	5'-GACTATGTTAAGATACGCGAGAGAGTGGCTCATTTCAAGCCATTAAGTATACCCAACTTAACTAATAATC-3'	74,8	6,1
8	5'-GACTATGTTAAGATACACGCACAAGTGGCTCATTTCAAGCCATTAGGTATCCGGAAATTAACTAATAATC-3'	**51,4**	**4,2**
9	5'-GACTATGTTAAAATACGCGAAGAAGTGGCTCATTTCAAGCCATTATGTATACAGAACTTAACTAATAATC-3'	92,3	7,6
12	5'-GACTATGTTAAGATACGCGAAAAAGTGGCTCATTTCAAGCCATTAAGTATACCCAACTTAACTAATAATC-3'	**122,7**	**10,1**
13	5'-GACTATGTTAATATACGCGAAGAAGTGGCTCATTTCAAGCCATTAAGTATTCAAAATTTAACTAATAATC-3'	55,5	4,6
17	5'-GACTATGTTAAAATACGCGAAGAAGTGGCTCATTTCAAGCCATTACGTATCCCTAATTTAACTAATAATC-3'	65,9	5,4
18	5'-GACTATGTTAAAATACGCGACGAAGTGGCTCATTTCAAGCCATTAGGTATGCCCAACTTAACTAATAATC-3'	22,3	1,8
23	5'-GACTATGTTAAAATACGCGAAGCAGTGGCTCATTTCAAGCCATTTGGTATTCTGAACTTAACTAATAATC-3'	46,2	3,8
26	5'-GACTATGTTAAGATACGCAAAAGAGTGGCTCATTTCAAGCCATTAAGTATACCCAACTTAACTAATAATC-3'	**41,1**	**3,4**
32	5'-GACTATGTTAAAATACGCGAAGAAGTGGCTCATTTCAAGCCATTATGTATGCCCAATTTAACTAATAATC-3'	100,5	8,2
33	5'-GACTATGTTAACATACACGA TTAAGTGGCTCATTTCAAGCCATTACGTATACTCGACTTAACTAATAATC-3'	**115,3**	**9,5**
37	5'-GACTATGTTAAGATACACGAGCAAGTGGCTCATTTCAAGCCATTACGTATACCGCACTTAACTAATAATC-3'	73,1	6,0
40	5'-GACTATGTTAACATACGCGACGCAGTGGCTCATTTCAAGCCATTTAGTATACACACGTTAACTAATAATC-3'	88,2	7,2
41	5'-GACTATGTTAAAATACACGAAGAAGTGGCTCATTTCAAGCCATTATGTATACCGGATTTAACTAATAATC-3'	**100,1**	**8,2**
42	5'-GACTATGTTAAGATACGCGAGTAAGTGGCTCATTTCAAGCCATTATGTATACCCAACTTAACTAATAATC-3'	76,8	6,3
43	5'-GACTATGTTAACATACGCTAAGGAGTGGCTCATTTCAAGCCATTATGTATTGCTAAATTAACTAATAATC-3'	**36,8**	**3,0**
65	5'-GACTATGTTAAAATACGCGACGAAGTGGCTCATTTCAAGCCATTATGTATACCCAACTTAACTAATAATC-3'	63,4	5,2
	CGA C A		

Randomized positions are underlined. Mutated positions are indicated in gray.

[a] N= LacZ expression normalized to the value of the wild-type element.

TableIV. Sequences of variants from a partially degenerated library of the localization element E2A (loops II + III) that interacted with She2p in a yeast three-hybrid screen

Clone	Sequence	LacZ expression	
		Miller units	N[a]
WT	5'-TTGCGAATAGAGA**CA**TTCT**ATCGAACAA**TTCCAAATCTAATGTAAGGAAACCATCT**A**AGAA**CAAAA**TCTC-3'	13,0	1,0
2	5'-TTGCGAATAGAGA**CA**TTCT**ATCGAGCTA**TTCCAAATCTAATGTAAGGAAACCATCT**C**AGAA**CAAAA**TCTC-3'	12,4	1,0
3	5'-TTGCGAATAGAGA**CA**TTCT**ATCGAAGGA**TTCCAAATCTAATGTAAGGAAACCATCT**A**AGAA**CAAAA**TCTC-3'	46,3	3,6
4	5'-TTGCGAATAGAGA**CA**TTCT**ATCGAACAA**TTCCAAATCTAATGTAAGGAAACCATCT**A**AGAA**CTAAA**TCTC-3'	23,6	1,8
10	5'-TTGCGAATAGAGA**CA**TTCT**AACGAAGAA**TTCCAAATCTAATGTAAGGAAACCATCT**A**AGAA**CAAAA**TCTC-3'	7,9	0,6
13	5'-TTGCGAATAGAGA**CA**TTCT**ACCGAACGC**TTCCAAATCTAATGTAAGGAAACCATCT**A**AGAA**CAAAA**TCTC-3'	15,0	1,2
15	5'-TTGCGAATAGAGA**CA**TTCT**AGCGAAGAA**TTCCAAATCTAATGTAAGGAAACCATCT**T**AGAA**CAAAA**TCTC-3'	20,2	1,6
30	5'-TTGCGAATAGAGA**CA**TTCT**ATCGAACGG**TTCCAAATCTAATGTAAGGAAACCATCT**A**AGAA**CAAAA**TCTC-3'	33,2	2,6
32	5'-TTGCGAATAGAGA**CA**TTCT**ATCGAGCGT**TTCCAAATCTAATGTAAGGAAACCATCT**C**AGAA**CAAAA**TCTC-3'	32,6	2,5
39	5'-TTGCGAATAGAGA**CA**TTCT**AGCGAATAA**TTCCAAATCTAATGTAAGGAAACCATCT**A**AGAA**CAAAA**TCTC-3'	16,0	1,2
52	5'-TTGCGAATAGAGA**CA**TTCT**ACCGAAGAA**TTCCAAATCTAATGTAAGGAAACCATCT**A**AGAA**CAAAA**TCTC-3'	41,0	3,2
55	5'-TTGCGAATAGAGA**CA**TTCT**AACGAACAT**TTCCAAATCTAATGTAAGGAAACCATCT**A**AGAA**CAAAA**TCTC-3'	32,2	2,5
64	5'-TTGCGAATAGAGA**CA**TTCT**ATCGAACGT**TTCCAAATCTAATGTAAGGAAACCATCT**A**AGAA**CCGAA**TCTC-3'	29,8	6,0
	CA A CGA C AAA		

Randomized positions are underlined. Mutated positions are indicated in gray.[a] N= LacZ expression normalized to the value of the wild-type element

TABLE V. Sequences of variants from a partially degenerated library of the localization element E2A (loop III) that interacted with She2p in a yeast three-hybrid screen.

Clone	Sequence	LacZ expression	
		Miller units	N[a]
WT	5'-TTGCGAATAGAGACATTCT**ATCGAACAA**TTCCAAATCTAATGTAAGGAA**ACC ATC TA**AGAACAAAATCTC-3'	**12,5**	**1,0**
1	5'-TTGCGAATAGAGACATTCT**ATCGAACAA**TTCCAAATCTAATGTAAGGAA**TTG AAT TG**AGAACAAAATCTC-3'	73,5	5,9
2	5'-TTGCGAATAGAGACATTCT**ATCGAACAA**TTCCAAATCTAATGTAAGGAA**GCATGC TC**AGAACAAAATCTC-3'	88,8	7,1
10	5'-TTGCGAATAGAGACATTCT**ATCGAACAA**TTCCAAATCTAATGTAAGGAA**TTT GGG TA**AGAACAAAATCTC-3'	**126,0**	**10,1**
12	5'-TTGCGAATAGAGACATTCT**AACGAAGGA**TTCCAAATCTAATGTAAGGAA**TCGTTG GC**AGAACAAAATCTC-3'	**115,0**	**9,2**
15	5'-TTGCGAATAGAGACATTCT**ATCGAACAA**TTCCAAATCTAATGTAAGGAA**TTC TGC TC**AGAACAAAATCTC-3'	107,4	8,6
19	5'-TTGCGAATAGAGACATTCT**ATCGAACAA**TTCCAAATCTAATGTAAGGAA**ATC ATC TT**AGAACAAAATCTC-3'	64,4	5,1
22	5'-TTGCGAATAGAGACATTCT**ATCGAACAA**TTCCAAATCTAATGTAAGGAA**ATT GCG TA**AGAACAAAATCTC-3'	96,2	7,7
25	5'-TTGCGAATAGAGACATTCT**ATCGAAGAG**TTCCAAATCTAATGTAAGGAA**ACCATC TA**AGAACAAAATCTC-3'	11,7	0,9
28	5'-TTGCGAATAGAGACATTCT**ATCGAACAA**TTCCAAATCTAATGTAAGGAA**TTT CTG TA**AGAACAAAATCTC-3'	118,9	9,5
34	5'-TTGCGAATAGAGACATTCT**ACCGAAGAA**TTCCAAATCTAATGTAAGGAA**ACC ATC TA**AGAACAAAATCTC-3'	**88,0**	**7,0**
43	5'-TTGCGAATAGAGACATTCT**ATCGAACAA**TTCCAAATCTAATGTAAGGAA**ATG GAC TG**AGAACAAAATCTC-3'	109,2	8,7
46	5'-TTGCGAATAGAGACATTCT**ATCGAACAA**TTCCAAATCTAATGTAAGGAA**TGC CTA TC**AGAACAAAATCTC-3'	94,2	7,5
47	5'-TTGCGAATAGAGACATTCT**ATCGAACAA**TTCCAAATCTAATGTAAGGAA**TTC GCT AA**AGAACAAAATCTC-3'	75,3	6,0
48	5'-TTGCGAATAGAGACATTCT**ATCGAACAA**TTCCAAATCTAATGTAAGGAA**CGG AAT CA**AGAACAAAATCTC-3'	**107,1**	**8,6**
49	5'-TTGCGAATAGAGACATTCT**ATCGAACAA**TTCCAAATCTAATGTAAGGAA**TGG AAC TC**AGAACAAAATCTC-3'	81,5	6,5
50	5'-TTGCGAATAGAGACATTCT**ATCGAACAA**TTCCAAATCTAATGTAAGGAA**TCG GTT TA**AGAACAAAATCTC-3'	73,7	5,9
51	5'-TTGCGAATAGAGACATTCT**TACGAACAA**TTCCAAATCTAATGTAAGGAA**ACC ATC TA**AGAACAAAATCTC-3'	61,9	4,9
53	5'-TTGCGAATAGAGACATTCT**ATCGAACAA**TTCCAAATCTAATGTAAGGAA**CGT ATA TA**AGAACAAAATCTC-3'	54,7	4,4
60	5'-TTGCGAATAGAGACATTCT**ACCGAAAAA**TTCCAAATCTAATGTAAGGAA**TTAGTC TA**AGAACAAAATCTC-3'	86,4	6,9
61	5'-TTGCGAATAGAGACATTCT**ATCGAAAAC**TTCCAAATCTAATGTAAGGAA**GTGTGC GT**AGAACAAAATCTC-3'	**18,9**	**1,5**
65	5'-TTGCGAATAGAGACATTCT**ATCGAACAA**TTCCAAATCTAATGTAAGGAA**ATGCTG TC**AGAACAAAATCTC-3'	32,2	2,6
67	5'-TTGCGAATAGAGACATTCT**ACCGAATAT**TTCCAAATCTAATGTAAGGAA**ACT ATG TA**AGAACAAAATCTC-3'	62,0	5,0
69	5'-TTGCGAATAGAGACATTCT**ATCGAACAG**TTCCAAATCTAATGTAAGGAA**ACC GAT TC**AGAACAAAATCTC-3'	93,9	7,5
71	5'-TTGCGAATAGAGACATTCT**ATCGAACAA**TTCCAAATCTAATGTAAGGAA**GTA GCC TA**AGAACAAAATCTC-3'	13,2	1,1
73	5'-TTGCGAATAGAGACATTCT**AACGAACAC**TTCCAAATCTAATGTAAGGAA**GTT AGC AT**AGAACAAAATCTC-3'	**87,3**	**7,0**
77	5'-TTGCGAATAGAGACATTCT**ATCGAACAA**TTCCAAATCTAATGTAAGGAA**TTT ATT GA**AGAACAAAATCTC-3'	53,1	4,2
	A CGAA A		

Randomized positions are underlined. Mutated positions are indicated in gray.

[a] N= LacZ expression normalized to the value of the wild-type element

TABLE VI. Sequences of variants from a partially degenerated library of the localization element E2B-D1 that interacted with She2p in a yeast three-hybrid screen

Clone	Sequence	LacZ expression	
		Miller units	N[a]
WT	5'-TGCATCTTCATCTCCATCTCCCTCCACACCGACGAAAAGTGGCAAGATGAGATCAAGATCATCCTCACCTG-3'	221,0	1,0
5	5'-TGCATCTTCATCTCCATCTACATCCACACCGACGAACAGTGGCAAGATGAGATCAAGATCATCCTCACCTG-3'	**242,5**	**1,1**
6	5'-TGCATCTTCATCTCCATCTCCCTCCACAACGACGCAAAGTGGCAAGATGAGATCAAGATCATCCTCACCTG-3'	116,7	0,5
10	5'-TGCATCTTCATCTCCATCTCCTTCCACAACGACGAACAGTGGCAAGATGAGATCAAGATCATCCTCACCTG-3'	131,5	0,6
12	5'-TGCATCTTCATCTCCATCTCCCTCCACACCGACGTTACGTGGCAAGATGAGATCAAGATCATCCTCACCTG-3'	**313,3**	**1,4**
20	5'-TGCATCTTCATCTCCATCTCCCTCCACACCGACGAAATGTGGCAAGATGAGATCAAGATCATCCTCACCTG-3'	151,7	0,7
26	5'-TGCATCTTCATCTCCATCTACCTCCACATCGACGCAAAGTGGCAAGATGAGATCAAGATCATCCTCACCTG-3'	126,4	0,6
56	5'-TGCATCTTCATCTCCATCTCCCTCCACACCGACGACAAGTGGCAAGATGAGATCAAGATCATCCTCACCTG-3'	341,3	1,5
62	5'-TGCATCTTCATCTCCATCTCCATCCACACCGACGACAAGTGGCAAGATGAGATCAAGATCATCCTCACCTG-3'	104,9	0,5
66	5'-TGCATCTTCATCTCCATCTCCCTCCACATCGACGTAAAGTGGCAAGATGAGATCAAGATCATCCTCACCTG-3'	223,0	3,2
69	5'-TGCATCTTCATCTCCATCTACCTCCACATCGACGAAAAGTGGCAAGATGAGATCAAGATCATCCTCACCTG-3'	**269,5**	**1,2**
73	5'-TGCATCTTCATCTCCATCTCCCTCCACATCGACGCAAAGTGGCAAGATGAGATCAAGATCATCCTCACCTG-3'	164,4	0,7
87	5'-TGCATCTTCATCTCCATCTCCTTCCACACCGACGAAAAGTGGCGAGATGAGATCAAGATCATCCTCACCTG-3'	**152,4**	**0,7**
91	5'-TGCATCTTCATCTCCATCTCCCTCCACACCGACGCAAAGTGGCAAGATGAGATCAAGATCATCCTCACCTG-3'	99,6	0,5
92	5'-TGCATCTTCATCTCCATCTCCCTCCACAACGACGCATAGTGGCAAGATGAGATCAAGATCATCCTCACCTG-3'	174,2	0,8
102	5'-TGCATCTTCATCTCCATCTACCTCCACACCGACGAAAAGTGGCAAGATGAGATCAAGATCATCCTCACCTG-3'	169,0	0,8
106	5'-TGCATCTTCATCTCCATCTCACTCCACCCCGACGTAAAGTGGCAAGATGAGATCAAGATCATCCTCACCTG-3'	**93,2**	**0,4**
112	5'-TGCATCTTCATCTCCATCTCACTCCACACCGACGCAATGTGGCAAGATGAGATCAAGATCATCCTCACCTG-3'	173,8	0,8
	T A CGACG CA		

Randomized positions are underlined. Mutated positions are indicated in gray.

[a] N= LacZ expression normalized to the value of the wild-type element.

TABLE VII. Sequences of variants from a partially degenerated library of the localization element E3 that interacted with She2p in a yeast three-hybrid screen

Randomized positions are underlined. Mutated positions are indicated in gray.
[a] N= LacZ expression normalized to the value of the wild-type element.

Clone	Sequence	LacZ expression	
		Miller units	N
WT	5'-AGAATTGATACA**TG**GATAACTGAATCTCTTTCAACTAATAAGAGACATTATC**ACG AAA CAA T**TGTA-3'	158,0	1,0
1	5'-AGAATTGATACA**CG**GATAACTGAATCTCTTTCAACTAATAAGAGACATTATC**TCG AAA ACA T**TGTA-3'	337,5	2,9
2	5'-AGAATTGATACA**TG**GATAACTGAATCTCTTTCAACTAATAAGAGACATTATC**ACG ATT CCA T**TGTA-3'	347,8	3,0
3	5'-AGAATTGATACA**AG**GATAACTGAATCTCTTTCAACTAATAAGAGACATTATC**TCG TAT ATG T**TGTA-3'	**333,8**	**2,9**
4	5'-AGAATTGATACA**AT**GATAACTGAATCTCTTTCAACTAATAAGAGACATTATC**TCT AGA CAA A**TGTA-3'	270,8	2,3
5	5'-AGAATTGATACA**AT**GATAACTGAATCTCTTTCAACTAATAAGAGACATTATC**TCC CAA CAT T**TGTA-3'	325,7	2,8
6	5'-AGAATTGATACA**CA**GATAACTGAATCTCTTTCAACTAATAAGAGACATTATC**ACC ATA CAT T**TGTA-3'	314,2	2,7
7	5'-AGAATTGATACA**TG**GATAACTGAATCTCTTTCAACTAATAAGAGACATTATC**ACA CAA CAA C**TGTA-3'	235,9	2,0
8	5'-AGAATTGATACA**GG**GATAACTGAATCTCTTTCAACTAATAAGAGACATTATC**GCG ACT CAT T**TGTA-3'	356,9	3,1
9	5'-AGAATTGATACA**TG**GATAACTGAATCTCTTTCAACTAATAAGAGACATTATC**ACG TTC CAA A**TGTA-3'	281,7	2,4
10	5'-AGAATTGATACA**TG**GATAACTGAATCTCTTTCAACTAATAAGAGACATTATC**TCG GCA CCT G**TGTA-3'	235,8	2,0
11	5'-AGAATTGATACA**TG**GATAACTGAATCTCTTTCAACTAATAAGAGACATTATC**GCA ACC CAA A**TGTA-3'	304,3	2,6
12	5'-AGAATTGATACA**TG**GATAACTGAATCTCTTTCAACTAATAAGAGACATTATC**TCT TAC GTA T**TGTA-3'	284,9	2,5
15	5'-AGAATTGATACA**TG**GATAACTGAATCTCTTTCAACTAATAAGAGACATTATC**ACG AAA AAC T**TGTA-3'	342,7	3,0
16	5'-AGAATTGATACA**AG**GATAACTGAATCTCTTTCAACTAATAAGAGACATTATC**TCG CAA CCA C**TGTA-3'	300,2	2,6
18	5'-AGAATTGATACA**TA**GATAACTGAATCTCTTTCAACTAATAAGAGACATTATC**TCC ACA TCA C**TGTA-3'	178,2	1,5
19	5'-AGAATTGATACA**CG**GATAACTGAATCTCTTTCAACTAATAAGAGACATTATC**TCG AGT CAC T**TGTA-3'	182,6	1,6
20	5'-AGAATTGATACA**CC**GATAACTGAATCTCTTTCAACTAATAAGAGACATTATC**ACG GCG CTT A**TGTA-3'	**183,1**	**1,6**
21	5'-AGAATTGATACA**TG**GATAACTGAATCTCTTTCAACTAATAAGAGACATTATC**TCG CCG TTA C**TGTA-3'	244,2	2,1
23	5'-AGAATTGATACA**AT**GATAACTGAATCTCTTTCAACTAATAAGAGACATTATC**GCC GAA CAA T**TGTA-3'	228,3	2,0
24	5'-AGAATTGATACA**TG**GATAACTGAATCTCTTTCAACTAATAAGAGACATTATC**ACG AAA CAT T**TGTA-3'	220,9	1,9
25	5'-AGAATTGATACA**TG**GATAACTGAATCTCTTTCAACTAATAAGAGACATTATC**TCG AAA TCG T**TGTA-3'	217,4	1,9
28	5'-AGAATTGATACA**TG**GATAACTGAATCTCTTTCAACTAATAAGAGACATTATC**CCG GTC TAC A**TGTA-3'	141,6	1,2
29	5'-AGAATTGATACA**TA**GATAACTGAATCTCTTTCAACTAATAAGAGACATTATC**ACG CAG CGA A**TGTA-3'	85,5	0,7
30	5'-AGAATTGATACA**TG**GATAACTGAATCTCTTTCAACTAATAAGAGACATTATC**TCA TCA AAT A**TGTA-3'	**139,5**	**1,2**
31	5'-AGAATTGATACA**AG**GATAACTGAATCTCTTTCAACTAATAAGAGACATTATC**ACG ATG TTG T**TGTA-3'	104,5	0,9
32	5'-AGAATTGATACA**GG**GATAACTGAATCTCTTTCAACTAATAAGAGACATTATC**ACG AAA CAA T**TGTA-3'	127,3	1,1
33	5'-AGAATTGATACA**TG**GATAACTGAATCTCTTTCAACTAATAAGAGACATTATC**ACG AAT AAA T**TGTA-3'	524,4	4,5
34	5'-AGAATTGATACA**CG**GATAACTGAATCTCTTTCAACTAATAAGAGACATTATC**ACG AAC AGA T**TGTA-3'	367,6	3,2
38	5'-AGAATTGATACA**TT**GATAACTGAATCTCTTTCAACTAATAAGAGACATTATC**ACG AAA CAA T**TGTA-3'	**387,8**	**3,4**
39	5'-AGAATTGATACA**TA**GATAACTGAATCTCTTTCAACTAATAAGAGACATTATC**GCG ACC CAA T**TGTA-3'	387,5	3,3
41	5'-AGAATTGATACA**AG**GATAACTGAATCTCTTTCAACTAATAAGAGACATTATC**ACG AAT GTA T**TGTA-3'	406,4	3,5
42	5'-AGAATTGATACA**AG**GATAACTGAATCTCTTTCAACTAATAAGAGACATTATC**CCG ACA TAC T**TGTA-3'	**702,1**	**6,1**
43	5'-AGAATTGATACA**TG**GATAACTGAATCTCTTTCAACTAATAAGAGACATTATC**ACG AAA TAG T**TGTA-3'	372,4	3,2
46	5'-AGAATTGATACA**TG**GATAACTGAATCTCTTTCAACTAATAAGAGACATTATC**CCG GTA CAC C**TGTA-3'	565,7	4,9
48	5'-AGAATTGATACA**TG**GATAACTGAATCTCTTTCAACTAATAAGAGACATTATC**TCG AGT GAC T**TGTA-3'	309,0	2,7
49	5'-AGAATTGATACA**TG**GATAACTGAATCTCTTTCAACTAATAAGAGACATTATC**ACG ATG AAA T**TGTA-3'	360,3	3,1
50	5'-AGAATTGATACA**GC**GATAACTGAATCTCTTTCAACTAATAAGAGACATTATC**ACG GAG CAG T**TGTA-3'	632,8	5,5
52	5'-AGAATTGATACA**TG**GATAACTGAATCTCTTTCAACTAATAAGAGACATTATC**GCG CAT CAA T**TGTA-3'	370,5	3,2
53	5'-AGAATTGATACA**AA**GATAACTGAATCTCTTTCAACTAATAAGAGACATTATC**ACG TTA CTA C**TGTA-3'	179,6	1,6

TABLE VIII: Yeast strains used in this study

Strain	genotype	source
K699	*MATα, ura3-1, leu2-3, his3-11, trp1-1, ade2-1*	(17)
K699 *she2*	K699 *she2::KAN*	this study
YBZ1	*MATa, ura3-52, leu2-3,112, his3-200, trp1-1, ade2, LYS2:(LexAop)-lacZ, LexA-MS2-MS2 coat (N55K)*	(16)
YCO1	YBZ1 *she2::KAN*	this study

Manuscript in preparation

4.Tethering eIF4G to the *ASH1* mRNA coding sequence by Khd1p: a novel paradigm for translational regulation

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ABSTRACT

In *S. cerevisiae*, the *ASH1* mRNA is localized at the bud tip of late-anaphase cells, resulting in the exclusive sorting of Ash1p to the daughter cell nucleus. While the mechanism behind the localization of this transcript has been well studied, the regulation of its translation is still poorly understood. We now report that Khd1p regulates the translation of the *ASH1* mRNA via a new mechanism. We found that the localization element E1 of the *ASH1* mRNA, which is present within the coding sequence of this transcript, acts with Khd1p to recruit the translation initiation factor eIF4G via the C-terminal RNA-binding domain of this factor. While the interaction between Khd1p and eIF4G has no effect on the formation of the eIF4F complex, it results in a decreased level of ribosome initiation on the *ASH1* mRNA. Deletion of Khd1p or disruption of the element E1 increased the expression of Ash1p *in vivo* and lead to a defective sorting of this factor. We propose that Khd1p defines a novel mechanism of translational regulation which acts by recruiting eIF4G within the coding sequence of an mRNA in order to interfere with ribosome initiation.

INTRODUCTION

The regulation of the translation of localized mRNAs allows cells to define properly the site and time of expression of specific proteins. Among the best known examples are the *Drosophila oskar* and *nanos* mRNAs which are translationally repressed when they are not properly localized (Gavis, 1994; Kim-Ha, 1995). This regulation is often mediated by trans-acting factors which interact with cis-acting elements in the 3' untranslated region (3'UTR) of the target mRNAs (Wilkie et al., 2003). For instance, the *nanos* mRNA contains stem-loop structures in its 3'UTR that are recognized by Smaug, an RNA-binding protein responsible in part for the translational repression of this transcript (Dahanukar, 1999; Smibert, 1996). Smaug recruits Cup, an eIF4E-binding protein which block the eIF4E-eIF4G interaction and inhibits ribosome initiation on the *nanos* mRNA (Nelson, 2004).

In *S. cerevisiae*, Ash1p serves as a model system for studying the asymmetric segregation of cell-fate determinants through mRNA localization (Chartrand et al., 2001; Darzacq et al., 2003). The asymmetric sorting of Ash1p to the daughter cell nucleus correlates with the localization of the *ASH1* mRNA to the distal tip of daughter cells during the anaphase of the cell cycle (Long et al., 1997; Takizawa, 1997) and results in the inhibition of mating-type switching in the daughter cell (Jansen, 1996; Sil, 1996). The core components of the *ASH1* mRNA localization machinery have been identified and were shown to form a complex, called the "locasome", which includes Myo4p, She2p and She3p (Bertrand, 1998; Bohl et al., 2000; Long et al., 2000; Munchow et al., 1999; Takizawa and Vale, 2000). Myo4p is a type V myosin involved in the transport of the *ASH1* mRNA to the bud tip (Bertrand, 1998). She3p has been found to interact with the tail of Myo4p and with She2p, therefore acting as a bridge between these two proteins

(Bohl et al., 2000). Finally, She2p was identified as a RNA-binding protein that binds specifically to the four *ASH1* mRNA localization elements (Bohl et al., 2000; Long et al., 2000; Olivier et al., 2005).

While the factors involved in *ASH1* mRNA sorting are well known, the translational regulation of this transcript is still poorly understood. A recent study has reported that the presence of cis-acting localization elements within the coding sequence of the *ASH1* mRNA played a role in the regulation of the translation of this transcript (Chartrand, 2002). Moreover, two trans-acting factors, Puf6p and Khd1p, have been recently suggested to be involved in *ASH1* mRNA translational control (Gu et al., 2004; Irie et al., 2002). A deletion of *PUF6*, which encode for a PUF family protein, increases the translation of the *ASH1* mRNA (Gu et al., 2004). Puf6p has been shown to bind to the localization element E3 of the *ASH1* mRNA and colocalizes with this transcript at the bud tip. Puf6p is present mostly within the nucleus, an uncommon distribution for a PUF family protein, which suggests a role for Puf6p in repressing the translation of the *ASH1* mRNA before it comes in contact with the translation initiation machinery. The other protein, Khd1p, interacts within the first 900 nucleotides of the *ASH1* mRNA and it also colocalizes with this transcript at the bud tip (Irie et al., 2002). Moreover, overexpression of Khd1p lead to a decreased synthesis of Ash1p, suggesting a possible role of Khd1p in the translational control of the *ASH1* mRNA.

We now report that Khd1p is a regulator of the translation of the *ASH1* mRNA and it acts on eIF4G1 via a new mechanism. A deletion of *KHD1* results in a two to three fold increase in the expression of Ash1p and in a decreased asymmetry in Ash1p distribution. Khd1p binds the terminal stem-loop of the localization element E1 of the *ASH1* mRNA and interacts with the C-terminal domain of eIF4G1. Interestingly, the C-

terminal domain of eIF4G1 is an RNA-binding domain important for translation efficiency, and it also interacts with the localization element E1. We propose a novel mechanism for translation regulation where Khd1p recruits eIF4G1 within the coding sequence of the *ASH1* mRNA via their common interaction with the localization element E1, which leads to a decreased recruitment of ribosomes on this transcript.

RESULTS

Deletion of *KHD1* results in an increased expression of Ash1p

To establish the regulating effect of Khd1p on *ASH1* mRNA translation, we compared the expression of Ash1p in a wild type versus a *khd1*- strain. Western blot analysis revealed that deletion of *KHD1* in a yeast strain resulted in a twofold increase in myc-tagged Ash1p expression (Fig 1A). To confirm that the resulting increase in Ash1p expression was not due to an increased *ASH1* mRNA level, we measured the level of this transcript by Northern blot. As shown in Figure 1B, *ASH1* mRNA levels are equal in a wild type and in a *khd1* strains, suggesting that Khd1p acts at the level of the translation of the *ASH1* mRNA. To compare the kinetic of Ash1p synthesis in a wild-type versus a *khd1* strain, we expressed the *ASH1* mRNA from a galactose-inducible promoter and measured the synthesis of Ash1p-myc by Western blot. After one hour of galactose stimulation, we observed a two to three fold increase in Ash1p synthesis in a *khd1* yeast strain compared to a wild-type strain (Fig 1C). Northern blot analysis showed that mRNA levels are similar in both strains after one hour of galactose induction (Figure 1D). To rule out the possibility that the mere binding of a protein within the coding sequence of the *ASH1* mRNA would explain such effect on Ash1p synthesis, we performed the same induction of *ASH1* mRNA in a yeast strain deleted of the She2

protein, which also bind within the coding sequence of *ASH1* (Bohl et al., 2000; Long et al., 2000). Such deletion did not resulted in an increased Ash1p expression, suggesting a specific effect of Khd1p on Ash1p synthesis (Figure 1C).

Khd1p was previously reported to interact with the *ASH1* mRNA within the first 900 nucleotides of this transcript (Irie et al., 2002). To confirm that the effect of Khd1p on Ash1p synthesis was caused by an interaction between the *ASH1* mRNA and Khd1p, we generated fusion proteins of Ash1p and a luciferase reporter. A fragment encompassing the nucleotides 1-900 of the *ASH1* mRNA coding sequence was cloned in frame with a luciferase protein (Fig 1E). Induction of the resulting Ash1-luciferase fusion revealed a two-fold increased expression in a *khd1* strain compared to a wild-type strain (Fig.1F), suggesting that this fragment of the *ASH1* mRNA is sufficient to confer regulation by Khd1p. The expression of an Ash1-luciferase fusion which contained the nucleotides 1-621 of the *ASH1* coding sequence was not affected by the deletion of the *KHD1* gene. However, an Ash1-luciferase fusion containing the nucleotides 1 to 150 and 600 to 900 of the *ASH1* coding sequence, showed a three fold increased expression in a *khd1* strain compared to a wild-type strain (Figure 1F). Altogether, these results point to a sequence between nucleotides 621 to 900 of the *ASH1* mRNA essential for the action of Khd1p as a translational regulator of this transcript. Interestingly, this region of the *ASH1* mRNA contains the localization element E1, which raised the possibility that Khd1p may interact with this stem-loop structure.

Khd1p binds the localization element E1 of the *ASH1* mRNA

The localization element E1 folds into a long stem-loop structure (Chartrand et al., 1999) and has been shown to contain a She2p-binding motif below its terminal stem (Olivier et al., 2005) (Figure 2A). To test the possibility of an interaction between Khd1p and the localization element E1, we performed an electrophoresis mobility shift assay (EMSA) using recombinant GST-Khd1p (67kDa) and *in vitro* transcribed localization element E1 RNA. As shown in Figure 2B, a complex was observed between Khd1p and the E1 RNA. This complex was specific since only a weak shift was observed between GST-Khd1p and the iron responsive element (IRE) RNA (Fig. 2B). Multiple bands were observed at higher protein concentration, suggesting a possible dimerization of Khd1p with RNA. Such dimerization of proteins with KH domains has been reported previously (Chen et al., 1997). In a competition assay, the Khd1p-E1 RNA complex was competed by an excess of unlabelled E1 RNA, while an E1 RNA with a mutation in the terminal loop competed weakly compared to wild-type E1 RNA (mutant M6; Fig.2C). These results show that Khd1p interacts directly and specifically with the localization element E1. Futhermore, GST clivage assay will be performed to assume that GST dimerization doesn't interfere with our results.

In order to confirm that the interaction between Khd1p and the element E1 is important for the translational regulation of the *ASH1* mRNA, we tested the effect of the presence of mutations in the localization element E1 on the expression of an Ash1-luciferase reporter protein. Using an Ash1-luciferase fusion containing the nucleotides 1-900 of the *ASH1* coding sequence, we observed a three-fold increase in luciferase expression when the element E1 is mutated in the terminal stem (mutant M5; Fig.2A). The same effect was observed with an Ash1-luciferase containing only the element E1

with a mutation in the terminal loop (mutant M6; Fig.2A). However, when these two mutants were expressed in yeast strains deleted of the *KHD1* gene, no further increase in luciferase activity was observed (Fig. 2D). Altogether, these results suggest that Khd1p acts through the terminal stem-loop of the element E1 in order to regulate the translation of the *ASH1* mRNA.

Several proteins with KH domains have already been described in the literature, and for some of them, the RNA motif they recognized has been identified. KH-domains have been shown to have a preference for UCA rich sequences in RNA (Musunuru and Darnell, 2004). Interestingly, the sequence of the terminal loop of the element E1 (including the last base-pair of the stem) contains two UCA motifs: UCAU and UCAA (Figure 2A). This observation supports the finding that Khd1p interact with this region of the localization element E1.

Deletion of *KHD1* results in an increased ribosome load on the *ASH1* mRNA and in a decreased asymmetric distribution of Ash1p.

Sucrose gradient fractionation showed that some amount of Khd1p is associated with translating ribosomes (Supplementary data, Figure 1), which support the possibility of a role of Khd1p in translation regulation. To determine at which level of *ASH1* mRNA translation Khd1p may act, we used sucrose gradients to purify the various ribosome fractions from yeast cells and revealed the distribution of the *ASH1* mRNA in these fractions using dot blots. As shown in Figure 3A, the *ASH1* mRNA is mostly in the monosome and lower polysomes fractions of the gradient in a wild-type yeast strain. However, when the *KHD1* gene is deleted, the *ASH1* mRNA was redistributed toward the higher polysome fractions of the gradient (Fig. 3B). Deletion of *KHD1* had no impact on

the distribution of the *ACT1* mRNA, suggesting that the effect of Khd1p is specific to the *ASH1* mRNA. Similar results were obtained when using RT-PCR for the detection of the *ASH1* mRNA in the fractions of the polysome gradient (data not shown). Altogether, these results suggest that Khd1p reduces the level of ribosome initiation on the *ASH1* mRNA and therefore reduces the ribosome load on this transcript.

Since it has already been shown that mutants increasing the translation of *ASH1* mRNA result in a decreased asymmetric distribution of Ash1p (Chartrand, 2002; Gu et al., 2004), we inquired about the effect of a deletion of *KHD1* on the localization of Ash1p. Using immunofluorescence, we found that while 80% of late-anaphase wild-type yeasts showed an asymmetric localization of Ash1p in the daughter cell nucleus, only 40% of *khd1* yeasts still had Ash1p segregated to the daughter cell nucleus (Table 1). These results are similar to the those obtained in strains where the secondary structure of all four localization was disrupted (Chartrand, 2002) and to a *puf6* strain (Gu et al., 2004). To determine if this defective sorting of Ash1p was caused by a decreased localization of the *ASH1* mRNA at the bud tip in a *khd1* strain, we verified the localization of this transcript by FISH. As shown in Table 1, we observed no defect in *ASH1* mRNA localization, suggesting that the regulation of Ash1p translation by Khd1p is important for the proper localization of this factor to the daughter cell nucleus.

Khd1p interacts with the C-terminal domain of eIF4G1 in an RNA-dependent manner

Large scale affinity purification assays have shown that Khd1p is associated *in vivo* with several proteins involved in translation initiation: eIF4E, eIF4G1 and 2, Cbp80 and the β subunit of eIF2 (Gavin AC, 2002). Among these proteins, eIF4E and eIF4G are

the two which are the most frequently targeted by translation regulation factors (Gingras AC, 1999). In order to identify the mechanism by which Khd1p regulates the translation of the *ASH1* mRNA, we characterized the interaction between Khd1p and these translation initiation factors. Using a yeast protein extract containing Khd1p-TAP in a GST-pulldown assay, we found that Khd1p-TAP was retained by an immobilized GST-eIF4G1 recombinant protein, but not by GST alone (Fig 4A). Treatment with RNAse A disrupted this complex, suggesting that the interaction is RNA-dependent. We were unsuccessful to detect an interaction between Khd1p and the cap binding protein eIF4E *in vitro* (data not shown), thus ruling out the possibility of Khd1p being able to sequester eIF4E.

In order to identify the Khd1p binding site on eIF4G1, we prepared recombinant GST fusions of deletion mutants of eIF4G1 and tested these deletions in GST-pulldown assays with the TAP-tagged Khd1 protein. Our results show that only the GST fusions that contain the C-terminal domain of eIF4G1, from amino acids 850 to 952, were capable of binding to Khd1p (Fig. 4B). Whereas Khd1p binds to the C-terminal domain of eIF4G1, this binding site does not overlap with any other binding sites of known eIF4G-binding factors, like eIF4E, Pab1p, Cbp80p or eIF4A (Prevot et al., 2003). However, we cannot rule out the possibility that the binding of Khd1p to eIF4G1 affects the recruitment of one of the translation initiation factors, leading to a reduced translation initiation. For instance, in the interaction between eIF4E and Cbp80 to eIF4G1, it has been shown that when one of these factors is bound to eIF4G1, the second factor has a decreased affinity for its binding site on eIF4G1 (Fortes et al., 2000). In order to verify this possibility, we used increasing amounts of purified eIF4E, Pab1p and eIF4A to compete the interaction between Khd1p and eIF4G1 *in vitro*. As shown in Figure 4C-F,

the interaction between Khd1p and eIF4G1 was not competed by either of these translation initiation factors, suggesting that the binding of Khd1p to eIF4G1 does not affect the formation of the translation initiation complex. Since a previous study has shown that the C-terminal domain of eIF4G1 is able to bind RNA (Berset et al., 2003), we investigated the ability of poly(U) RNA to interfere in the Khd1p-eIF4G1 interaction. Using the GST-pulldown assay, increasing concentrations of poly(U) RNA was found to compete the binding of Khd1p to eIF4G1 (Fig. 4F). This result underlines the importance of RNA in the interaction between eIF4G1 and Khd1p.

The C-terminal domain of eIF4G1 interacts with the localization element E1

Since the C-terminal domain of eIF4G1 has been shown to bind RNA (Berset et al., 2003) and since the Khd1p-eIF4G1 interaction is RNA-dependent, the possibility that the C-terminal domain of eIF4G1 can bind the localization element E1 was investigated. To determine if eIF4G1 can interact directly with the localization element E1, we used purified GST-eIF4G1 and ^{32}P labelled localization element E1 RNA in a EMSA assay. As shown in Figure 5A, increasing concentration of GST-eIF4G1 (132 kDa) resulted in a shift in the migration of the E1 RNA, indicating a direct interaction. A weaker interaction was observed using the Iron Responsive Element (IRE) RNA, suggesting that eIF4G1 has a higher affinity for the E1 RNA. To verify that the interaction between the element E1 and eIF4G1 is dependent on the C-terminal domain of eIF4G1, we did the same band shift assay with purified GST-eIF4G1$_{850\text{-}952}$ (eIF4G1-Cterm) and found that a complex was indeed formed between the C-terminal domain of eIF4G1 and the E1 RNA (Figure 5B). Unlike the full-length eIF4G1, the C-terminal domain is less specific *in vitro* since it interacted with the IRE RNA at a similar affinity than the element E1 RNA.

In order to confirm these results *in vivo*, we verified the interaction between the C-terminal domain of eIF4G1 (eIF4G-Cterm) and the localization element E1 in a yeast three-hybrid assay. As demonstrated in Figure 5C, this domain of eIF4G1 showed a specific interaction with the localization element E1 *in vivo*. This interaction is disrupted by a mutation in the terminal stem of the element E1 (mutant E1-M5, fig 2D). eIF4G-Cterm did not show any interaction with the localization element E2B-D1, which fold in a stem-loop structure like the element E1, or with an unstructured region of the element E2B (E2B-D2). Deletion of the *KHD1* gene did not affect the interaction between eIF4G-Cterm and the element E1, suggesting that this interaction is not mediated via Khd1p. Altogether, these results show that the C-terminal domain of eIF4G1 binds directly to the localization element E1 and this interaction is specific *in vivo*.

DISCUSSION

In the current study, we present several evidences that Khd1p is a regulator of *ASH1* mRNA translation and it acts via a new mechanism of translational control. Previous evidences on the role of Khd1p in the control of *ASH1* mRNA translation came from the overexpression of this factor, which lead to a reduction of the level of Ash1p in yeast (Irie et al., 2002). Also, Irie et al. have shown that Khd1p binds and colocalizes with the *ASH1* mRNA at the bud tip, and that the *KHD1* locus genetically interacted with a *myo4/she1* mutant gene, which suggest that Khd1p affects the asymmetric distribution of Ash1p at a different step than the She proteins (Irie et al., 2002). We now report that the deletion of the *KHD1* gene resulted in an increased synthesis of Ash1 protein, which support the previous observations. Khd1p interacts directly with the terminal stem-loop structure of the localization element E1. It also interacts with the C-terminal domain of

eIF4G1, which contains a RNA-binding domain. We have shown that the complexes Khd1p-E1 and eIF4G-E1 are formed *in vivo* and *in vitro*, and regulate the translation of the *ASH1* mRNA by reducing the level of ribosome initiation on this transcript.

The finding that Khd1p regulates the translation of the *ASH1* mRNA raises the question of the function of this regulation in the proper sorting of the Ash1 protein. Previous findings rule out the possibility that Khd1p may repress the translation of unlocalized *ASH1* mRNA, like the *Drosophila* Bruno or Smaug proteins do with the *oskar* and *nanos* mRNAs, respectively (Dahanukar, 1999; Kim-Ha, 1995), since delocalized *ASH1* mRNA is still properly translated (Chartrand, 2002; Long et al., 1997). Another possibility is that Khd1p may repress the translation of the *ASH1* mRNA during its transport in order to avoid any premature synthesis of Ash1 protein before the localization of this transcript, and release the mRNA when it gets localized. This way, Khd1p controls the level of Ash1p produced and avoid any undesirable leakage of Ash1p to the mother cell before cytokinesis. Such observation has already been reported: using an *ASH1* mRNA with all four localization elements in the 3'UTR, Chartrand et al. observed a decreased asymmetric distribution of Ash1p even if this *ASH1* mRNA was still properly localized (Chartrand, 2002). By reducing the level of *ASH1* mRNA translation using a short stem-loop cloned in the 5'UTR of this transcript, the proper sorting of Ash1p was restored. In this case, it is possible that by moving the localization element E1 in the 3'UTR, Khd1p was unable to reduce the translation of the *ASH1* transcript, which resulted in an increased production of Ash1p and in its leakage to the mother cell.

In this study, we report that Khd1p acts on translation through its interaction with eIF4G. The role of eIF4G in translation is crucial and altering the function of this protein greatly affects the cell ability to synthesize proteins. Therefore, eIF4G or the interaction between eIF4G and other translation initiation factors are frequent targets of translation regulation factors (Gingras AC, 1999; Prevot et al., 2003). The interaction between eIF4G and eIF4E is competed by proteins like Cup, eIF4EBPs and Maskin (Mader et al., 1995; Stebbins-Boaz, 2000; Wilhelm et al., 2003). The rotavirus protein NSP3 binds eIF4G and competes for PABP in order to enhance the translation of viral transcripts (Piron, 1998). Many picornaviruses express proteases that cleave eIF4G in order to privilege cap-independent viral RNA translation, hence shutting down the host cell protein expression (Gradi A., 2003). All these mechanisms of translational repression have one aim in common: to inhibit the formation of a functional eIF4F initiation complex on the mRNAs. Unlike the previous examples, the interaction between Khd1p and eIF4G1 does not seem to affect the formation of the eIF4F initiation complex: first, we showed that the Khd1p-eIF4G complex is not competed by other eIF4G-binding partners; second, translation of the *ASH1* mRNA still occurs in the presence of Khd1p, although at a decreased level.

This study also revealed that Khd1p is an unconventional regulator of translation. First, unlike most known translational repressors or activators which interact with mRNAs in their 5' or 3' untranslated regions (Moor et al., 2005; Pickering and Willis, 2005), Khd1p is instead recruited within the coding sequence of the mRNA it regulates, without having any function for translation activation or inhibition. To our knowledge,

this is the only known translational regulator to do so. Second, Khd1p interacts with the C-terminal domain of eIF4G1, a domain important for translation efficiency (Berset et al., 2003), but not a common target of translation regulation factors. Unlike the mammalian eIF4G, the yeast eIF4G C-terminal domain does not bind the kinase Mnk1, but instead it has been found to bind RNA (Berset et al., 2003). A previous study suggested that this RNA-binding domain may be involved in the recruitment of the ribosomes to the translated mRNAs by interacting with ribosomal RNA (Berset et al., 2003). Our results suggest instead that this RNA-binding domain of eIF4G interacts with the translated mRNA and that it has more specificity for some RNA motifs. While the RNA-binding domain of eIF4G is used as a target for translational control of the *ASH1* mRNA, for most yeast mRNAs, it may be used to anchor or position the C-terminal domain of eIF4G within the 5'UTR of the transcripts in order to properly define the landing site of the 40S small subunit.

Therefore, we propose a novel mechanism of translational regulation in which the localization element E1 and Khd1p recruits eIF4G within the coding sequence of the *ASH1* mRNA (Figure 6). Since eIF4G can be seen as a "launching pad" used by eIF4A and the 40S small subunit to position their landing site within the 5'UTR of mRNAs, the Khd1p-E1 complex may modify the landing site of the 40S small subunit by diverting the eIF4G "launching pad" toward the coding sequence of the mRNA. This may result in initiation of translation at inappropriate initiation codons, in another reading frame, or in premature termination. This mechanism has analogies to the ribosome "shunting" in adenoviral mRNAs, where the interaction between eIF4G and the viral 100K protein with the tripartite leader structure in the 5'UTR of viral mRNAs promotes the direct recruitment of the 40S subunit at the initiation codon, bypassing large segments of the

5'UTR (Xi et al., 2004). Interestingly, the viral 100k protein promotes shunting by interacting with the C-terminal domain of eIF4G (Xi et al., 2004). Moreover, bringing eIF4G close to a strong stem-loop structure, like the element E1, would decrease the efficiency of scanning by the 40S subunit. Indeed, this situation is similar to the introduction of a stem-loop structure close to the 5' capped end of an mRNA, which has been previously shown to decrease the efficiency of translation initiation (Vega Laso et al., 1993).

MATERIAL AND METHODS

Growth media and yeast strains

Yeast cells were grown in either synthetic growth media lacking the nutrients indicated or rich media (Rose, 1990). Transformation was performed according to the protocol of Gietz and Schiestl (Schiestl, 1989). Yeast gene disruption cassette was created by PCR amplification of the *loxP*-KAN-*loxP* construct in plasmid pUG6 and primers specifics for the gene of interest (Guldener et al., 1996). Specific disruption was confirmed by PCR analysis of genomic DNA. Integration of myc-tag to endogenous proteins was done accordingly to Longtine et al. (Longtine, 1998). Yeasts strains used in this study are described in Table 2.

Plasmid constructions

Plasmid YIP128-*GAL1prom-ASH1-MYC* is described in Chartrand et al. (2002). Plasmids containing *ASH1*-luciferase fusions were made from the *Firefly* luciferase of the pGL3 plasmid (Clontech). All luciferase fusions are in-frame with the Ash1 protein. The luciferase fusion containing the $ASH1_{1\text{-}900}$ fragment was obtained by subcloning the luciferase gene in the NcoI and SalI sites of YIP128-*Gal1prom-ASH1-MYC*. The

luciferase fusion containing the *ASH1*$_{1-621}$ fragment was created by subcloning a *GAL1prom-ASH1*$_{1-621}$ fragment generated by PCR with Pst1-Nco1 sites in the YIP128-*Gal1prom-ASH1*$_{1-990}$*-Luc* plasmid digested with Pst1-Nco1. Constructions containing the localization element E1 (WT and M6 mutant) from *ASH1* were made by PCR from a pGEM-E1 plasmid with primers containing AvrII and NcoI sites and subcloned in the YIP128-*Gal1prom-ASH1*$_{1-990}$*-Luc* digested with AvrII/NcoI. The *KHD1* open reading frame was cloned in the pGADT7 plasmid by recombination in yeast. After plasmid purification from yeast cells, the pGADT7-*KHD1* plasmid was transformed in bacteria. The pGEX-*KHD1* plasmid was created by EcoR1-XhoI digestion of the pGADT7-*KHD1* plasmid and subcloning in the pGEX-5X-3 plasmid digested with EcoR1-XhoI. GST fusions of eIF4G were generated by PCR amplification. The pTIF4631 plasmid, containing the eIF4G1 gene under the control of the *TIF4632* promoter, is a generous gift from M. Ashe (UMIST, UK). Construction of the eIF4G1$_{\Delta 2600}$ deletion was obtained by PCR amplification of the *TIF4631* open reading frame from nucleotide 1 to 2600. The PCR product was subcloned in the pTIF4631 plasmid previously digested with EcoRI and BamH1. Plasmid for the dual luciferase assay was generated from the pESC-HIS plasmid (Stratagene). This plasmid has both *Renilla* and *Firefly* luciferase, each under the control of a galactose promoter. The *Renilla* luciferase was obtained by PCR from the plasmid pHR-LUC-N1 (gift from Michel Bouvier) and cloned at the EcoR1 site of pESC-HIS to generate pESC(HR-LUC). The *ASH1*$_{1-900}$*-Luc* gene was cloned at the BamH1 site of the pESC(HR-LUC).

Ash1p kinetics and Western blots

Yeast cells were grown in 600 ml of non-inducible, non-repressive medium (2% lactic acid, 3% glycerol) up to an OD_{600} of 0.2-0.4, induced with galactose and 50 ml samples

were taken at different time points, to which NaN_3 (6 mM final concentration) was added to stop all reactions. Yeast cells were processed with 500 µl of glass beads mixed with 500 µl of sample buffer (60 mM Tris pH 6.8, 0.5mM PMSF, 0.5 mM Benzamidine, pepstatin, leupeptin, aprotinin, 10% glycerol, 2% SDS, 5% ß-mercaptoethanol and 0.0025% Bromophenol Blue). The tubes were vortex 45 seconds and put on ice 45 seconds, repeated 4 times. After a centrifugation of 5 minutes, the supernatant was recovered. For the Western blot, 20 µl of yeast extracts was loaded on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. Ash1p-myc was detected using a mouse anti-myc antibody (9E10; Roche) and Pgk1p was detected with a mouse anti-Pgk1 antibody (Molecular Probes), following by a HRP-labelled goat anti-mouse secondary antibody (Jackson Laboratories) and revealed with the ECL kit (AmershamPharmacia). Western blots were quantified using the Scion Image software (NIH Image). The results are the average of at least three independent experiments.

Northern Blots

RNA was purified from 50mL of yeast culture at an OD_{600} of 0.8 according to (Schmitt et al., 1990). Northern Blots were performed according to Sambrook and Russell (Sambrook, 2001). *ASH1* and *ACT1* probes were labelled with [α-^{32}P] dCTP using Ready-To-Go DNA Labelling Beads -dCTP (Amersham).

Fluorescence in situ hybridization and immunofluorescence

Yeast cells were processed for fluorescent in-situ hybridization and immunofluorescence according to the protocols described in (Chartrand, 2000). For in-situ hybridization, yeast spheroplasts were hybridized with a pool of Cy3-conjugated *ASH1* DNA oligonucleotide probes. For immunofluorescence, a 1:50 dilution of a mouse anti-myc 9E10 antibody

(Roche) was used as primary antibody. For the secondary antibody, a 1:1000 dilution of a Donkey anti-Mouse Oregon Green-conjugated antibody (Jackson Laboratories) was used.

Sucrose gradients and dot blots

Yeast cultures were grown in the selection medium and reached stationary phase ($D.O._{600}$ 1.0 to 1.5). Polysome disassembly was inhibited by adding 100ug/ml cycloheximide (Sigma) for 15 minutes on ice. Cells were harvested by centrifugation 10 minutes at 4000 rpm, washed twice with water (10 ml) containing 100ug/ml cycloheximide and lysed with glass beads and 1X lysis buffer (25mM Tris HCl pH 7.2, 50mM KCl, 30mM $MgCl_2$, 5mM β-mercaptoethanol, 200ug/ml cycloheximide, 2ug/ml aprotonin, 1mM PMSF, 0,5ug/ml leupeptin, 2,9 ug/ml E64, 1ug/ml antipaine, 0.2ug/ml chymostatine and 20mM VRC). Cells supernatant was transferred into an Eppendorf tube and centrifuged 10 minutes at 6500 rpm. at 4°C, and a second time at 14000 rpm. Finally, 300 ul to 500 ul of supernatant were loaded on a 10-50 % sucrose gradient (10 mM Tris pH 7.4, 70 mM NH_4Cl, 4mM MgOAc) and centrifuged at 36000 rpm. for 2.5 hours at 4°C with a Beckman SW41 rotor (210 000G). For each gradient, 21 fractions of 500 µl were collected in Eppendorf tubes while the gradient profile was monitored at OD_{254}. Each fraction was phenol extracted and the RNA was precipitated with ethanol.

Dot blot assay was performed on sucrose gradient fractions. Briefly, samples were first denatured at 65°C for 5 minutes and then applied on Hybond-N membrane (Amersham). Samples crosslinked by UV were washed 2 hours at 68°C with hybridization solution (0,5M $NaHPO_4$, 7% SDS, 1mM EDTA). Finally, the probe was added for 12 hours at 68°C with hybridization solution.

GST pulldowns

GST fusions of eIF4G1 were purified according to (Tarun, 1996). Briefly, transformed BL21 cells were grown to an OD_{600} of 0.6 in 1L and induced with 0.5mM IPTG for 4 hours. Cells were then centrifuged at 3700rpm for 20min, washed with Buffer C (150mM NaCl, 16mM Na_2HPO_4, 4mM NaH_2PO_4), centrifuged again and resuspended in 10mL which were aliquoted in 2mL and frozen at -80°C. Aliquot containing recombinant GST-eIF4G1 was thawed, brought to 0.1% Triton X-100, 87.5µg/mL PMSF, 5 µg/mL pepstatin, 5 µg/mL aprotinin, 5 µg/mL leupeptin and sonicated 10 sec then put on ice 5 times. Extract was cleared by centrifuging 5min at 3700rpm and supernatant was incubated 1.5 hours at 4°C with 120µL of glutathion-sepharose-4B (Amersham) and washed three times with buffer C, 0.1% Triton X-100. Strain S288C-Khd1-TAP was grown to an OD_{600} of 1 and an 100 OD_{600} were broken with glass beads in 1 ml of buffer A (PBS 1X, 0.1% triton X-100, 87.5µg/mL PMSF, 5 µg/mL pepstatin, 5 µg/mL aprotinin, 5 µg/mL leupeptin). Extract was cleared by centrifugation for 1min at 13000rpm. The supernatant was applied on immobilised GST-eIF4G1 for 2 hours at 4°C. Beads were washed 5 times in buffer A and eluted by boiling in loading buffer for 3 min. Eluted proteins were loaded on a 10% SDS-PAGE gel and transferred to Hybond-ECL nitrocellulose membrane (Amersham). The membrane was first incubated with a Goat anti-mouse-HRP (1:1000) (Amersham) and secondly with a Rabbit anti-Goat-HRP (1:1000) (Sigma) and revealed with the ECL-kit (Amersham). Recombinant His tagged Pab1p was purified according to (Amrani et al., 1997). Recombinant His-tagged eIF4A was purified according to (He et al., 2003). Recombinant eIF4E was purified according to a protocol from Jon Lorsch.

Electrophoretic mobility shift assays

To produce *in vitro* transcribed RNA, plasmids pGEM-E1 and pGEM-IRE were linearized with EcoR1, while plasmid pGEM-E1(M6) was linearized with Pst1, and transcribed with T7 RNA polymerase in the presence of [α-^{32}P]CTP (Jorgen, 1998). The transcripts were purified on 6% denaturing polyacrylamide gels, then extracted, and desalted on G25 Sephadex spin columns (Roche). For unlabelled RNAs, the transcripts were purified, after treatment with DNAse1, by phenol/chloroform extraction, ethanol precipitation and desalting on G25 Sephadex spin columns. GST-Khd1p was expressed and purified according to the protocol published by Pharmacia. For electrophoretic mobility shift assays, the ^{32}P-labelled RNA was denatured by heating at 85°C for 2 minutes in the binding buffer and allowed to fold at room temperature for 10 minutes. A total of 10 000 CPM of labelled RNA (~ 1 ng) were added to the binding buffer (10 mM Hepes pH. 7.4, 150 mM KCl, 1 mM DTT, 5 mM $MgCl_2$, 4 % glycerol, 15 U RNAse Inhibitor (Pharmacia)) prior to the addition of various concentration of recombinant protein, up to a final volume of 20 µl. The reactions were incubated at 4°C for 30 minutes, then 2 µl of 10 mg/ml heparin was added and incubated for 10 more minutes at 4°C to prevent non-specific interactions. The samples were separated on a 4% non-denaturing gel at 120V for 4hrs at 4°C, dried and exposed overnight with Kodak films. For competition experiments, unlabelled RNAs were added prior to the addition of the protein.

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FIGURE LEGENDS

Figure 1: Deletion of *KHD1* results in an increased expression of Ash1p. (A) Detection of endogenous levels of Ash1p and Pgk1p in a wild-type strain (lane 1), *khd1* strain (lane 2), and *ash1* strain (lane 3) by Western blot. (B) Endogenous levels of *ASH1* mRNA and *ACT1* mRNA in a wild-type strain (lane 1), *khd1* strain (lane 2), and *ash1* strain (lane 3), detected by Northern blot. (C) Kinetics of Ash1p synthesis after galactose induction in a wild-type strain (♦), *khd1* strain (▲), and *she2* strain (■). (D) mRNA levels of *ASH1* mRNA normalized to *ACT1* mRNA after one hour of induction in WT, *khd1*, and *she2* strains. (E) Schematic representation of the *ASH1* constructions used for luciferase assays. All the *ASH1* fragments are in frame with the *Firefly* luciferase ORF. (F) Relative luciferase activity normalized to total protein concentration. Labels 1-900, 1-621, and E1 refer to regions of the *ASH1* gene fused to luciferase. Black columns correspond to WT strain while grey columns are for *khd1* strain.

Figure 2: Khd1p binds the localization element E1. (A) Secondary structure of the localization element E1. The nucleotides are numbered starting from the adenosine of the start codon as +1. Regions mutated in mutants M5 and M6 are labelled. (B) Electrophoresis mobility shift assay for GST-Khd1 with localization element E1 and the IRE RNA. Increasing concentration of recombinant GST-Khd1p (0, 0.3, 0.6, 1.5, 3, 9 μM) was added to ^{32}P labelled RNA. The free RNA is indicated by an asterisk. (C) Competition of the Khd1-E1 RNA complex by unlabelled E1 RNA and a M6 mutated E1 RNA (0.22 uM, 0.73 uM and 1.47 uM). Increasing concentration of recombinant GST-Khd1p (0, 1.5, 9 μM) was added to P^{32} labelled E1 RNA without competitor RNA. For

constant concentration of GST-Khd1p (15µg) was combined to an increasing concentration of unlabelled RNA (10x, 50x, 150x). (D). Relative luciferase activity normalized to protein concentration of *ASH1* mutants E1-M5 and E1-M6 compared to wild-type localization element E1 in wild-type (black) and *khd1* (grey) strain. 1-900 and E1 refer to regions of the *ASH1* gene fused to luciferase.

Figure 3: Deletion of *KHD1* results in a redistribution of *ASH1* mRNA to the higher polysome fractions. Dotted lines indicated the peak fractions of *ASH1* mRNA. (A) Sucrose gradient and relative distribution of *ASH1* mRNA and *ACT1* mRNA in a WT strain. (B) Sucrose gradient and relative distribution of *ASH1* mRNA and *ACT1* mRNA in a *khd1* strain.

Figure 4: Khd1p interacts with eIF4G1 in a RNA-dependent manner. (A) GST pulldown assays of yeast extract containing Khd1p-TAP with recombinant GST-eIF4G1. Lane 1: Yeast protein extract with untagged Khd1p. Lane 2: Yeast protein extract containing Khd1p-TAP. Lane 3: Pulldown with GST. Lane 4: Pulldown with GST-eIF4G1. Lane 5: RNAse A treated extract and pulldown with GST-eIF4G1. (B) GST pulldown assays of yeast extract containing Khd1p-TAP with various deletions of recombinant GST-eIF4G1. eIF4G1 and its interaction domains are schematically represented. Numbers represent amino acids of eIF4G1 in fusion with GST. Lane 1: input yeast extract. Lane 2: pulldown with GST. Lane 3: pulldown with GST-eIF4G1 deletions. (C-F) eIF4G1-binding proteins do not affect the formation of the Khd1p-eIF4G1 complex. Western Blots for Khd1p-TAP after pulldown with GST-eIF4G1. Interaction was subjected to increasing amount of competitors. (C) eIF4E: Yeast extract with untagged Khd1p (lane

10.0µg of recombinant Pab1p (lanes 5, 6, 7 and 8). (E) eIF4A: Yeast extract with untagged Khd1p (lane 1), Yeast extract containing Khd1p-TAP (lane 2), pulldown with GST (lane 3), pulldown with GST-eIF4G1 (lane 4), pulldown with GST-eIF4G1 and 5, 10, and 15µg of recombinant eIF4A (lanes 5, 6, and 7). (F) Poly(U) RNA: Yeast extract with untagged Khd1p (lane 1), Yeast extract containing Khd1p-TAP (lane 2), pulldown with GST (lane 3), pulldown with GST-eIF4G1 (lane 4), pulldown with GST-eIF4G1 and 5, 10, and 15µg of poly(U) RNA (lanes 5, 6, and 7).

Figure 5: eIF4G1 interacts with the localization element E1 RNA. (A) eIF4G1 interacts with the localization element E1 RNA *in vitro*. Electrophoresis mobility shift assay of recombinant GST-eIF4G1 with P^{32}-labelled E1 RNA and IRE RNA. Increasing concentration of recombinant GST-eIF4G1 (0, 30, 60, 150, 300, 900 nM) was added to P^{32} labelled RNA. Free RNA is indicated by an asterisk. (B) The C-terminal domain of eIF4G1 interacts with the localization element E1 *in vitro*. Electrophoresis mobility shift assay of recombinant GST fusion of C-terminal domain of eIF4G1 with P^{32}-labelled E1 RNA and IRE RNA. Increasing concentration of recombinant GST-eIF4G1-Cterm (0, 0.1, 0.2, 0.5, 1.1, 3.2 nM) was added to P^{32} labelled RNA. Free RNA is indicated by an asterisk. (C) The C-terminal domain of eIF4G1 interacts with the localization element E1 *in vivo* in a yeast three-hybrid assay. Yeast strain YBZ1 expressing the MS2 coat protein fused to LexA was transformed with a vector expressing the C-terminal domain of eIF4G1 fused to the GAL4 activation domain, and a vector expressing a RNA fusion with tandem MS2 binding sites. An interaction between the eIF4G-Cterm-GAL4 fusion and the hybrid RNA allows the activation of the reporter gene (*HIS3*) and growth on medium lacking histidine. MS2-RNA fusions contained the element E1 WT and E1-M5, the

coat protein fused to LexA was transformed with a vector expressing the C-terminal domain of eIF4G1 fused to the GAL4 activation domain, and a vector expressing a RNA fusion with tandem MS2 binding sites. An interaction between the eIF4G-Cterm-GAL4 fusion and the hybrid RNA allows the activation of the reporter gene (*HIS3*) and growth on medium lacking histidine. MS2-RNA fusions contained the element E1 WT and E1-M5, the domains 1 and 2 of the element E2B (E2B-D1 and E2B-D2). The three-hybrid assay was performed in the YBZ1 strain with wild-type (left) or deleted (right) *KHD1* gene.

Figure 6: Model of the mechanism of action of Khd1p on *ASH1* mRNA translation. (A) Initiation of translation in a normal mRNA. (B) Regulation of translation of the *ASH1* mRNA by Khd1p. The localization element E1, located ~700 nucleotides after the start codon, interacts with the C-terminal domain of eIF4G1. Khd1p interacts with both eIF4G1 and the element E1, and stabilizes this interaction. eIF4G1 recruits the 40S small subunit and promotes its landing near the localization element E1. This may result in premature termination or initiation in another frame by the ribosome. Another possibility is that the strong secondary structure of the element E1 inhibits the scanning of the 40S subunit. In all cases, it results in a decreased synthesis of full-length Ash1p.

TABLE 1: *ASH1* mRNA localization and Ash1p distribution in *KHD1* wild-type and knockout yeast cells[a].

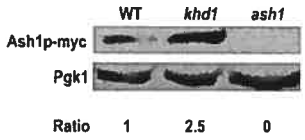
genotype	*ASH1* mRNA			Ash1p	
	bud tip	bud	delocalized	asymmetric	symmetric
KHD1	70	20	10	85±3	15±3
khd1	70	18	12	43±3	57±3

[a]. Values are in %. Scoring of *ASH1* mRNA localization and Ash1p distribution was performed as in Chartrand et al. (2002).

TABLE 2: Yeast strains used in this study.

strain	genotype	source
K4452-*cla4*	*Matα, ura3, leu2, his3, trp1, HO-ADE2, HO-CAN1, cla4Δ*	Chartrand, 2002
K4452-*cla4, khd1*	K4452, *khd1::KAN*	this study
K6278-*cla4*	*Mat a, ura3-1, leu2-3, his3-11, trp1-1, ade2-1, ash1::TRP1, cla4Δ*	Chartrand, 2002
K6278-*cla4, khd1*	K6278, *khd1::KAN*	this study
K699	*Mat a, ura3-1, leu2-3, his3-11, trp1-1, ade2-1,*	Jansen, 1996
K699-*khd1*	K699, *khd1::KAN*	this study
K699-*she2*	K699, *she2::KAN*	this study
K5552	K699, *ASH1::ASH1-myc9*	Long, 1997
K5552-*khd1*	K5552, *khd1::KAN*	this study
S288C	*Mat a, his3Δ1, leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystem
S288C-Khd1-TAP	S288C *KHD1-TAP::HIS3*	Open Biosystem
YBZ1	*MAT**a**, ura3-52, leu2-3, 112, his3-200, trp1-1, ade2, LYS2:: (LexAop)-HIS3, ura3 :: (lexA-op)-lacZ, LexA-MS2 coat (N55K)*	Hook, 2005
YBZ1-*khd1*	YBZ1, *khd1::KAN*	this study
YMK328	*Mat a, ade2, his3, leu2, trp1, ura3, tif4631::LEU2 tif4632::ura3*, p[TIF4632 *URA3 CEN*]	Mark Ashe

A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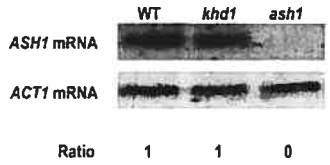
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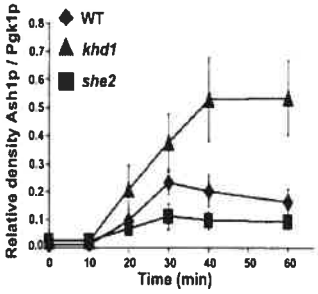
ash1
ASH1 mRNA
ACT1 mRNA
Ratio 1 1 0

C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khd1
she2
Relative density Ash1p / Pgk1p
Time (min)

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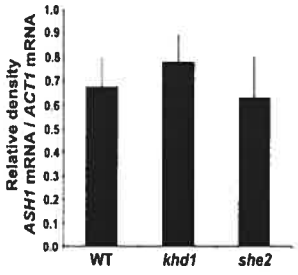
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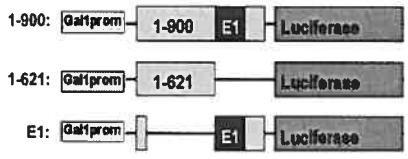
ASH1 mRNA / ACT1 mRNA
WT
khd1
she2

E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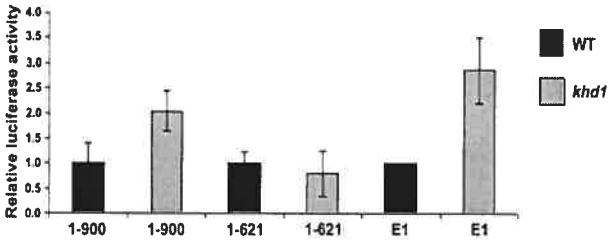
Luciferase

F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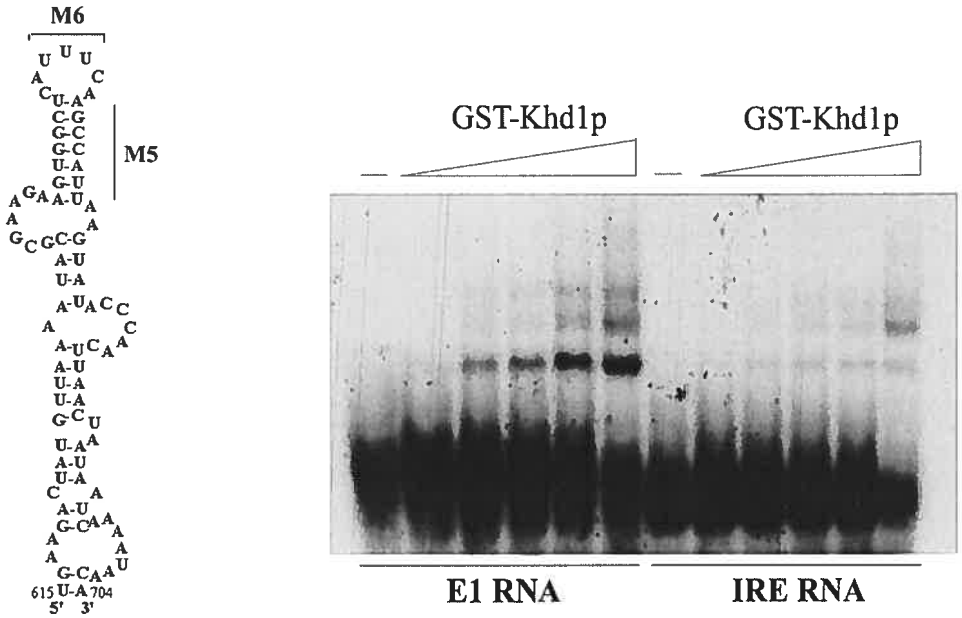
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E1 RNA
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D
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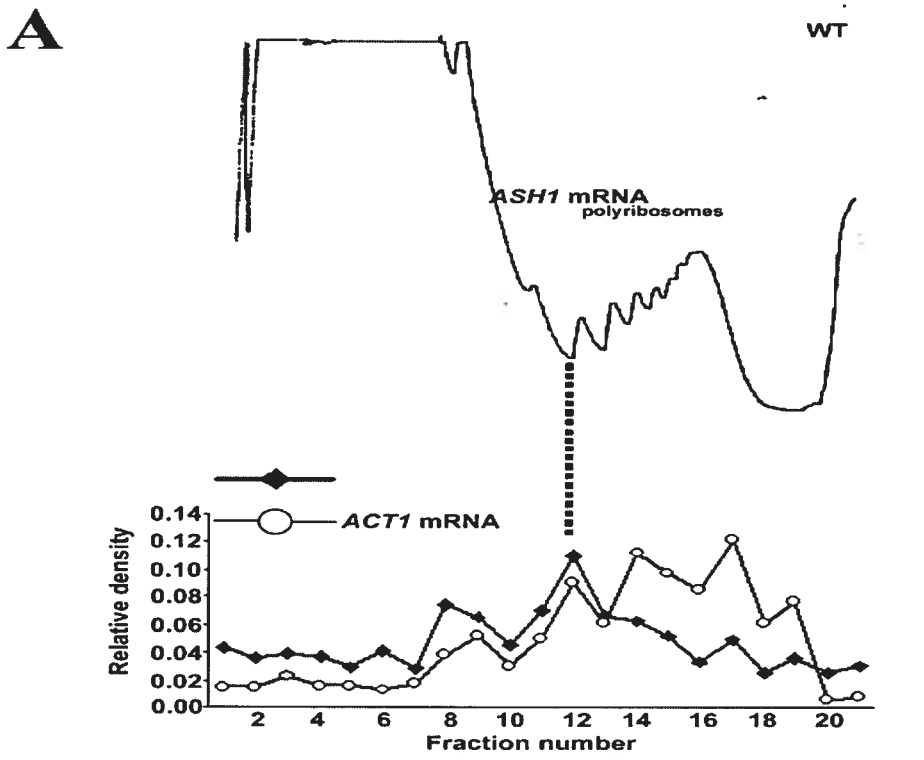
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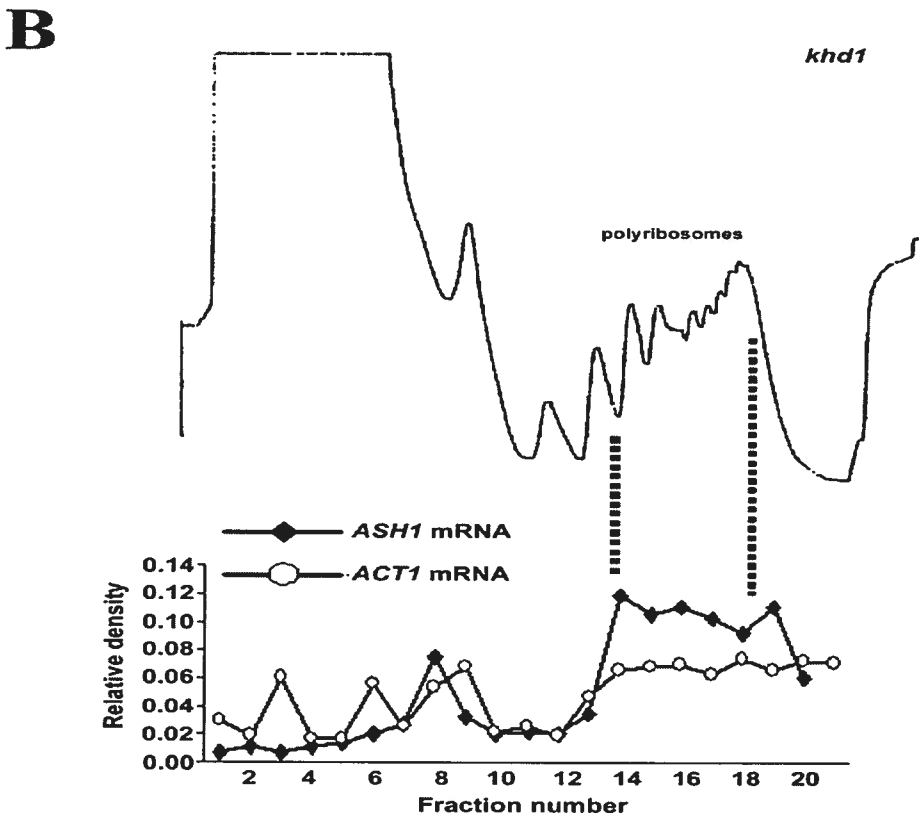
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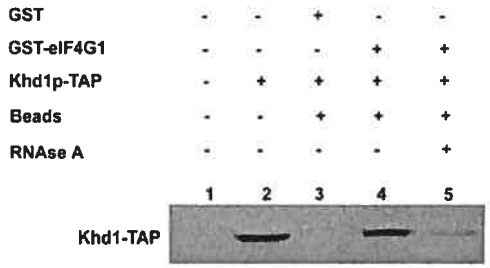
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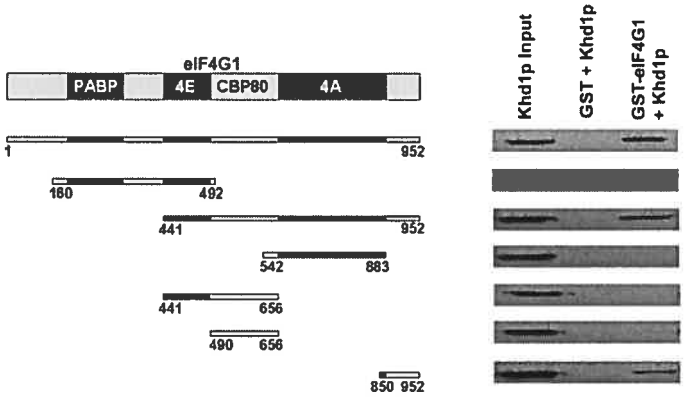
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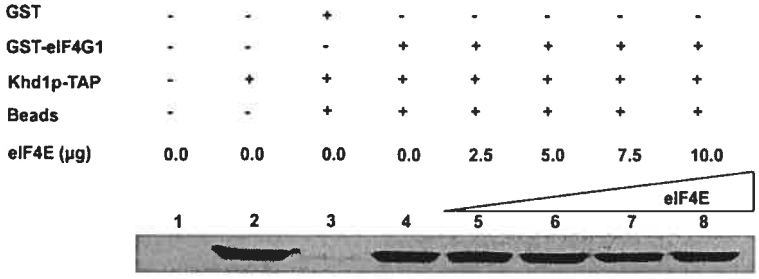
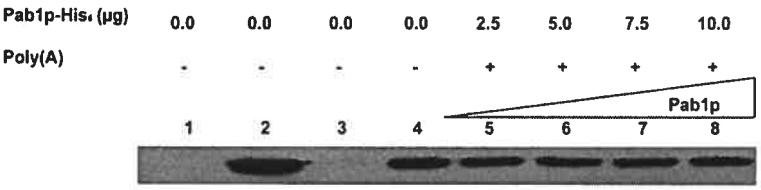
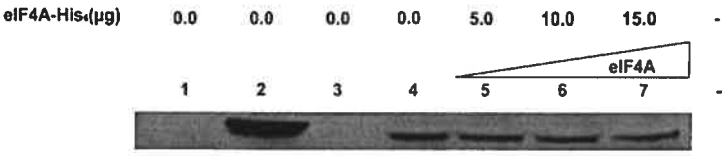

GST
GST-eIF4G1
Khd1p-TAP
Beads
eIF4E (µg)
0.0 0.0 0.0 0.0 2.5 5.0 7.5 10.0
eIF4E
1 2 3 4 5 6 7 8

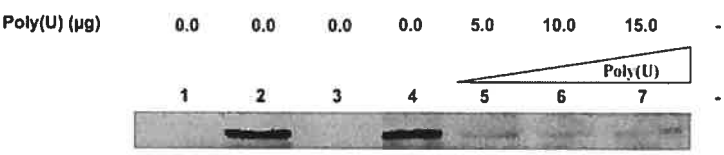
D

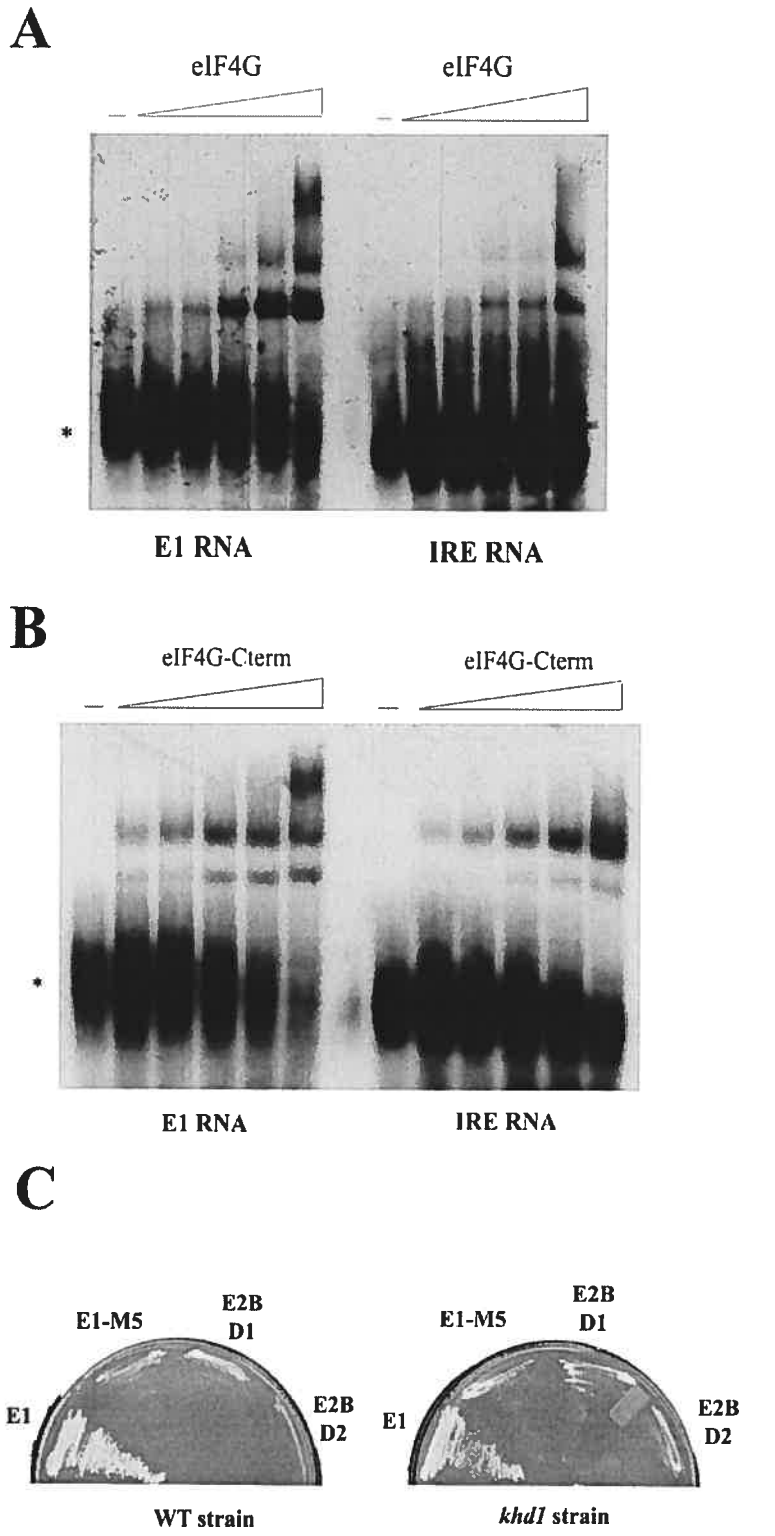

Pab1p-His6 (µg)
0.0 0.0 0.0 0.0 2.5 5.0 7.5 10.0
Poly(A)
Pab1p
1 2 3 4 5 6 7 8

E

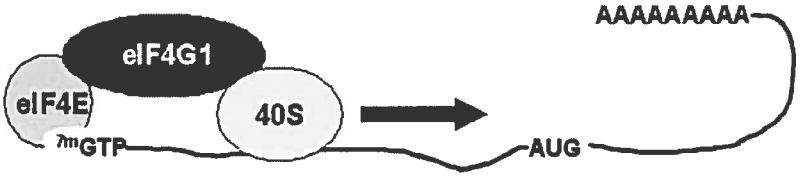

eIF4A-His6(µg)
0.0 0.0 0.0 0.0 5.0 10.0 15.0 -
eIF4A
1 2 3 4 5 6 7 -

F

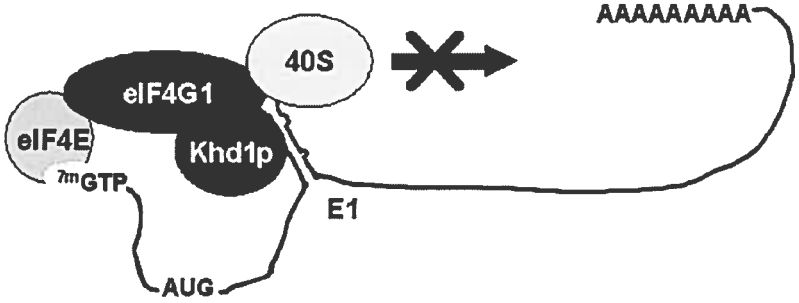

Poly(U) (µg)
0.0 0.0 0.0 0.0 5.0 10.0 15.0 -
Poly(U)
1 2 3 4 5 6 7 -


A
eIF4G
eIF4G
*
E1 RNA
IRE RNA
B
eIF4G-Cterm
eIF4G-Cterm
*
E1 RNA
IRE RNA
C
E1-M5
E2B D1
E1
E2B D2
WT strain
E1-M5
E2B D1
E1
E2B D2
khd1 strain

A


AAAAAAAAA
eIF4G1
eIF4E
40S
^{7m}GTP
AUG

B


AAAAAAAAA
40S
eIF4G1
eIF4E
Khd1p
^{7m}GTP
E1
AUG

Supplementary material

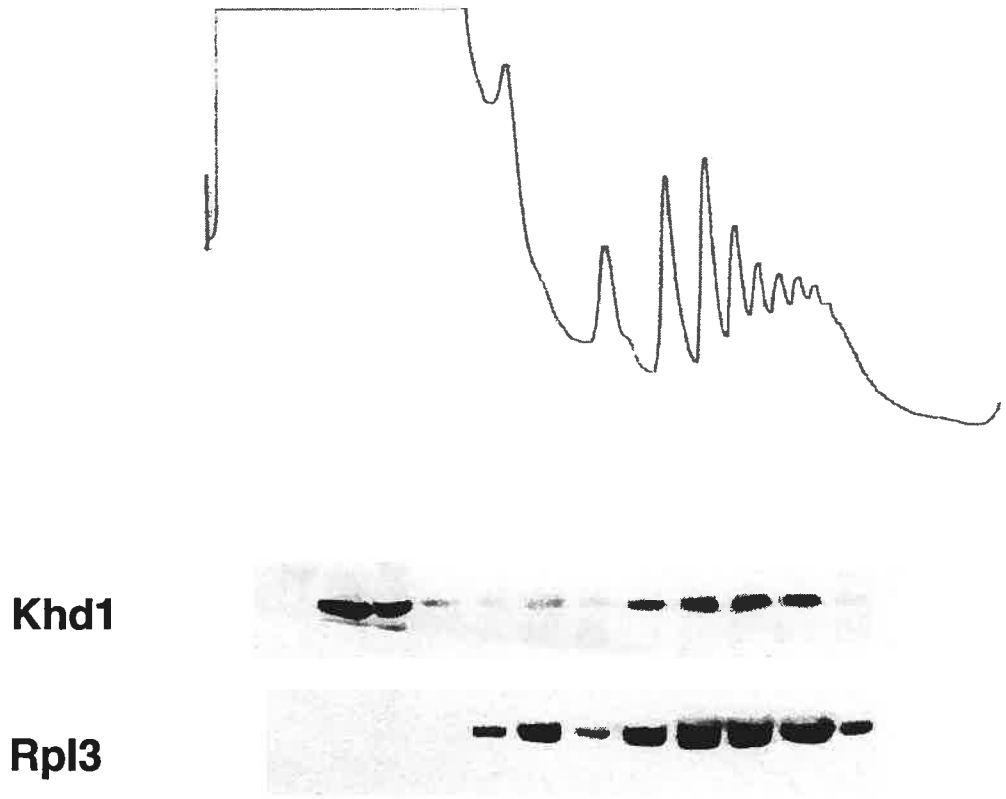


Figure 1 : Distribution of Khd1p in the fractions of a sucrose gradient. Rpl3 is a marker of the large ribosomal subunit.

5. DISCUSSION

5.1 Reconnaissance par She2p d'un motif présent dans les éléments de localisation de l'ARNm *ASH1*

Les études effectuées sur les éléments de localisation de l'ARNm *ASH1,* grâce à la technique de triple-hybride chez la levure, ont permis d'identifier un motif minimal commun entre ces 4 éléments. Le motif d'ARN lié par She2p est constitué d'un trinucléotide CGA ainsi que d'une cytosine, séparés par une tige de quatre à cinq pare de base (**Figure 1 d'Olivier *et al*, 2005**). La distance entre chacune de ces cytidine nucléotidiques est estimée à 28 Å lorsque le modèle est représenté en trois dimensions (**Figure 6 d'Olivier *et al*, 2005**). Ces caractéristiques sont présents une fois sur trois des quatre éléments de localisation (soit E1, E2A et E2B).

Par contre, l'élément de localisation E3 est quelque peu différent. On y retrouve deux cytosines présentes de part et d'autre d'une tige de cinq nucléotides conservant une distance de 28 Å entre chaque cytosine comme les autres éléments de localisation. La cytosine conservée, soit celle du côté 5' dans le cas de l'élément de localisation E3, se retrouve non liée dans la tige plutôt que dans une boucle interne. D'autre part, la séquence CGA ne se retrouve pas du même côté de la boucle par rapport à la tige (soit du côté 3' de l'ARN) comparativement aux autres éléments de localisation. Le fait que la cytosine seule ne soit pas intégrée dans une boucle pourrait engendrer une structure tridimensionnelle différente des autres éléments de localisation tout en laissant la cytosine bien exposée, à une distance de 28Å du triplet CGA. Ceci pourrait maintenir l'interaction entre la cytosine et la protéine She2, et expliquer l'importance de cette cytosine malgré le fait qu'elle ne soit pas dans le même environnement que dans les autres éléments de localisation. Le fait que le CGA soit vers l'extrémité 3' de l'ARN, plutôt qu'en 5' comme chez les autres éléments de localisation n'est pas un facteur à négliger. L'analyse des structures secondaires des ARNm *YMR171C* et *IST2* a révélé que le motif CGA peut être présent sur chacune des extrémités de la tige de quatre à cinq nucléotides et que leur présence est nécessaire pour que la protéine She2 se lie à l'ARNm (**Figure 7 d'Olivier *et al*, 2005**). Il serait donc possible que la séquence GA du CGA soit

requise pour interagir avec la protéine She2 ou pour mieux exposer la cytosine, qui elle interagirait avec cette protéine.

Les études de Sheppard *et al* en 2003 ont permis d'identifier 22 ARNm de levure qui interagissent avec la protéine She2. Cette découverte a permis de montrer que le locasome est utilisé par plusieurs ARNm afin d'être localisé au niveau du bourgeon des levures. Par contre, aucun motif d'interaction de ces ARNm avec She2p, à l'exception de l'ARNm *ASH1*, n'avait été identifié à ce jour. Nos études sur le motif d'ARN liant She2p nous ont permis d'identifier ce motif sur quelques uns de ces ARNm, tels que *YRM171C* et *IST2*. Récemment, le criblage d'une librairie constitué de fragments de l'ARNm *CLB2* par la technique de triple-hybride a permis d'identifier le motif impliqué dans son interaction avec la protéine She2. L'étude de la structure secondaire nous a permis de retrouver une fois de plus le même motif minimal identifié sur les éléments de localisation de l'ARNm *ASH1* (résultats non publiés). Ces résultats confirment une fois de plus l'importance du motif d'ARN liant She2p dans la localisation des ARNm au bourgeon.

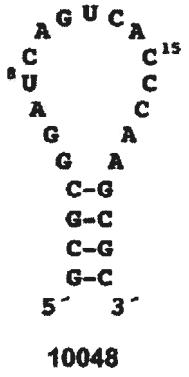
Les résultats obtenus soulèvent des questions quant à la reconnaissance du motif minimal par la protéine She2. La structure tridimensionnelle de la protéine She2 montre que cette protéine forme un homodimère pour exposer une série d'acides aminés basiques nécessaires pour interagir avec l'ARN (**Niessing *et al*, 2004**). Cette surface basique qui recouvre les acides aminés R44 à K57 se replie en une structure hélice-boucle-hélice et couvre une distance de 27 Å, ce qui est la même distance qu'entre la cytosine et le triplet CGA dans le motif d'ARN. Par cette analogie, il serait possible de croire que la structure tridimensionnelle des éléments de localisation pourrait se lier à cette région riche en acides aminés basiques de la protéine She2. Cependant, Niessing *et al.* suggèrent que l'homodimérisation de la protéine She2 ne permet de lier toutefois qu'un seul ARNm, malgré la présence de deux sites potentiels. Ils ont proposé que l'ARNm ciblé par le dimère de She2 adopterait une structure particulière qui rend l'interaction avec deux ARNm impossible. Par contre, nos essais de retardement sur gel d'électrophorèse (EMSA) ont plutôt suggéré que le ratio d'ARN lié aux protéines She2 varierait en fonction de la longueur des ARN. Par exemple, dans le cas de l'élément de localisation E2B, long de 75 nucléotides, un seul complexe est observé, alors qu'avec l'élément de localisation E2A, qui contient 103 nucléotides, on peut observer deux complexes (**Figure**

2 d'Olivier *et al*, 2005), ce qui est compabible avec quelques motifs déterminé lors de recherches précédentes (**Tableau II**). Toutefois, nos études de retard sur gel n'ont pas permis d'établir la stoichiométrie exacte de ces complexes.

5.2 Régulation traductionnelle de l'ARNm *ASH1* par Khd1p

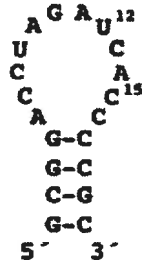
Nous avons montré que la protéine Khd1 se lie à des nucléotides de la séquence UCAUUUCA présente sur la boucle terminale de l'élément E1 pour réguler la traduction de l'ARNm *ASH1*. La comparaison de cette séquence avec d'autres protéines à domaine KH permet d'observer des éléments communs avec la séquence consensus UCAY proposée (**Tableau II**). En effet, en plus d'avoir la séquence UCAY dans la séquence d'interaction de la protéine Khd1, on peut observer l'absence complète de guanine dans la séquence cible. Il a été démontré que l'affinité des protéines à domaine KH pour son ARNm cible est toujours élevée tant et aussi longtemps que la séquence CA est conservée et qu'il n'y ait aucune guanine dans la séquence cible (**Figure 11, Jensen *et al*, 2000**). En plus de montrer que la protéine Khd1 interagit avec l'élément de localisation E1 et qu'il réduit la quantité de ribosomes présents sur l'ARNm *ASH1*, la protéine Khd1 se lie de façon ARN dépendante au domaine C-terminal de la protéine eIF4G. Cette région contient un domaine riche en arginine et sérine reconnu pour sa capacité à lier l'ARN et est importante pour la fonction de eIF4G **(Figure 12).**

Figure 11 : Comparaison des substitutions possibles à la séquence UCAY reconnue par les protéines à domaines KH (Munusuru *et al*, 2004). Les termes « mild », « moderate » et « severe » déterminent la quantification de la perte d'interaction entre la protéine à domaine KH avec son ARNm cible lorsque la séquence UCAY est différente par un nucléotide.



10048

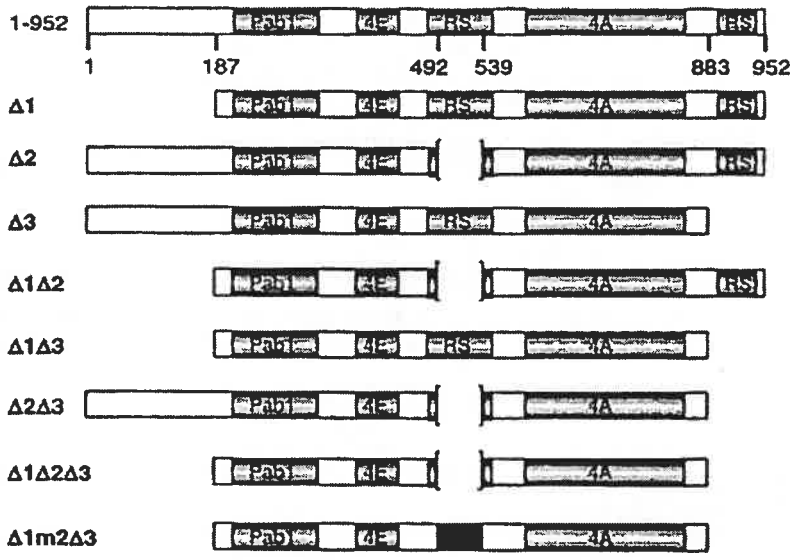
	mild	moderate	severe
U8	C	A	G
C9			G,A,U
A10		U,C	G
G11		A,U,C	
U12	C	G,A	
C13			G,A,U
A14			G,U,C
C15		A,U	G



	mild	moderate	severe
U12	C	G,A	
C13			G,A,U
A14			G,U,C
C15	U	A	G

Figure 12 : Importance des domaines RS dans la fonction de eIF4G (Berset *et al.* 2003)

A



eIF4G	stimulation of translation in vitro
eIF4G1 1-952	++
Δ1	++
Δ2	++
Δ3	+
Δ1Δ2	-
Δ1Δ3	+
Δ2Δ3	-
Δ1Δ2Δ3	-

5.3 Comparaison de la fonction de la protéine Khd1 avec d'autres facteurs de régulation traductionnelle.

Le fait que la protéine Khd1 soit impliquée dans le mécanisme de régulation de la traduction soulève des questions quant à son rôle dans la distribution asymétrique de l'ARNm *ASH1*. Plusieurs protéines observées chez la Drosophile se lient aux ARNm afin d'inhiber la traduction durant plusieurs stages du développement. Par exemple, la protéine Cup inhibe complètement la traduction de l'ARNm *Oskar* durant les sept premiers stages du développement (**Wilhelm *et al.* 2003**), la protéine Bruno se lie au fragment 3'UTR de l'ARNm *Oskar* afin d'inhiber sa traduction dans des endroits non désirés du cytoplasme (**Lie *et al*, 1999; Castagnetti *et al*, 2000**). La protéine Nanos empêche l'allongement de la queue poly (A) de l'ARNm *Hunchback* (**Wreden *et al*, 1997**). Par contre, la protéine Khd1 ne semble pas remplir de rôle similaire aux protéines découvertes chez la Drosophile. En effet, il serait plus probable que la déadénylation de la queue de poly(A) de l'ARNm *ASH1* se fasse grâce à la protéine Puf6. En effet, la protéine Puf6 est membre de la famille Pumilio, reconnue pour leur capacité à réguler la traduction en s'associant à d'autres protéines telles que CPEB, Nanos et Brat, et influençant la longueur de la queue de poly(A) (**Nakahata *et al*, 2001; Edwards *et al*, 2001**). De plus, la protéine Khd1 ne réprime pas de façon transitoire la traduction de l'ARNm *ASH1* puisqu'il est possible d'observer l'apparition de la protéine Ash1 quelques instants après la synthèse de son ARNm, en présence ou non de la protéine Khd1 (résultats non publiés). Finalement, il est impossible que la protéine Khd1 inhibe la traduction ou même stimule la dégradation de l'ARNm *ASH1* lorsque ce dernier n'est pas localisé. En effet, la délétion de la protéine She2 est suffisante pour observer une distribution symétrique de l'ARNm et la protéine Ash1 malgré la présence de la protéine Khd1 (**Long *et al*, 2000; Chartrand et al, 2002**). Si la protéine Khd1 possèderait un rôle au niveau du contrôle de la traduction des ARNm non localisé, la localisation de la protéine Ash1 ne serait pas observé. Il semblerait plutôt que la présence de la protéine Khd1 réduirait l'efficacité de recrutement de la sous-unité ribosomale 40S sur l'ARNm *ASH1*, causant ainsi une diminution de la traduction durant le transport. Par contre, une

fois que le ribosome a débuté sa traduction, la protéine Khd1 ne peut pas intervenir lors de l'assemblage du ribosome ainsi que durant la traduction de l'ARNm.

Nous ne pouvons pas négliger la possibilité que la protéine Khd1 pourrait empêcher les protéines eIF4G et eIF4A de recruter et d'orienter adéquatement la sous-unité ribosomale 40S sur l'ARNm *ASH1*. Ce mécanisme pourrait amener une traduction erronée et causer la dégradation des protéines obtenues. Ce système de changement de cadre de lecture de traduction est fréquent chez les virus, qui utilisent ce système afin de traduire plusieurs protéines virales à partir d'un même brin d'ARNm (**Piron *et al*, 1998, Gradi *et al*, 2003**).

5.4 Khd1p est unique par sa capacité à se lier dans la région codante d'un ARNm et le domaine RS de la protéine eIF4G

Pour permettre une inhibition de la traduction, la plupart des protéine connues se lient soit du côté 3'UTR de l'ARNm, comme c'est le cas avec CPEB, Bruno et Puf6 (**Gu *et al*, 2004; Wu *et al*, 1998; Lie *et al*, 1999**), ou au niveau 5' UTR comme observé avec IRE-BP (**Rouault *et al*, 2002**). Puisque la protéine Khd1 est la seule protéine à ce jour qui régule la traduction d'un ARNm à l'intérieur de la région codante, sa présence dans cette région constitue un obstacle supplémentaire aux ribosomes et aux hélicases par un mécanisme jamais observé auparavant. La présence de la protéine dans la région codante constitue donc un avantage supplémentaire pour obtenir une meilleure régulation de la traduction.

Contrairement à toute attente, la protéine Khd1 requière le domaine C-terminal de la protéine eIF4G, où aucune protéine responsable de l'initiation de la traduction n'est connue pour se lier à cet endroit. Étant donné que la formation du complexe eIF4F est requise pour être en mesure d'initier la traduction, plusieurs protéines inhibitrices compétitionnent pour les sites d'interaction entre eIF4E et eIF4G, comme c'est le cas avec les protéines eIF4EBP, eIF4EHP, Cup, Maskin et même chez les virus (**Nelson *et al*, 2004; Mader *et al*, 1995**). Il a aussi été observé que l'interaction entre eIF4G et PABP peut être inhibée pour empêcher la traduction (**Svitkin *et al*, 2001; Kuyumcu *et al*, 2004**). Dans chacun des cas, ces compétitions empêchent la circularisation de l'ARNm, ce qui rend l'initiation de la traduction inefficace. On observe aussi des modifications de

la longueur de la queue de poly(A) afin d'empêcher la fixation de la protéine PABP, comme c'est le cas avec les protéines ORB, YPS, Bruno et les protéines de la famille Pumilio, dont Puf6p (**Lie *et al*,1999; Chang *et al*, 1999; Nakahata *et al*, 2001; Edwards *et al*, 2001).** Dans le cas de la protéine Khd1, nous avons montré qu'il n'y a aucun phénomène de compétition envers les protéines responsables de l'initiation de la traduction liant eIF4G (**Figure 4 de Paquin *et al***). Par contre, en se liant à l'extrémité C-terminale de la protéine eIF4G, la protéine Khd1 interagit avec l'un de ses deux domaines RS. Il a été montré qu'une mutation ou une délétion d'au moins un des domaines RS de eIF4G entraîne une diminution de l'efficacité et même une inhibition de la traduction des ARNm (**Figure 12**). La liaison de la protéine Khd1 avec un de ces deux domaines RS pourrait être une voie de régulation à considérer afin d'expliquer le mécanisme de régulation de la traduction de l'ARNm *ASH1*. Ces domaines RS seraient utilisés pour lier directement les ARNm et permettre d'initier la traduction avec une meilleure efficacité (**Berset *et al*, 2003**). L'effet de compétition engendré par la protéine Khd1 sur le domaine C-terminal de la protéine eIF4G permettrait donc de réduire l'efficacité de traduction de l'ARNm *ASH1* durant son transport vers le bourgeon en inhibant l'interaction domaine RS-ARNm.

5.5 Modèle de régulation de la traduction par la protéine Khd1

Les résultats obtenus dans le laboratoire ont permis de proposer un modèle d'un nouveau mécanisme de régulation de la traduction de l'ARNm *ASH1*. En effet, nous proposons que la protéine Khd1 se lie à la tige-boucle terminale de l'élément de localisation E1 ainsi qu'à l'extrémité C-terminale de la protéine eIF4G de façon ARN dépendant pour former un complexe ternaire (**Figure 6 de Paquin *et al***). La protéine Khd1 fixée à ces deux endroits serait suffisamment stable pour réduire le nombre de ribosomes recrutés sur l'ARNm *ASH1* et ainsi diminuer l'efficacité de traduction de l'ARNm. L'effet de Khd1p combiné avec la présence des structures secondaires des éléments de localisation situés dans la séquence codante ainsi que la participation de la protéine Puf6 au niveau 3'UTR permet de réguler la traduction de l'ARNm *ASH1*. Grâce à ces facteurs, il y a donc moins de ribosomes présents sur l'ARNm *ASH1* et ces derniers possèdent une efficacité de traduction réduite. Cette régulation conjointe de la traduction

durant le transport de l'ARNm *ASH1* vers l'extrémité de la levure fille en bourgeonnement est suffisante pour permettre une distribution asymétrique de la protéine Ash1. Par ses interactions avec l'extrémité C-terminale de la protéine eIF4G ainsi qu'avec l'élément E1 présent dans la séquence codante, la protéine Khd1 possède un mécanisme de régulation de la traduction jamais étudié à ce jour. Il serait fort possible que dans les années futures, d'autres exemples de régulation de la traduction passant par une interaction avec la séquence codante d'un ARNm soient identifiés. Par contre, le modèle ne tient pas compte de la possibilité de l'influence de la protéine Puf6 sur la régulation de l'ARNm *ASH1* puisque son mécanisme d'action est encore nébuleux.

6. PERSPECTIVES

Malgré l'avancement du projet, quelques expériences restent à être effectuées afin de compléter l'analyse du mécanisme proposé. Il faudrait exprimer dans la levure une protéine eIF4G délétée de son domaine C-terminal pour observer si la protéine Khd1 peut maintenir son effet régulateur sur l'ARNm *ASH1*. De plus, Il serait intéressant de déplacer l'élément de localisation E1 au niveau du 5'UTR de l'ARNm *ASH1* afin de déterminer si la liaison entre les protéines eIF4G-Khd1 est toujours possible. Si c'est le cas, il faudrait regarder si l'effet de régulation de la protéine Khd1 ne serait pas inversé, c'est-à-dire qu'elle aiderait la protéine eIF4G à recruter des ribosomes sur l'ARNm et agirait comme activateur de la traduction. Par la suite, il faudrait déterminer si le mécanisme d'action de la protéine Puf6 nécessiterait la présence de la protéine Khd1 ou si elle agit de façon indépendante, ce qui nous permettrait d'avoir une idée quant à la complémentarité possible entre les protéines Khd1 et Puf6.

7. CONCLUSIONS

La localisation d'ARNm est un mécanisme complexe bien orchestré par une combinaison de plusieurs protéines qui exécutent des fonctions bien définies. Dans le cas de l'ARNm *ASH1*, la localisation permet d'inhiber le changement de type sexuel de la levure fille, facilitant la fusion de celle-ci avec la levure mère. Dans notre cas, le transport de l'ARNm *ASH1* assuré par le locasome participe aussi à la régulation de la traduction de l'ARNm. En effet, la présence des éléments de localisation dans la séquence codante de cet ARNm diminue de moitié l'efficacité de la traduction, grâce à la stabilité des structures secondaire des éléments liés par la protéine She2. Le motif commun de l'interaction protéine-ARN déterminé par l'article d'Olivier *et al* (2005) semble être utilisé par plusieurs ARNm localisés par la protéine She2, comme par exemple *IST2, YMR171C* et *CLB2*. Par contre, le motif de reconnaissance par la protéine She2 ne se retrouve par nécessairement dans le séquence codante, ce qui permet une localisation d'ARNm sans pour autant avoir un effet sur la traduction de celle-ci.

La combinaison d'autres facteurs tels que la présence de la protéine Khd1 permet d'augmenter l'efficacité de localisation de la protéine Ash1 au noyau de la cellule fille en contribuant de façon complémentaire à la diminution de la traduction de l'ARNm *ASH1*. Il a été démontré durant ma maîtrise que Khd1p possède la capacité de se lier à la fois à l'élément de localisation E1 ainsi qu'à la protéine eIF4G au niveau de la région C-terminale riche en Arginine (R) et en Sérine (S). Le complexe ainsi formé permet à la protéine Khd1 d'effectuer deux types de régulation; il diminuerait le recrutement des sous-unités 40S sur l'ARNm et réduirait l'efficacité d'interaction du domaine RS pour l'ARNm. Cette région de la protéine eIF4G est reconnue pour favoriser la traduction des ARNm (Berset *et al*, 2003). Il est a noter que la régulation par Khd1p n'est pas une inhibition complète mais bien une contribution au ralentissement de la traduction durant le transport assuré par le locasome. Ce mécanisme observé par la protéine Khd1 semble

être une nouvelle voie dans le domaine de la régulation de la traduction d'un ARNm. Pour le moment, la protéine Khd1 semble être lié seulement sur l'élément de localisation E1, mais il serait possible d'observer d'autres domaines d'interactions sur différents ARNm dont la traduction serait régulée.

En combinant les deux mécanismes, nous pouvons observer que la localisation de l'ARNm *ASH1* est la réalisation de plus d'un facteur. En effet, une fois que la protéine Khd1 ralentit l'apport des sous-unités 40S et diminue l'efficacité de traduction médié par la protéine eIF4G, la protéine She2 contribue a ralentir les ribosomes qui ont été assemblés sur l'ARNm *ASH1* par la stabilisation des structures secondaires des éléments de localisation. De plus, durant cette combinaison de régulation de la traduction, le locasome assure le transport de l'ARNm *ASH1* vers le bourgeon où il pourra être finalement traduit afin d'inhiber le changement de type sexuel de la levure fille. Il reste évidemment d'autres facteurs à considérer dans ce réseau de régulation, tels que la protéine Puf6 qui se lie à l'élément de localisation E3 afin de réguler la traduction. Par contre, étant donné que son mécanisme est inconnu, il est impossible de mentionner si Puf6p fait partie intégrante du mécanisme de régulation provenant des protéines She2/ Khd1 ou si elle agit par une voie complètement indépendante. Quoiqu'il en soit, le mécanisme de régulation de l'ARNm *ASH1* a connu d'importantes découvertes qui vont contribuer à mieux comprendre le mécanisme de transport et de régulation des ARNm.

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