Evidence for a nuclear passage of nascent polypeptide-associated complex subunits in yeast

Jacqueline Franke¹, Barbara Reimann², Enno Hartmann³, Matthias Köhler¹, 4 and Brigitte Wiedmann²,*

¹Max Delbrück Center for Molecular Medicine, Robert-Rösle-Straße 10, D-13122 Berlin, Germany
²Department of Biochemistry, Humboldt University, Charité, Hessische Straße 3-4, D-10115 Berlin, Germany
³Center for Biochemistry and Molecular Cell Chemistry, Georg-August University, Heinrich-Düker-Weg 12, D-37073 Göttingen, Germany
⁴Franz-Volhard-Klinik, Humboldt University, Charité, Willbergstraße 50, D-13122 Berlin, Germany

*Author for correspondence (e-mail: brigitte.wiedmann@charite.de)

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SUMMARY

The nascent polypeptide-associated complex (NAC) has been found quantitatively associated with ribosomes in the cytosol by means of cell fractionation or fluorescence microscopy. There have been reports, however, that single NAC subunits may be involved in transcriptional regulation. We reasoned that the cytosolic location might only reflect a steady state equilibrium and therefore investigated the yeast NAC proteins for their ability to enter the nucleus. We found that single subunits of yeast NAC can indeed be transported into the nucleus and that this transport is an active process depending on different molecular import factors. Translocation into the nucleus was only observed when binding to ribosomes was inhibited. We identified a domain of the ribosome-binding NAC subunit essential for nuclear import via the importin Kap123p/Pse1p-dependent import route. We hypothesize that newly translated NAC proteins travel into the nucleus to bind stoichiometrically to ribosomal subunits and then leave the nucleus together with these subunits to concentrate in the cytosol.

Key words: NAC, Nuclear transport, Saccharomyces cerevisiae

INTRODUCTION

The nascent polypeptide-associated complex (NAC) is an evolutionarily conserved protein complex. Three NAC subunits are present in yeast: Egd2p, Btt1p and Egd1p. Egd2p is homologous to αNAC proteins in mammals (Shi et al., 1995) and plants (Wiedmann et al., 1994) and a protein family in Archea (J.F. et al., unpublished). Btt1p and Egd1p are homologues of the mammalian βNAC (BTF 3) (Hu et al., 1994; Parthun et al., 1992; Wiedmann et al., 1994). Whereas Egd2p and Egd1p are expressed at about stoichiometric levels compared with ribosomes, the concentration of Btt1p is about 100-times lower (George et al., 1998; Reimann et al., 1999). The proteins form tight heteromeric complexes even at 500 mM salt. After cell fractionation, more than 95% of the Egd2p is found bound to ribosomes via Egd1p or Btt1p (J.F. et al., unpublished; Reimann et al., 1999).

Several functions have been attributed to NAC or its subunits. The entire complex at the ribosome protects nascent polypeptides from premature interactions with cytosolic proteins. The NAC complex also inhibits the binding of ribosomes that translate non-secretory proteins to translocation sites in the membrane of the endoplasmic reticulum (Wang et al., 1995; Wiedmann et al., 1994; Lauring et al., 1995a; Lauring et al., 1995b; Möller et al., 1998; Wiedmann and Prehn, 1999). NAC is associated with the ribosomes and binds to emerging nascent polypeptides before any other cytosolic protein. NAC releases the nascent chain when a binding domain for cytosolic proteins such as the signal recognition particle (SRP) or chaperones is completed (Wang et al., 1995). Furthermore, NAC may function in cotranslational protein import into mitochondria (George et al., 1998; Fünschilling and Rospert, 1999).

In addition to the functions of ribosome-bound NAC in the cytosol, single NAC subunits may interact with DNA thereby regulating transcription (Parthun et al., 1992; Moreau et al., 1998). Such a function would imply that at least some NAC protein is located transiently within the nucleus and that the NAC subunits contain signals for their active translocation into the nucleus. Indeed, the NAC α-subunit has been found in the nuclei of serum-deprived osteoblastic cells in mice (Yotov, et al., 1998). However, verification of this finding has been difficult (Beatriz et al., 2000). NAC subunit translocation into nuclei should require import receptors.

According to our present knowledge, nuclear import substrates bind to soluble receptors of the importin β family either directly or with the help of specific adapter proteins (importin α) and translocate through the nuclear pore driven by the concentration gradient of Ran-GTP (Wozniak et al., 1998; Görlich and Kutay, 1999). Most presently known substrates bear a so called ‘classical’ nuclear localization signal (NLS). These proteins interact with their receptor importin β via adapter molecules of the importin α family. Other substrates contain a NLS which allows their direct interaction with one of the various import receptors. Some proteins need a dimer of two different importins for efficient transport. For example, histone H1 requires importin β and importin 7 (Jäkel et al., 1999). Other proteins can use more than one import pathway due to their ability to bind to different import receptors. The yeast ribosomal protein L25...
is imported by Yrb4p (also known as Kap123p) and Pse1p (also known as Kap121p) (Rout et al., 1997; Schlenstedt et al., 1997; Seedorf and Silver, 1997). The human homologue, L23a, can even be imported by four different receptors, namely importin 5, importin 7, importin β, and transportin (Jäkel and Görlich, 1998). We report here, that non-ribosome-associated yeast NAC subunits can translocate into the nucleus in vivo. Using in vitro assays and in vivo experiments, we demonstrate that this transport is specific and can be mediated by several of the known import factors.

**MATERIALS AND METHODS**

**Plasmids and yeast strains**

Standard yeast methods and media were used. The NAC-knockout mutants are based on strain W303, as described previously (Reimann et al., 1999). The Δkapl23 and pse1-1 strains were kindly provided by P. Silver (Harvard Medical School, Boston, MA). The srp1-31 and srp1-49 strains were from M. Nomura (University of California, Irvine, CA).

GFP fusion plasmids of *EGD1* and *EGD2* were constructed by cloning 500 base pairs upstream of ATG and the coding region in frame to two C-terminal copies of modified GFP in pRS414 (Sikorski and Hieter, 1989; Cormack et al., 1997). To yield truncated versions of *EGD1* the promoter region of *EGD1* was cloned via a Clal restriction site to the coding region of *EGD1* without its N-terminal 33 (ΔN11-egd1), 42 (ΔN14-egd1), 81 (ΔN27-egd1) or 132 (ΔN44-egd1) base pairs into pRS415, which already contained the *EGD2* gene. The new ATG start codon was introduced with oligonucleotides that were designed to fuse the promoter with the shortened coding region. These mutated genes were cloned via SacI and EcoRI restriction sites in frame into pRS414-2GFP in order to create GFP fusion proteins. The plasmid pRS414-2GFP was a gift of U. Lenk (MDC, Berlin). All constructs were sequenced, and the expression of fusion proteins was verified by SDS/PAGE and western blot analysis.

**Recombinant expression of proteins**

Coding regions of *EGD1* and *EGD2* were cloned via PCR into BamHI and HindIII sites of pQE 30 vector (Qiagen). Expression of the His-tagged proteins was induced by IPTG in *Escherichia coli* M15 at 25°C for 4 hours. HIS-Egd2p was purified under native conditions according to the QAExpress protocol. Briefly, harvested cells of a 1 litre culture were resuspended in 100 mM sodiumphosphate buffer, pH 7.8, 300 mM NaCl and lysed by freeze/thawing and sonication. The lysate was centrifuged (30 minutes, 10,000 g) and the supernatant (OD_{540} = 1) was loaded onto a pre-equilibrated Ni-NTA-column (8 ml 50% Ni-NTA-resin). The protein was eluted by 50 mM Hepes, pH 3.5 and neutralized immediately. His-Egd1p was purified under denaturing conditions according to the QAExpress protocol. The difference to the His-Egd2p purification was that the cells were resuspended in 100 mM sodiumphosphate buffer, pH 8.0, 8 M urea. His-Egd1p was renatured bound to the Ni-NTA-resin by a gradient of 8-0 M urea in 100 mM sodiumphosphate buffer, pH 8.0 and then eluted as described above.

Preparation of recombinant import factors occurred as described earlier: C-terminal His-tagged importin α1/Rch1 and Srp1p (Görlich et al., 1995), α3, α4, α5/5hSrp1 and α7 (Köhler et al., 1999), importin β, nucleoplasmin, nucleoplasmin core, human Ran, Schizosaccharomyces pombe Rna1p, murine RanBP1 and NTF2 (Kutay et al., 1997). Expression clones for importin β, nucleoplasmin, nucleoplasmin core and NTF2 and recombinant protein of human Ran, Schizosaccharomyces pombe Rna1p and murine RanBP1 were kindly provided by D. Görlich (ZMBH, Heidelberg).

**In vitro nuclear protein import assay**

Import assays were performed as described previously (Adam et al., 1990; Jäkel and Görlich, 1998, Köhler et al., 1999). Briefly, HeLa cells were grown on three-well microscopy slides (Roth) to 40-80% confluency, washed once in ice-cold PBS, and permeabilized for 8 minutes in ice-cold 20 mM Hepes-KOH (pH 7.5), 150 mM potassium acetate, 5 mM magnesium acetate, 0.5 mM EGTA, 250 mM sucrose, 30 μg digitonin (Sigma) per ml. The cells were incubated for 8 minutes on slides with 20 μl of import mixture at room temperature. The import reaction was stopped by fixation with 3% paraformaldehyde/PBS. After washing in PBS, the slides were mounted with Vectashield mounting medium (Vector). Import mixtures contained an energy regenerating system (0.5 mM ATP, 0.5 mM GTP, 10 mM creatine phosphate, 50 μg creatine kinase/ml), core buffer (2 μg nucleolapsmin core/ml, 20 mM Hepes-KOH (pH 7.5), 150 mM potassium acetate, 5 mM magnesium acetate, 250 mM sucrose), 0.5 mM EGTA, 3 μM RanGDP, 0.2 μM Rna1p, 0.3 μM RanBP1 and 0.4 μM NTF2. The assay was adjusted to 10% with reticulocyte lysate, and 1 μM importin β and 2 μM of importin α were added.

Fluorescence labelling of recombinant proteins was performed with Texas red or fluorescein 5′-maleimide as described previously (Kutay et al., 1997). Fluorescence microscopy was performed with a confocal microscope (MRC 1024, BioRAD) equipped with a 63x oil-immersion objective (Nikon Diaphot). Images were processed by Adobe Photoshop.

**Fluorescence microscopy of yeast cells**

Cultures of exponentially growing yeast cells were incubated at 30°C (or 25°C for temperature sensitive mutants). To visualize nuclei, Hoechst 33258 (Sigma) was added to a final concentration of 10 μM for 10 minutes prior to microscopy. Fluorescence microscopy was performed with living cells using a Zeiss Axioplan 1 microscope equipped with a NEOFULAR 100x/1.30 oil-immersion objective lens (Zeiss). GFP fluorescence was obtained using the FITC channel. Stained nuclei were visualized in the UV channel. Photographs were taken with a CCD camera (PCP computer optics, Kelheim) and the Axiovision 1 software (Zeiss). Images were processed by Adobe Photoshop.

**Cell fractionation**

Cells were grown to middle log-phase on SD, spheroplasted with Zymolyase 100T, homogenized in IP-buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 5 mM Mg(OAc)2, 1 mM PMSF, and protease inhibitor mix (Sigma)) at about 0.5-1 g fresh weight/ml buffer in a Brown-homogenizer (Brown, Melsungen, Germany). Cell debris, left over cells and nuclei were sedimented by two 15 minute centrifugation steps at 3,000 g. The supernatant was cleared of mitochondria by a 15 minute centrifugation at 10,000 g. A 30 minute centrifugation at 400,000 g yielded a supernatant free of endoplasmic reticulum. Ribosomes were separated from cytosol by sedimentation at 400,000 g in a Beckman TL100.4 rotor for 30 minutes. The volume of fractionation samples was adjusted so that it equalled the original volume and electrophoresis lanes were directly comparable.

**Co-immunoprecipitation**

The 40,000 g supernatant of a cell fractionation was used for immunoprecipitation. 2 ml were incubated 2-4 hours with 3 μl antibody against Egd1p and/or Egd2p at 6°C on a roller (antibodies provided by M. Wiedmann, MSKKC, New York). Antigen-antibody complexes were collected with 50 μl 50% protein A-sepharose in IP-buffer, washed extensively with IP-buffer, and solubilized by boiling in 50 μl 2× SDS sample buffer. Proteins were separated on 12% SDS polyacrylamide gels. Western blot detection was performed using the ECL system according to the instructions of the manufacturer (Amersham Pharmacia Biotech).
RESULTS

Yeast NAC proteins can be transported into nuclei in vivo

Under steady state conditions most of the yeast NAC proteins are in a complex tightly associated with cytosolic ribosomes. However, one cannot exclude the existence of low amounts of free NAC proteins or NAC that is not or not yet ribosome-bound in the cell. Therefore, we sought in vivo conditions that increased the amount of such molecules in the cell to determine their location. Egd2p is associated with the ribosome by Egd1p and to a lesser extent by Btt1p. Thus, we expressed a fusion protein of Egd2p with two copies of the green fluorescence protein (Egd2p-2GFP) in a NAC-knockout strain and looked for the location of that protein.

Fig. 1A shows the location of Egd2p-2GFP in a Δegd2 background. This strain, which resembles the wild-type phenotype (Fig. 1A, top two images), was compared with the knockout strain (Fig. 1A, bottom two images). There was no co-localization of fluorescence with the nuclear staining Hoechst in wildtype. However, when Egd2p-2GFP was the only NAC subunit left in the cell, the nuclei showed an intense green fluorescence in addition to a cytoplasmic staining. Also, overexpression of Egd2p-2GFP in wild-type yeast caused nuclear fluorescence (data not shown) indicating again that free Egd2p-2GFP can translocate into the nucleus. In each case the vacuoles were free of Egd2p-2GFP.

Next, we tested the location of Egd1p-2GFP. As expected from our cell fractionation experiments, full length Egd1p-2GFP was found exclusively in the cytosol in wild-type cells and also in the NAC-knockout strain (Fig. 1B). Therefore, we searched for EGD1 mutants that are deficient in ribosome binding and shortened the coding region subsequently at its N-terminus. Removal of the first 11 N-terminal amino acids...
(ΔN11-Egd1p-2GFP) abolished the association with ribosomes. Only traces of truncated Egd1p-2GFP were left in the ribosomal fraction (Fig. 2A). The interaction of truncated Egd1p versions with the Egd2p was not affected, as shown by co-immunoprecipitation (Fig. 2B). The N-terminal shortened Egd1p-2GFP accumulated in the nucleus of NAC-knockout cells (compare Fig. 1B,C) and wild-type cells (not shown). In summary, these experiments proved the ability of both proteins (Egd1p, Egd2p) to enter the nucleus.

The in vitro import of yeast NAC into nuclei is energy dependent and mediated by different importin α proteins

We next investigated the nuclear import of these proteins in more detail. First, we tested whether or not yeast NAC proteins can be transported into the nucleus via the ‘classical’ importin α/importin β-mediated import pathway using an in vitro import assay (Adam et al., 1990; Jäkel and Görlich, 1998, Köhler et al., 1999). Basically, HeLa cells were permeabilized and the cytosol was washed out. Fluorescein labelled recombinant Egd1p or Egd2p and the components necessary for proper import including different human α-importins and the α-importin homologue of yeast Srp1p were added to a standard in vitro import reaction. The fluorescence signal in the nuclei of the samples containing importins α1/Rch1, α3, α7 or yeast α-importin Srp1p demonstrates that both Egd2p and Egd1p were actively transported into nuclei (Fig. 3a,b,d,e,m,n,p,q). The human β-importin alone (Fig. 3s,t) could not mediate transport of yeast NAC proteins. Several lines of evidence indicate that the import of the yeast NAC subunits is due to a specific binding to their import receptors. First, not all α-importins could promote nuclear import of the yeast proteins. Importins α4 and α5 failed to import Egd1p or Egd2p (Fig. 3g,h,j,k) even though they could transport nucleoplasmin, a typical import substrate (Fig. 3i,l). Second, nucleoplasmin competed with the Egd2p for interaction with importin α1 (see Fig. 4), indicating that the import of Egd2p requires an empty NLS-binding site on its import adapter importin α1. Furthermore, there was no transport in the absence of energy (Fig. 4i). Together, these experiments show that yeast NAC subunits in vitro can enter the nuclei of human tissue culture cells with components of the ‘classical’ import pathway.

In vivo transport of Egd1p into nuclei is inhibited in nuclear protein import mutants

We then asked how yeast NAC proteins are imported in vivo and searched for import mutants that could decrease the intense nuclear accumulation of ΔN11-Egd1p-2GFP. To evaluate the role of the importin α/importin β-mediated transport, we looked for localization of ΔN11-Egd1p-2GFP in mutants of SRP1: srp1-31 and srp1-49 (Loeb et al., 1995; Shulga et al., 1996, Tabb et al., 2000). We observed no effect in srp1-49 cells but a slight increase of cytosolic staining in srp1-31 cells (Fig. 5A) after a 3 hour shift to the nonpermissive temperature. The relatively small effect can be explained by the already existing strong nuclear accumulation of ΔN11-Egd1p-2GFP at permissive temperature. Another possible reason could be that other

Fig. 3. In vitro nuclear import assay of recombinant Egd1p and Egd2p. Recombinant Egd1p and Egd2p were labelled with fluorescein-5′-maleimide. Dependency of in vitro nuclear import of Egd1p on different importin α proteins (left); import of Egd2p (middle); import of the standard substrate nucleoplasmin (NPL) by the different importin α proteins (right). The images s, t and u represent the negative controls (import assay without α importin). HeLa cells were permeabilized, the cytosol was washed out and recombinant import factors, energy mix, and labelled substrate were added. After fixation the fluorescence of the cells was observed by confocal microscopy.
Deletion of the first 27 amino acids of Egd1p abolishes Kap123p and Pse1p dependent nuclear import

We examined the further deleted Egd1p-2GFP to define the domain that is responsible for nuclear import by Kap123p and/or Pse1p. Nuclear accumulation of ΔN14-Egd1p-2GFP compared with ΔN11-Egd1p-2GFP was reduced and the protein localized in the cytoplasm. Removal of 27 amino acids increased this effect slightly (Fig. 6A). Deletion up to 44 amino acids showed the same distribution as that seen in ΔN27-Egd1p-2GFP (data not shown). However, nuclear import was not completely abolished. We always detected truncated Egd1p-2GFP in nuclei. The remaining nuclear staining of ΔN14-Egd1p-2GFP and ΔN27-Egd1p-2GFP was also observed in Δkap123 and in the pse1-1 mutant - even at nonpermissive temperature (Fig. 6B). Therefore, we assume that the region responsible for Kap123p/Pse1p-dependent import is located between amino acids 11 and 27.

DISCUSSION

NAC has been associated with both cytosolic and nuclear functions. Although its cytosolic location is well established, conflicting results have been reported regarding the presence of NAC in the nucleus (Yotov et al., 1998; Beatrix et al., 2000). We show here, for the first time, NAC proteins inside the nucleus in growing yeast. We employed mutant yeast strains and demonstrated that yeast NAC subunits may reside in the nucleus when they cannot bind to ribosomes. In the case of Egd2p-2GFP, we removed Egd1p and Btt1p, the two ribosome-binding proteins. We also overexpressed Egd2p-2GFP. Ribosomal binding of the Egd1p was abolished when 11 amino acids of the N-terminus were removed. Whereas truncated Egd1p-2GFP accumulated strongly inside the nucleus, Egd2p was equally distributed between nucleus and cytosol. We did not evaluate the reason for this difference. However, we assume that the difference is related to folding artifacts in Egd2p-2GFP caused by the C-terminal fusion to GFP or the absence of its binding partner Egd1p.

Although the NAC subunits were small enough to enter the nucleus via diffusion, we found evidence for active transport mechanisms. In our in vitro transport assay revealed that full length Egd2p, a homologue of mammalian αNAC, as well as Egd1p, a BTF3 homologue, can be transported into the nucleus by distinct importin α-proteins, the mammalian α1, α3, α7 and the yeast importin α Srp1p. The mammalian importins α4 and α5 failed to import the yeast NAC proteins although they carried other cargos such as nucleoplasm in the same assay. Competition of Egd2p-2GFP with nucleoplasmin confirmed the specificity of the transport process. The weaker fluorescence signal found for import with yeast importin α Srp1p in this assay had also been observed for other cargos (Köhler et al., 1999).

The fact that the NAC proteins do not possess a classical NLS as described previously (Makkerh et al., 1996) does not contradict the observed interaction with importin α proteins. Other proteins that lack a classical NLS, such as RanBP3, are also known to be imported via the importin α/importin β-dependent pathway (Welch et al., 1999). We believe that the result obtained for yeast NAC proteins in vitro is likely to reflect the in vivo situation in mammals, since human αNAC protein can be co-immunoprecipitated with HeLa-cell lysate using anti-importin α1/Rch1 antibodies (data not shown).

Our presumption of an active transport via the importin α/importin β-dependent pathway was confirmed by in vivo
experiments employing temperature sensitive mutants of SRP1. It was shown that in the srp1-31 mutant the nuclear import of classical NLS-bearing substrates and of the ribosomal protein L11b was impaired (Loeb et al., 1995; Shulga et al., 1996; Stage-Zimmermann et al., 2000). ΔN11-Egd1p-2GFP accumulated in the nucleus in wild-type cells but in srp1-31 cells the truncated protein also localized in the cytoplasm at nonpermissive temperature. In addition, we employed the srp1-49 mutant, which exhibits defects in protein degradation and, to a lesser extent, in nuclear transport (Tabb et al., 2000) and could not detect any significant difference compared with the wildtype. We propose two explanations for the rather moderate effect of the SRP mutations. First, a decrease in nuclear accumulation could not occur because transport back to the cytosol was not possible. Only the import of newly synthesized ΔN11-Egd1p-2GFP was blocked. Second, there may exist alternative import pathways. We tested this hypothesis by employing yeast nuclear import mutants of ribosomal proteins, namely Kap123p and Pse1p.

Furthermore, we delineated the domain of Egd1p responsible for the interaction with importins Kap123p/Pse1p. Whereas deletion of the first 11 amino acids abolished ribosome binding and led to a nuclear accumulation of Egd1p, a further deletion of the next 3 amino acids drastically reduced the Kap123p/Pse1p-dependent import of Egd1p. This region contains a KLXKL motif that is found in all Egd1p homologues in the database. Additional deletion of amino acids 15-27, a region that also bears a highly conserved cluster of basic amino acids, enhanced the import defect only slightly. Both motifs are not present in L25, the TATA-binding protein or Pho4, proteins known to be imported by Pse1p/Kap123p (Schaap et al., 1991; Kaffman et al., 1998; Pemberton et al., 1999). Our finding indicates a close proximity or even partial overlap of the ribosome binding domain and one of the nuclear localization signals in Egd1p. However, nuclear import was not abolished completely when the first 27 amino acids were missing. Therefore, we assume that Egd1p bears several nuclear import signals that trigger nuclear transport by different pathways. One obvious candidate would be the Srp1p (yeast importin α)-dependent pathway. This assumption could not be tested because in the available temperature-sensitive mutants of SRP1, ΔN14-Egd1p-2GFP is transported into nuclei efficiently at permissive temperature. Thus, no difference in location of this protein was detectable after the temperature shift before the cells were dead (data not shown).

What is the reason for transport of NAC proteins into the nucleus? A possible transcriptional function of yeast Egd1p as described by other authors had been deduced from DNA gel shift experiments and moderately reduced amounts of some mRNA species in an EGD1/BTT1-knockout strain (Parthun et al., 1992; Hu and Ronne, 1994). However, the simultaneous deletion of all NAC subunits in yeast has no additional phenotype (Reimann et al., 1999) and firm direct proof for a function of Egd1p as transcription factor is still missing.

There are other processes that could demand a transient nuclear localization of NAC subunits. Key components of translation, such as the ribosomal subunits, 5S-RNPs and SRP
Transient nuclear passage of NAC

their synthesis in the cytosol to assemble with ribosomal subunits and are exported together with these subunits. In contrast to the SSB proteins, NAC subunits are not exported by the NES (nuclear export signal)-dependent factor Crm1p (J.F. et al., unpublished). Interestingly, efficient import of yeast NAC subunits requires both Kap123p and Pse1p, similar to what has been found for ribosomal proteins. We believe that the first efficient binding of NAC to ribosomal subunits occurs in the nucleus. This interpretation would imply that yeast NAC does not leave the ribosome when it releases the nascent polypeptide. The assumption is supported by our observation that ER-bound ribosomes bear NAC and that binding of in vitro translated yeast NAC to ribosomes is inefficient. One cannot exclude the possibility that in the absence of sufficient ribosomal binding sites the nuclear NAC is available for transcriptional processes. However, as all our data are consistent with the idea of a co-regulated NAC assembly with ribosomal subunits, we propose that the subunits of NAC enter the nucleus primarily to bind to their final destination, the ribosome.

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