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HEL/UAP56 Binds Cotranscriptionally to the Balbiani Ring Pre-mRNA in an Intron-Independent Manner and Accompanies the BR mRNP to the Nuclear Pore

Eva Kiesler, Francesc Miralles,[2] and Neus Visa[1]
Department of Molecular Biology
and Functional Genomics
University of Stockholm
SE-10691 Stockholm
Sweden

Summary

The splicing factor UAP56/HEL/Sub2p is essential for mRNA export [1-4]. It has been proposed [1,2] that UAP56/HEL/Sub2p interacts with the pre-mRNA during splicing and recruits the export factor Aly/REF/Yra1 (reviewed in [5]) to the spliced mRNA. However, UAP56/HEL/Sub2p also participates in the transport of intronless mRNAs, and thus its role in export is not necessarily coupled to splicing [2-4]. Here, we characterize the HEL protein of *Chironomus tentans* and we analyze in situ the interaction of HEL with a natural export substrate, the Balbiani ring pre-messenger ribonucleoprotein (BR pre-mRNP, reviewed in [6]). Using immunoelectron microscopy, we show that HEL binds to the BR pre-mRNP cotranscriptionally and that incorporation of HEL into the pre-mRNP is independent of the location of introns along the BR pre-mRNA. We also show that HEL accompanies the BR mRNP to the nuclear pore and is released from the BR mRNP during translocation to the cytoplasm. Aly/REF is also released from the BR mRNP during translocation but after dissociation of HEL. In summary, we have shown that binding of HEL to the BR pre-mRNA occurs independently of splicing, and we have established the point in the export pathway at which HEL and Aly/REF interact with the mRNP.

Results and Discussion

In a library screening to be reported elsewhere, a cDNA encoding the HEL/UAP56 homolog of the dipteran *Chironomus tentans* was isolated and sequenced (Accession Number AJ428513). The HEL protein of *C. tentans* (Ct-HEL) is 90% identical to *Drosophila melanogaster* HEL and 85% identical to human UAP56. Moreover, Ct-HEL contains the seven sequence motifs that are characteristic of DExD/H box RNA helicases (reviewed in [7]).

Antibodies raised against *D. melanogaster* HEL [8] recognized Ct-HEL in Western blots of *C. tentans* proteins (see the Supplementary Material available with this article online) and recognized recombinant Ct-HEL expressed in *Escherichia coli* (data not shown). Thus, the anti-HEL antibodies could be used for in situ studies in *C. tentans*. Staining of *C. tentans* tissue culture cells (Figure 1A) showed that Ct-HEL was mainly nuclear and distributed in a speckled manner. Double-labeling experiments allowed us to compare the location of Ct-HEL with that of hnRNP-like proteins such as Aly/REF [9, 10] and Hrp36 [11]. Hrp36 and Aly/REF were distributed evenly throughout the nucleus and were highly colocalized. The location of Ct-HEL was much more peripheral, perhaps reflecting a role of Ct-HEL in the late steps of intranuclear transport (see below).

The BR pre-mRNA has all the features of a typical protein-coding transcript and constitutes a useful experimental system for in situ studies of mRNA (reviewed in [6]). The association of proteins with the nascent BR pre-mRNA can be studied by immunolabeling of polytene chromosomes. Immunofluorescent staining of *C. tentans* chromosomes with anti-HEL and anti-REF antibodies revealed intense labeling of BR puffs (Figure 1B), which suggests that Aly/REF and HEL bind to nascent BR pre-mRNA cotranscriptionally. This is consistent with previous observations of the association of HEL with transcriptionally active genes in *D. melanogaster* [8] and with the reported genetic interaction between Sub2p and RNA polymerase II-associated factors [3].

The BR pre-mRNA is 35- to 40-kb long and contains four introns (Figure 2A). Introns 1–3 are spliced cotranscriptionally, whereas intron 4 is excised mainly posttranscriptionally [12–14]. The synthesis of the BR pre-mRNP particles can be visualized using transmission electron microscopy (EM). The active BR transcription units, represented schematically in Figure 2B, show a distinct polarity defined by the size and the structure of the nascent pre-mRNPs along the gene [15]. Full-length loops are not observed in the sections used for EM, but partial loop segments can be assigned to a defined position along the gene based on their morphology. The possibility of identifying the different parts of the BR transcription unit allowed us to map the binding of Ct-HEL to the BR pre-mRNA. All parts of the BR loop were labeled by the anti-HEL antibody (Figure 2C). The labeling in the proximal segment indicated that Ct-HEL binds to the newly synthesized pre-mRNAs shortly after transcription initiation. We also concluded that each BR pre-mRNP particle incorporates multiple copies of Ct-HEL, as shown by the fact that most of the pre-mRNPs were labeled with multiple gold markers. Gold markers on the same BR pre-mRNP were often more than 45 nm apart (arrows in Figure 2C) and, therefore, could not be explained by multiple antibody molecules binding to a single HEL molecule.

It has been proposed (reviewed in [16]) that HEL binds to the pre-mRNA during splicing and remains associated with the pre-mRNP after intron removal as part of the exon-exon junction complex (EJC). If this is so, the number of HEL molecules per pre-mRNA molecule is proportional to the number of introns excised from the pre-mRNA. To test this hypothesis, we quantified the progressive association of Ct-HEL to the BR pre-mRNP. We calculated the number of gold markers per pre-mRNP in the middle and distal segments of the BR gene as a relative measure of the number of Ct-HEL molecules

[1]Correspondence: neus.visa@molbio.su.se
[2]Present address: Cancer Research UK, 44 Lincoln's Inn Fields, London WC2A 3PX, United Kingdom.

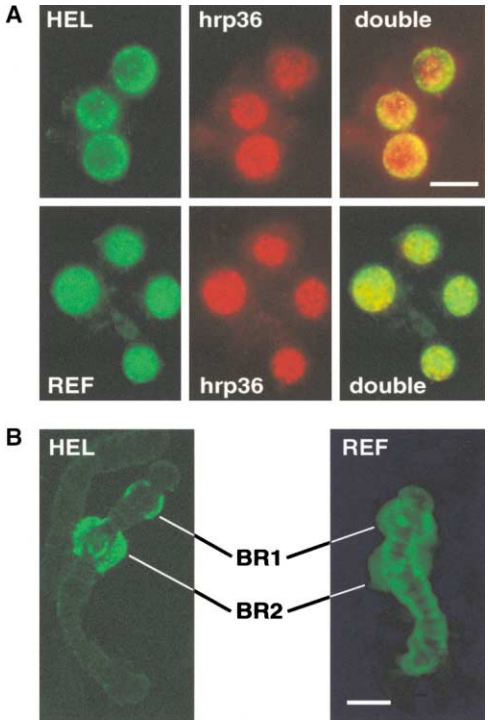

Figure 1. Location of HEL and Aly/REF in *C. tentans*

(A) HEL and Aly/REF are nuclear proteins in *C. tentans*. *C. tentans* tissue culture cells were double labeled with anti-Hrp36 antibody and either anti-HEL or anti-REF antibodies. The scale bar represents ~5 μm.

(B) HEL and Aly/REF are chromosomal proteins associated with transcriptionally active loci in *C. tentans* polytene chromosomes. Salivary gland polytene chromosomes were stained with anti-HEL or anti-REF antibodies following standard procedures. The large BR puffs in chromosome IV, BR1 and BR2, are stained with both antibodies. The scale bar represents ~10 μm.

per BR pre-mRNP. The proximal segment was excluded from this analysis due to the difficulty in identifying individual pre-mRNPs in this region. The average density of labeling per pre-mRNP was 50% higher in the distal segment of the gene than in the middle segment, which could not be correlated with the intron-exon structure of the BR pre-mRNA. Each pre-mRNP in the middle segment of the BR loop has already undergone three splicing events [12, 13]. Intron 4 is located ~600 nt upstream of the polyadenylation site and ~1200 nt from the transcription termination site [14]. The entire distal segment is about 12-kb long (one third of the length of the BR gene), and thus only 5%–10% of the distal BR pre-mRNPs contain intron 4. If intron 4 was spliced cotranscriptionally and HEL was added stochiometrically to the pre-mRNP in each splicing event, 5%–10% of the distal pre-mRNPs would show labeling values ~30% higher than those in the middle segment of the gene. In the context of the whole distal segment, this intron-related increase would be almost negligible and could not account for the 50% difference observed between the middle and the distal parts of the gene.

Interestingly, the increase in labeling density observed during transcription of the BR gene was nearly proportional to the increase in the length of the nascent pre-mRNA (histogram in Figure 2C). The average relative lengths of the BR pre-mRNAs in the proximal, middle, and distal segments of the BR gene are 16%, 50%, and 83%, respectively, of the full length of the BR pre-mRNA. According to these theoretical ratios, the increase in the RNA length from the middle segment to the distal segment was 66%, close to the observed 50% increase of Ct-HEL immunolabeling. We also compared the pre-mRNPs in the distal segment with the full-length nucleoplasmic BR mRNPs, and again the increase of labeling (13%) was very similar to the increase in the length of the nascent transcript (16%). These observations indicated that Ct-HEL binds progressively to the newly synthesized pre-mRNA regardless of the position of introns.

We extended our immunoelectron microscopy analysis to characterize the association of Aly/REF with the BR pre-mRNPs. Aly/REF was also detected in the three segments of the BR transcription unit (Figure 2D). However, quantitative comparisons between middle, distal, and nucleoplasmic BR pre-mRNPs did not reveal any increase in labeling density, which is compatible with the proposed binding of Aly/REF to the pre-mRNA during splicing as part of the EJC [17]. The slight reduction observed in the labeling density (histogram in Figure 2D) does not necessarily reflect a decrease in the number of Aly/REF molecules bound to each BR pre-mRNP but is probably due to the concealment of Aly/REF epitopes during pre-mRNP packaging, as described for other hnRNP proteins [18].

The results reported above show that the interactions of Aly/REF and HEL with the BR pre-mRNP follow different patterns. Furthermore, considering previous reports about the role of UAP56 in splicing [19], we conclude that HEL can interact with the pre-mRNA in two different ways, one related to its role as a splicing factor and another one that is independent of splicing.

Quantitative immunoelectron microscopy was also applied to map the dissociation of Ct-HEL from the BR mRNP (Figure 3). The percentages of labeled BR mRNPs were calculated for three consecutive transport stages: nucleoplasmic BR mRNPs, BR mRNPs docked at the nuclear pore complex (NPC), and translocating BR mRNPs. Most of the nucleoplasmic (88%) and docked (69%) BR particles were labeled by the anti-HEL antibody, whereas only 10% of the translocating BR particles were positive (Figure 3D). Thus, we concluded that Ct-HEL accompanies the BR particle to the NPC, remains associated with the BR mRNP during the docking step, and dissociates from the mRNP complex early in the translocation process.

We also analyzed the association of Aly/REF with the BR mRNPs (Figure 3C). A total of 73% of the nucleoplasmic BR mRNPs and 80% of the docked BR mRNPs were labeled by the anti-REF antibody (Figure 3D). Interestingly, the dissociation of Aly/REF was significantly delayed in comparison with the dissociation of Ct-HEL, as shown by the fact that 43% of the translocating BR particles were labeled by the anti-REF antibody, whereas only 10% were positive for HEL (Figure 3D). These results are in agreement with the proposal that binding of TAP/NXF-1 to Aly/REF causes the dissociation of HEL from the mRNP export cargo and triggers the translocation of the mRNP through the NPC [2, 16].

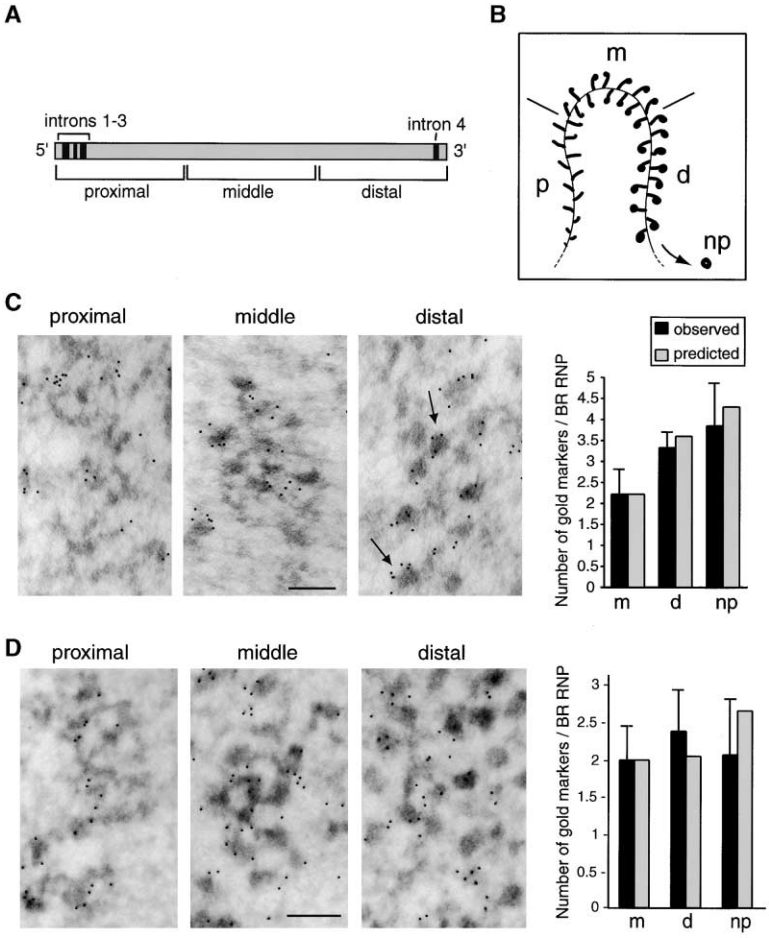

Figure 2. Cotranscriptional Binding of HEL and Aly/REF to BR Pre-mRNPs

(A) The exon-intron structure of the BR gene.

(B) A schematic representation of the BR transcription unit showing the progressive growth of the pre-mRNPs along the gene and their release into the nucleoplasm (np) upon transcription termination. For analytical purposes, the BR transcription unit is divided in three segments: proximal (p), middle (m), and distal (d).

(C) Immunoelectron microscopy localization of HEL in the BR transcription unit. Cryo-sections of salivary gland cells were labeled with anti-HEL using 6-nm gold markers conjugated to the secondary antibody as described previously [11]. Examples of labeling in proximal, middle, and distal segments are shown. The arrows point to BR particles labeled by multiple gold markers located more than 45 nm apart (see text for details). The histogram shows the average number of gold markers/BR pre-mRNP (black bars) observed in the middle segment of the BR gene (m), in the distal segment (d), and in nucleoplasmic (np) BR mRNPs. Error bars indicate standard deviations. The gray bars represent the predicted increase in the number of gold markers/BR pre-mRNP, assuming progressive incorporation of HEL into the pre-mRNP during transcription proportionally to the pre-mRNA length.

(D) Immunoelectron microscopy localization of Aly/REF in the BR transcription unit, as in (C). The gray bars in the histogram represent the predicted number of gold markers/BR pre-mRNP, assuming stochiometric incorporation of Aly/REF into the pre-mRNP during splicing as part of the EJC.

The scale bars represent 100 nm.

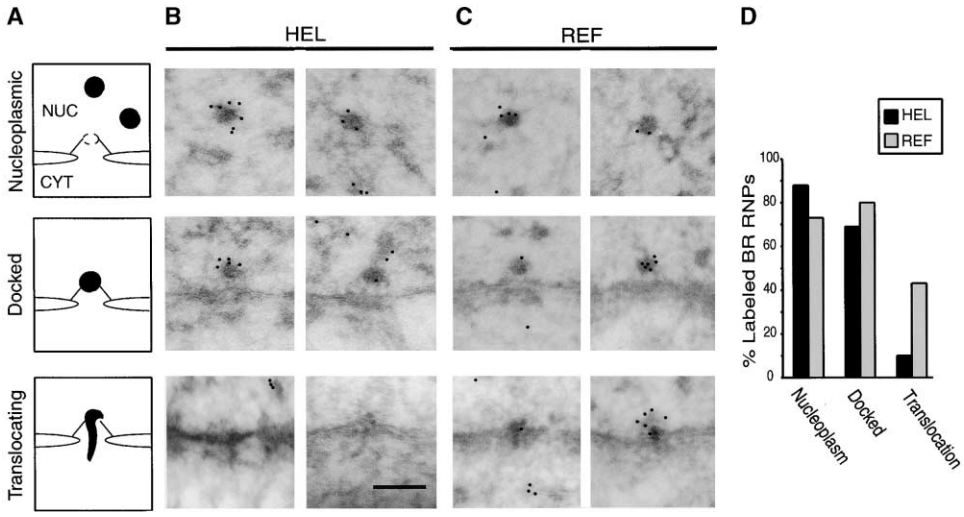

Figure 3. Dissociation of HEL and Aly/REF from BR mRNPs

(A) A schematic representation of BR mRNPs in three successive stages of nucleocytoplasmic transport.

(B and C) Cryo-sections of salivary glands immunostained as in Figure 2 with either anti-HEL or anti-REF antibodies. For each antibody, two examples of labeling in each transport stage are shown. The scale bar represents 100 nm.

(D) The histogram summarizes the percentages of labeled BR mRNPs observed with anti-HEL (black bars) and anti-REF (gray bars) antibodies in the nucleoplasm, when docked, and during translocation.

The BR mRNPs undergo a striking reorganization during nucleocytoplasmic transport, both in terms of conformational modifications and of changes in protein composition. At least two different hnRNP proteins, hrp23 and hrp45, leave the BR mRNP sequentially in the proximity of the NPC (reviewed in [6]), while other proteins come into contact with the BR mRNP at the nuclear pore [20]. Moreover, BR pre-mRNPs interact transiently with large fibrillar structures in the nucleoplasm and need to be released before translocation to the cytoplasm can take place [21]. It is likely that these complex rearrangements are facilitated by remodeling factors with the capacity to promote the exchange of RNA binding partners, such as RNA helicases [7, 22]. At least three RNA helicases are associated with the BR mRNPs: HEL, Dbp5 [23], and hrp84 (J. Zhao, D. Nashchekin, N.V., and B. Daneholt, unpublished data). All three are added cotranscriptionally to the BR pre-mRNP and are released at different time points during or after transport of the BR mRNP to the cytoplasm. We need to perform further biochemical work to establish the specific role(s) of each one in the export of the BR mRNPs and to define the step(s) of mRNA biogenesis in which each helicase is implicated.

Supplementary Material

Supplementary Material including full Experimental Procedures and two additional figures is available at http://images.cellpress.com/supmat/supmatin.htm.

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