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REF1/Aly and the additional exon junction complex proteins are dispensable for nuclear mRNA export

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The metazoan proteins UAP56, REF1, and NXF1 are thought to bind sequentially to mRNA to promote its export to the cytoplasm: UAP56 is thought to recruit REF1 to nascent mRNA; REF1 acts as an adaptor protein mediating the association of NXF1 with mRNA, whereas NXF1 translocates the mRNA across the nuclear pore complex. REF1 is a component of the exon–exon junction complex (EJC); thus, the EJC is thought to play a role in the export of spliced mRNA. NXF1 and UAP56 are essential for mRNA export. An essential role for metazoan REF1 or the additional EJC proteins in this process has not been established. Contrary to expectation, we show that REF1 and the additional components of the EJC are dispensable for export of bulk mRNA in *Drosophila* cells. Only when REF1 and RNPS1 are codepleted, or when all EJC proteins are simultaneously depleted is a partial nuclear accumulation of polyadenylated RNAs observed. Because a significant fraction of bulk mRNA is detected in the cytoplasm of cells depleted of all EJC proteins, we conclude that additional adaptor protein(s) mediate the interaction between NXF1 and cellular mRNAs in metazoa. Our results imply that the essential role of UAP56 in mRNA export is not restricted to the recruitment of REF1.

Introduction

Recent studies in yeast and higher eukaryotes have led to the elucidation of an evolutionarily conserved pathway for the export of bulk mRNA to the cytoplasm. mRNA export is mediated by a heterodimeric export receptor, distinct from the importin β–like family of nuclear transport receptors (Conti and Izaurralde, 2001; Dreyfuss et al., 2002; Lei and Silver, 2002; Reed and Hurt, 2002). The larger subunit of the heterodimer (ca. 70 kD) belongs to the conserved family of NXF proteins, which includes yeast Mex67p, *Caenorhabditis elegans*, *Drosophila melanogaster* (Dm),* and human NXF1 (also known as TAP; Herold et al., 2000). The small subunit, p15 (ca. 15 kD), is conserved in metazoa but not in *Saccharomyces cerevisiae*. Instead, the protein Mtr2p is predicted to be functionally analogous to p15 in this organism (Katahira et al., 1999).

Binding of the NXF1:p15 heterodimers to mRNA export cargoes is thought to be mediated by adaptor proteins. Among these are members of the REF protein family (Stutz et al., 2000). REFs bind directly RNA and NXF1 or Mex67p, facilitating the recruitment of NXF1 or Mex67p to cellular mRNAs. Yra1p (one of the *S. cerevisiae* members of this family) is essential for mRNA export in yeast cells (Strässer and Hurt, 2000; Stutz et al., 2000; Zenklusen et al., 2001). A murine member of the family (called Aly) partially restores growth of a Yra1p-deleted strain, indicating a functional conservation (Strässer and Hurt, 2000). Furthermore, evidence for a role of human and murine REFs in mRNA export has been reported (Zhou et al., 2000; Koffa et al., 2001; Rodrigues et al., 2001).

In higher eukaryotes, REFs, together with the splicing coactivators SRm160 and RNPS1, the Y14:mago-nashi (Y14:MGN) heterodimer and the acute myeloid leukemia-associated protein DEK bind preferentially to spliced mRNAs as components of a multiprotein complex (the exon–exon junction complex [EJC]), deposited by the spliceosome upstream of splice junctions (Blencowe et al., 1998; Mayeda et al., 1999; Kataoka et al., 2000, 2001; Le Hir et al., 2000a,b, 2001a,b; McGarvey et al., 2000; Zhou et al., 2000; Kim et al., 2001). The EJC is thought to play a role in postsplicing mRNA metabolism by influencing the transport, translation or stability of spliced mRNAs (for review see Dreyfuss et al., 2002; Reed and Hurt, 2002).

A role for the EJC in mRNA export is suggested by the observation that some of its components (REF1 and Y14) interact with NXF1 (Stutz et al., 2000; Kim et al., 2001; Rodrigues et al., 2001). Consistently, the EJC has been

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*Abbreviations used in this paper: Dm, *Drosophila melanogaster*; dsRNAi, double-stranded RNA interference; EJC, exon–exon junction complex; NPC, nuclear pore complex.

Key words: Aly; EJC; mRNA export; NXF1; REF1

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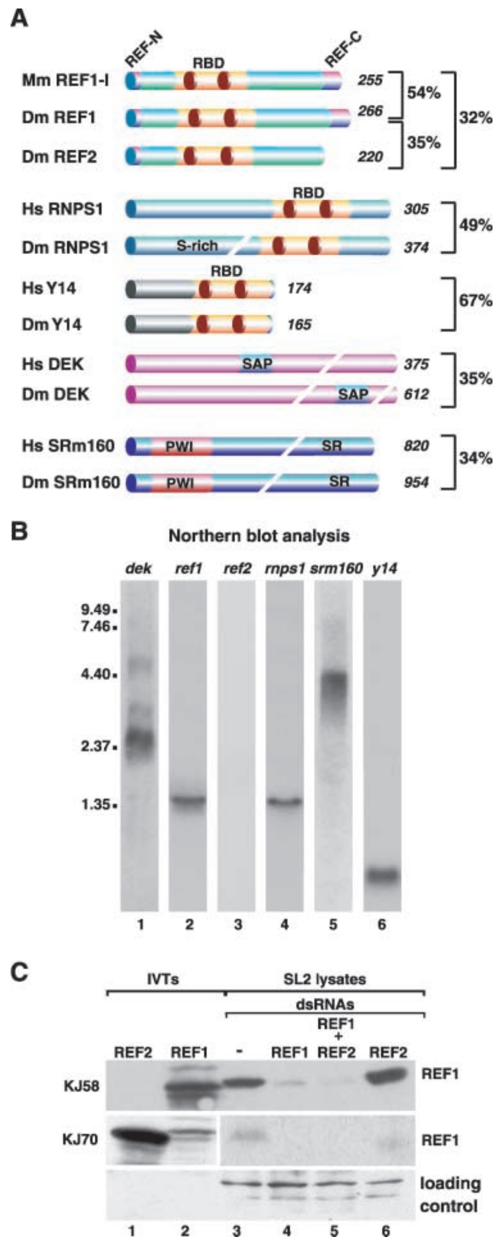

Figure 1. **Expression and domain organization of *Drosophila* EJC proteins.** (A) Schematic sequence relationships among *Drosophila* and vertebrate components of the EJC. The numbers in italics indicate the length of the amino acid sequence. The sequence identities between *Drosophila* and human proteins are indicated on the right. PWI, a motif characterized by a conserved PWI sequence (Blencowe and Ouzounis, 1999); RBD, RNP-type RNA binding domain (the RNP1 and RNP2 motifs are shown in red); SAP, a putative DNA binding motif (Aravind and Koonin, 2000); SR-domain, Serine and Arginine rich domain. The REF proteins are characterized by the presence of two conserved motifs at their NH_2 and COOH termini known as REF-N (N) and REF-C (C) boxes (Stutz et al., 2000). The REF-C box is missing in Dm REF2. RNPS1 is characterized by an RBD and an NH_2-terminal sequence rich in serines (S-rich). Despite sequence similarities and a similar domain organization, Dm DEK is almost twice the size of human DEK. (B) Detection of Dm *dek, ref1, ref2, rnps1, srm160,* and *y14* mRNAs by Northern blot analysis. 25 μg of total RNA from SL2 cells was loaded per lane. For *ref2,* no signal was detected even after prolonged exposures. (C) SL2 cells were transfected with dsRNA specific for Dm REF1 (lane 4), Dm REF2 (lane 6), or a mixture of both dsRNAs (lane 5). Protein samples from total lysates of untreated or depleted cells were analyzed by Western blot using two anti-REF antisera (KJ58 and KJ70). 1 μl aliquots of unlabeled Dm REF1 or Dm REF2 synthesized in vitro in *E. coli* lysates were analyzed in parallel (lanes 1 and 2).

shown to facilitate the recruitment of NXF1 to spliced mRNAs (Le Hir et al., 2001a). These and other observations led to the proposal that the splicing process facilitates the export of spliced mRNA products by recruiting EJC components directly and NXF1 indirectly (Zhou et al., 2000; Kim et al., 2001; Le Hir et al., 2001a). However, the fact that apart from REFs there are no obvious homologues of EJC proteins in *S. cerevisiae* suggests that additional or different mechanisms may account for the association of Yra1p, and hence Mex67p with spliced mRNAs in this organism. More importantly, a splicing-independent mechanism for the recruitment of these proteins by cellular mRNAs is suggested by the observation that Yra1p, Dm NXF1:p15, and *S. cerevisiae* Mex67p:Mtr2p export both intron-containing and intronless mRNAs (Hurt et al., 2000; Strässer and Hurt, 2000; Vainberg et al., 2000; Herold et al., 2001; Wilkie et al., 2001).

Yra1p and human REF1 interact with the DEAH-box helicase Sub2p (known as UAP56 in vertebrates; Luo et al., 2001; Strässer and Hurt, 2001). Although originally implicated in splicing (for review see Linder and Stutz, 2001), studies in yeast, *Drosophila* cells, and *Xenopus laevis* oocytes indicate that Sub2p/UAP56 also plays an essential role in mRNA export (Gatfield et al., 2001; Jensen et al., 2001; Luo et al., 2001; Strässer and Hurt, 2001). The interactions of Yra1p with Sub2p and Mex67p are mutually exclusive (Strässer and Hurt, 2001). Based on these observations and on the association of Sub2p with transcription elongation factors (Strässer et al., 2002) it has been proposed that Sub2p associates with nascent mRNAs cotranscriptionally and recruits Yra1p. The mRNA could then be rendered export competent by the displacement of Sub2p by Mex67p (Strässer et al., 2002).

The conservation of Sub2p, Yra1p, Mex67p, and their interactions, together with functional studies in *Xenopus* oocytes and mammalian cells, has led to the likely hypothesis that export of mRNAs in higher eukaryotes follows a similar pathway as in yeast (Reed and Hurt, 2002). Accordingly, the helicase UAP56 would bind mRNAs cotranscriptionally and recruit the adaptor protein REF1. Before export, NXF1:p15 heterodimers would bind to REF1 and thereby release UAP56. However, whereas in *S. cerevisiae*, Sub2p, Yra1p, and Mex67p are essential for mRNA export, in higher eukaryotes, only *C. elegans* NXF1 and the *Drosophila* proteins NXF1, p15, and UAP56 have so far been shown to be essential for this process (Tan et al., 2000; Gatfield et al., 2001; Herold et al., 2001; Wilkie et al., 2001; Wiegand et al., 2002).

We have investigated the export pathway of bulk mRNA in cultured *Drosophila* cells. Using double-stranded RNA interference (dsRNAi), we demonstrate that the *Drosophila* homologues of all known components of the vertebrate EJC are dispensable for the export of bulk mRNA. A partial accumulation of polyadenylated RNAs is observed within the nucleus of cells depleted of REF1 or simultaneously depleted of REF1 and RNPS1. This accumulation is increased by depleting Y14 and SRm160 as well, but it is not as dramatic as that observed when NXF1 or UAP56 are depleted. Because

a significant fraction of bulk mRNA is detected in the cytoplasm of cells simultaneously depleted of REF1, RNPS1, SRm160, and Y14, we conclude that mRNA export proceeds in their absence. This indicates that the recruitment of NXF1:p15 heterodimers by cellular mRNAs is mediated by additional adaptor protein(s) in higher eukaryotes.

Results

Conservation of EJC proteins in *Drosophila*

We have shown previously that depletion of Dm NXF1 and Dm UAP56 from *Drosophila* Schneider cells (SL2 cells) inhibits cell proliferation and results in a strong nuclear accumulation of poly(A)$^+$ RNA, whereas depletion of Dm Y14:MGN heterodimers impairs cell viability without affecting bulk mRNA export (Gatfield et al., 2001; Herold et al., 2001; Le Hir et al., 2001b). To investigate the export pathway of bulk mRNA further, we sought to deplete from SL2 cells the *Drosophila* homologues of REF and other known EJC proteins by dsRNAi (Clemens et al., 2000; Hammond et al., 2000). Using BLAST searches (Altschul et al., 1997), we identified two genes encoding members of the REF protein family, *ref1* and *ref2,* and single *dek*, *rnps1*, and *srm160* genes in available *Drosophila* genomic sequences (Adams et al., 2000). Comparison of the proteins encoded by these genes with their homologues in other metazoan species indicates that the overall domain organization is conserved (Fig. 1 A). Northern analysis revealed that *dek*, *ref1*, *rnps1*, *y14*, and *srm160* are expressed in SL2 cells (Fig. 1 B). In contrast, *ref2* mRNA is not expressed at detectable levels in this cell line (Fig. 1 B).

The expression of DEK, RNPS1, and SRm160 was confirmed by Western blotting using antibodies raised to the recombinant proteins (see below; Fig. 2). Because REF1 and REF2 could have partially redundant functions, it was important to confirm that *ref2* is not expressed in SL2 cells. Previously, we raised two distinct rabbit antisera (KJ58 and KJ70) against the recombinant RBD domain of murine REF1 (Rodrigues et al., 2001). Fortuitously, we found that KJ58 recognizes in vitro translated Dm REF1 but not Dm REF2, whereas KJ70 strongly crossreacts with in vitro synthesized Dm REF2 but only very weakly with Dm REF1 (Fig. 1 C, lanes 1 and 2). When used in Western blots, KJ58 labels a major band with the expected mobility of REF1 in SL2 cell lysates (Fig. 1 C, lane 3). Indeed, this band corresponds to REF1, as it is strongly reduced when expression of the *ref1* gene is silenced by dsRNAi (Fig. 1 C, lanes 4 and 5). KJ70 very weakly labels a single band, which corresponds to REF1 but not to REF2, as this band is no longer detected in extracts isolated from REF1 depleted cells (Fig. 1 C, lanes 4 and 5). Together with the Northern blot analysis, these results indicate that REF2 is not expressed at detectable levels in SL2 cells. More importantly, these results underline the specificity of dsRNAi, as a dsRNA corresponding to the coding sequence of Dm REF2 does not silence the expression of *ref1*.

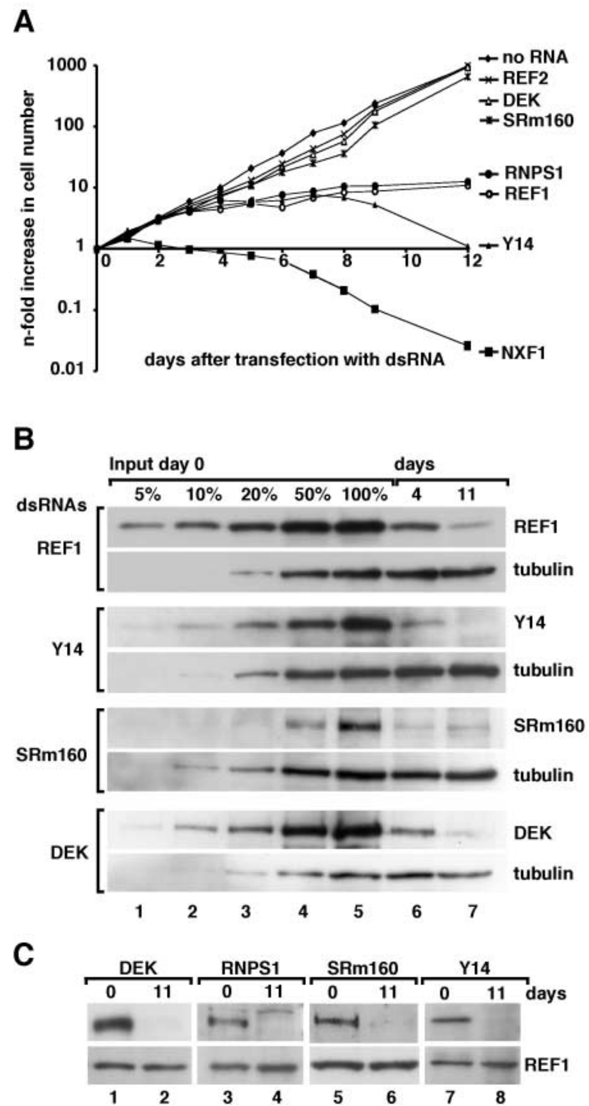

Figure 2. ***Drosophila* cells depleted of EJC proteins display diverse growth phenotypes.** (A) SL2 cells growing in suspension were transfected with dsRNAs specific for DEK, NXF1, REF1, REF2, RNPS1, SRm160, and Y14. Cell numbers were determined every day up to 12 d after transfection. On day 7, cells were retransfected with the corresponding dsRNAs, with the exception of NXF1-depleted cells. Data are given as the *n*-fold increase in cell numbers relative to the amount of cells used for transfection on day 0 (where *n* represents a number on the y axis). Note that on the y axis the scale is logarithmic. (B) Aliquots of cells shown in panel A were collected on days 4 and 11 and analyzed by Western blotting with antibodies raised against the recombinant proteins. The steady-state expression level of targeted proteins was reduced, whereas the expression level of the unrelated protein tubulin is not affected (lanes 5–7). In lanes 1–4, dilutions of the samples isolated on day 0 were loaded to analyze the efficiency of the depletion. (C) SL2 cells were transfected with dsRNAs specific for DEK, RNPS1, SRm160, and Y14 as indicated above the panels. 11 d after transfection cell lysates were analyzed by Western blotting with antibodies specific for the corresponding proteins. The expression level of REF1 is shown in the bottom panels.

Depletion of REF1 or of individual EJC proteins leads to different effects on cell proliferation

To deplete endogenous EJC proteins, SL2 cells were transfected with dsRNAs corresponding to the respective coding sequences. As a control, a dsRNA corresponding to GFP or to REF2 was transfected, since *ref2* is not expressed in SL2 cells. Cells depleted of REF1 and RNPS1 proliferate more slowly than untreated cells or cells transfected with REF2

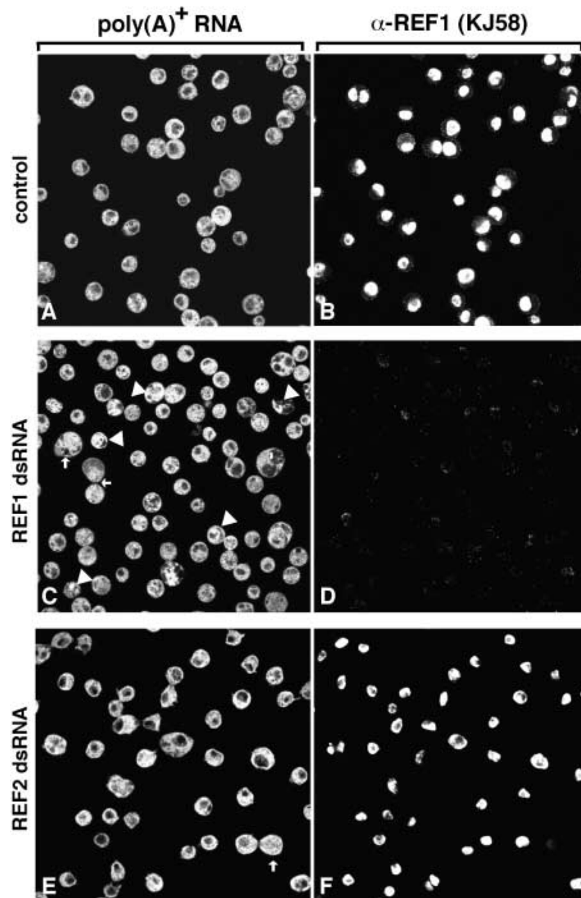

Figure 3. **REF1/Aly is dispensable for mRNA export in *Drosophila* cells.** SL2 cells treated with GFP (control), REF1, or REF2 dsRNAs were fixed 11 d after transfection and stained with antibodies that specifically recognize Dm REF1 (KJ58). Cells were double labeled by FISH with a Cy3-labeled oligo(dT) probe (poly[A]$^+$ RNA). The arrowheads point to cells in which a nuclear accumulation of poly(A)$^+$ RNA was observed. These represent <20% of the cell population. The arrows point to cells in which the nucleus is not in the focal plane.

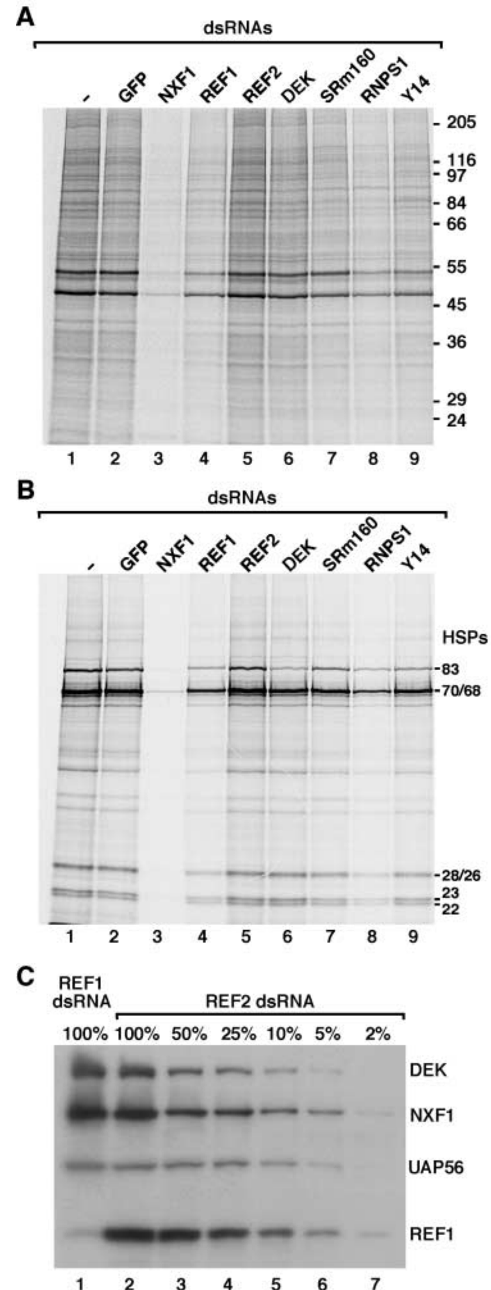

Figure 4. **Depletion of EJC proteins reduces, but does not abolish, incorporation of [^{35}S]Met into newly synthesized proteins.** (A and B) SL2 cells were transfected with GFP, REF1, REF2, DEK, SRm160, RNPS1, and Y14 dsRNAs. With the exception of cells depleted of NXF1, which were collected 4 d after transfection, depleted cells were collected 11 d after transfection. Cells were kept at 25°C (A) or subjected to a 1 h heat shock at 37°C (B). At this temperature, the heat-shock proteins (HSPs) are induced, whereas translation of nonheat shock proteins is inhibited. Cells were pulse labeled with [^{35}S]methionine for 1 h. Total lysates from equivalent numbers of cells were analyzed by SDS-PAGE followed by Coomassie blue stain and fluorography. Equal amounts of total protein were loaded per lane as judged by Coomassie staining. The efficacy of the depletions was tested by Western blot (not depicted). (C) SL2 cells were transfected with REF1 or REF2 dsRNAs. Aliquots of cells were collected 11 d posttransfection and analyzed by Western blotting with antibodies raised against the proteins indicated on the right. The steady-state expression level of REF1 was reduced, while the expression levels of DEK, NXF1, and UAP56 were not affected (compare lanes 1 and 2). In lanes 3–7, dilutions of the control sample were loaded to assess the efficiency of the depletion.

dsRNA (Fig. 2 A). This reduction of cell proliferation is significant compared to control cells, but is not as dramatic as that observed when Dm NXF1 (Fig. 2 A) or Dm UAP56 are depleted (Gatfield et al., 2001). Depletion of Y14 compromised cell viability (Le Hir et al., 2001b). In contrast, depletion of DEK and SRm160 had no apparent effect (Fig. 2 A).

The efficiency of the depletions was tested by Western blot (Fig. 2 B). 4 d after transfection, the steady-state expression levels of REF1, DEK, SRm160, and Y14 were <20% the levels detected in untreated cells (Fig. 2 B, lanes 6 vs. 3). 11 d after transfection, the residual levels of the proteins were <5% of those observed in control cells (Fig. 2 B, compare lanes 7 and 1). Transfection of dsRNAs corresponding to Y14, DEK, RNPS1, and SRm160 had no effect on the expression of REF1 (Fig. 2 C).

REF1 is not essential for nuclear export of bulk mRNA

The intracellular distribution of bulk poly(A)$^+$ RNA in control cells and in cells depleted of EJC proteins was investigated by oligo(dT) in situ hybridization. Depletion of REF1 or RNPS1 occasionally resulted in a partial accumulation of

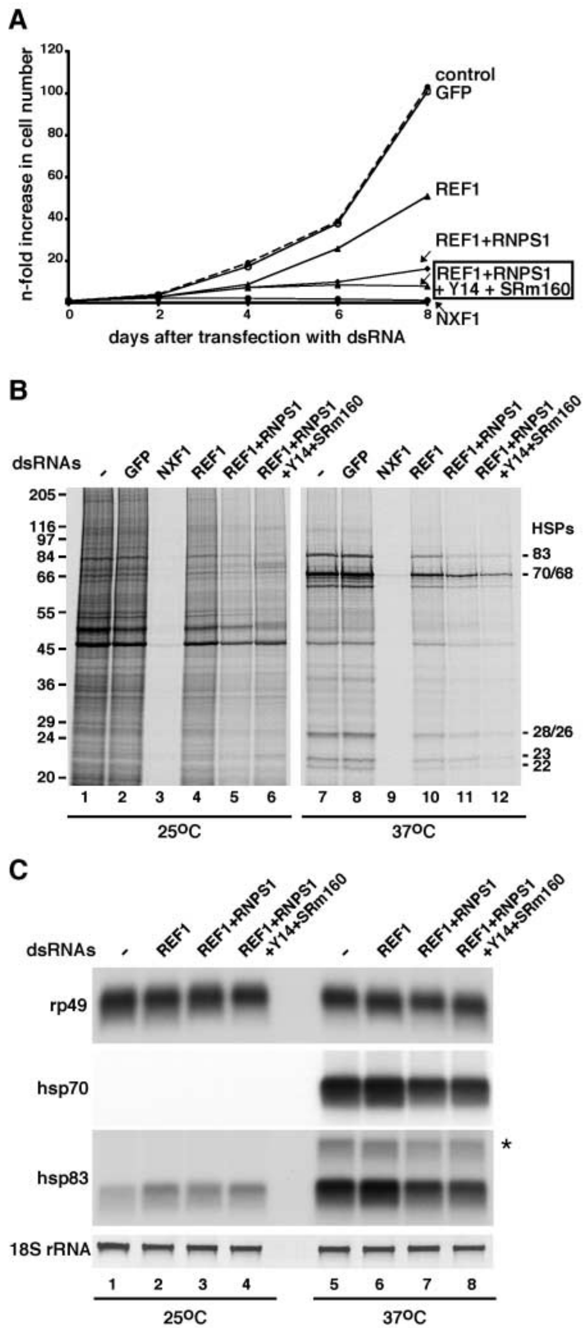

Figure 5. **Codepletion of EJC proteins reduces protein synthesis.** (A) SL2 cells were transfected with GFP, NXF1, or REF1 dsRNAs or with a mixture of dsRNAs as indicated. Cell numbers were determined every two days up to 8 d after transfection. On day 4, cells were retransfected with the corresponding dsRNAs, with the exception of NXF1-depleted cells. Data are given as the *n*-fold increase in cell numbers relative to the amount of cells used for transfection on day 0 (where *n* represents a number on the y axis). (B) SL2 cells were transfected with GFP, NXF1, or REF1 dsRNAs or with a mixture of dsRNAs as indicated above the lanes. 8 d after transfection, cells were kept at 25°C (lanes 1–6) or subjected to a 1 h heat shock at 37°C (lanes 7–12) and pulse-labeled as described in Figure 6. Total cell extracts were analyzed by SDS-PAGE followed by Coomassie stain and fluorography. Equal amounts of total proteins were loaded per lane as revealed by Coomassie staining. (C) 8 d after transfection, cells from the experiment shown in B were either kept at 25°C (lanes 1–4) or subjected to a 1 h heat shock at 37°C (lanes 5–8). 10 μg of total RNA was analyzed by Northern blot using probes specific for rp49, hsp70 or hsp83 mRNAs (Herold et al., 2001). The bottom panel shows the corresponding rRNA stained with ethidium bromide. The asterisk indicates the position of the unspliced hsp83 precursor mRNA that accumulates at 37°C.

poly(A)$^+$ RNAs within the nucleus. This accumulation was observed in <20% of the cells although indirect immunofluorescence showed that REF1 was efficiently depleted in the entire cell population (Fig. 3, C and D). No significant nuclear accumulation of poly(A)$^+$ RNAs was observed in cells depleted of DEK, SRm160 or Y14 (unpublished data).

The lack of a general mRNA export block after depletion of REF1 or other EJC proteins is further confirmed by the observation that depletion of some of these proteins reduces, but does not abolish the incorporation of [^{35}S]Met into newly synthesized proteins 11 d after transfection (Fig. 4 A). Moreover, induction of the heat shock response was not completely inhibited (Fig. 4 B). The strongest inhibitory effects were consistently observed after depletion of REF1 and RNPS1 (Fig. 4, A and B, lanes 4 and 8). As expected, no inhibition of protein synthesis was observed in cells depleted of REF2, DEK, and SRm160 (Figs. 4, A and B, lanes 5–7).

The possibility that undetectable levels of REF2 would compensate for the absence of REF1 is very unlikely for the following reasons. First, simultaneous depletion of both REF1 and REF2 does not exacerbate the slow growth phenotype observed when only REF1 is depleted. Second, suppression of REF2 in a cell population depleted of REF1 neither increases the proportion of cells exhibiting nuclear accumulation of poly(A)$^+$ RNA nor leads to a stronger inhibition of protein synthesis (unpublished data).

Furthermore, the lack of a strong export inhibition after REF1 depletion cannot be explained by a compensatory overexpression of NXF1, UAP56, or any one of the EJC proteins, as the endogenous levels of these proteins are not affected in REF1-depleted cells (Fig. 4 C; unpublished data). In summary, REF1 and the *Drosophila* homologues of mammalian EJC proteins may contribute to, but are not essential for bulk mRNA export.

Simultaneous depletion of REF1 and RNPS1 partially inhibits protein synthesis

The mild inhibition of protein synthesis after depleting REF1, RNPS1, or Y14, and the lack of phenotype of DEK and SRm160 could be explained if their role in bulk mRNA export (or in any other post-transcriptional mRNA process) were partially redundant. To address this question, we simultaneously inactivated more than one gene product by dsRNAi. With the exception of DEK, whose depletion combined with that of the other proteins does not exacerbate the phenotype observed following single depletions, codepletions of all other EJC proteins in all combinations result in a stronger inhibition of cell proliferation (Fig. 5 A, unpublished data). These data suggest that *Drosophila* SRm160, REF1, RNPS1, and Y14 function in a common pathway, but argue against a functional interaction between these proteins and DEK.

Codepletions of EJC proteins (except DEK) also lead to greater reduction of protein synthesis. The strongest effect

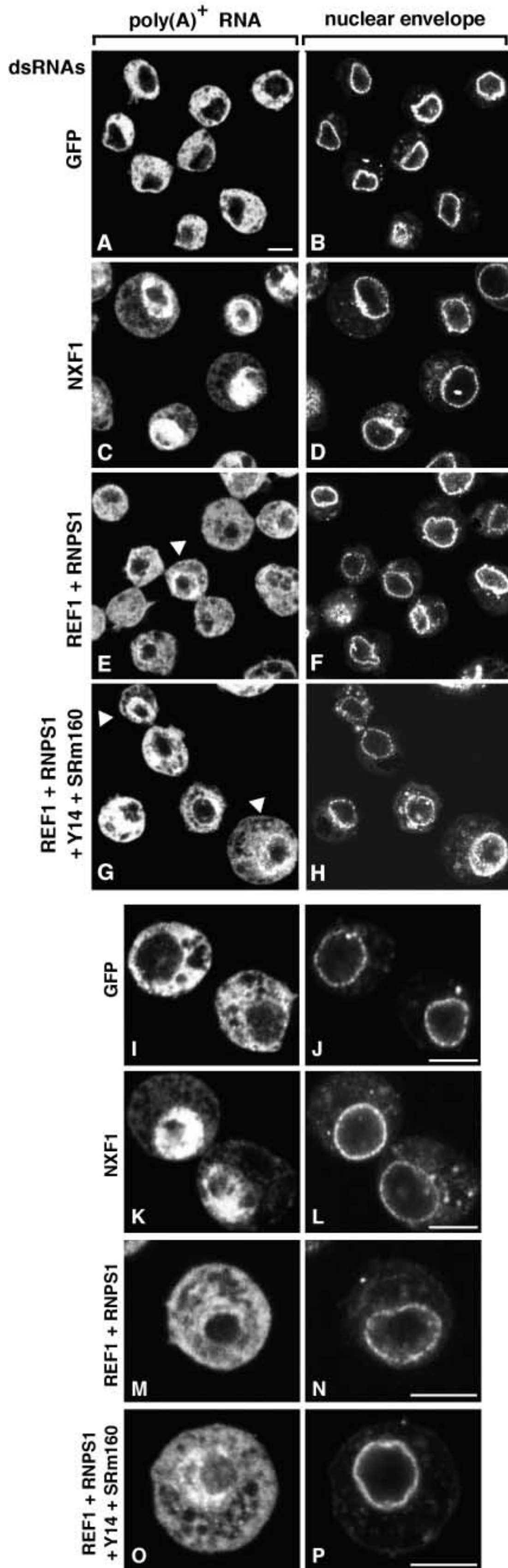

Figure 6. **Codepletion of EJC proteins leads to a partial nuclear accumulation of poly(A)+ RNA.** (A–H) SL2 cells were transfected with dsRNA corresponding to GFP, NXF1, and mixtures of dsRNAs. These mixtures consisted of dsRNAs corresponding to REF1 and RNPS1 or to REF1, RNPS1, SRm160, and Y14 as indicated. Cells depleted of NXF1 were fixed 5 d after transfection. Cells depleted of EJC proteins and control cells were fixed 8 d after transfection. Poly(A)+ RNA was detected by FISH with a Cy3-labeled oligo(dT) probe (A, C, E, G). The nuclear envelope was stained with Alexa 488-wheat germ agglutinin conjugates (B, D, F, H). The arrowheads point to cells in which a significant nuclear accumulation of poly(A)+ RNA was observed. Bar, 5 μm. (I–P) Representative examples of patterns of poly(A)+ RNA distribution in SL2 cells shown in A. Inhibition of mRNA export is complete in ~94% of cells depleted of NXF1 (K), but partial with an equal distribution of the oligo(dT) signal between the nucleus and the cytoplasm in ~20% of cells depleted of REF1 and RNPS1 (M). Depletion of all EJC proteins resulted in a stronger inhibition of mRNA export but a cytoplasmic signal was still detected (O). This phenotype is observed in ~40% of the cell population. Bars, 2.5 μm.

is observed when REF1 and RNPS1 are codepleted (Fig. 5 B, lanes 5 and 11). This inhibitory effect is slightly increased when, in addition to REF1 and RNPS1, Y14, and SRm160 are simultaneously suppressed (Fig. 5 B, lane 12). However, this reduction of protein synthesis is not as strong as that observed in the absence of NXF1 (Fig. 5 B, lanes 3 and 9), indicating that mRNA export is not completely inhibited.

To determine whether the partial inhibition of protein synthesis is caused by a block to transcription or splicing, we analyzed the steady-state expression level of rp49 mRNA, which is an abundant mRNA in SL2 cells. Northern blot analysis revealed that 8 d after addition of dsRNAs, the expression levels of rp49 mRNA are not affected in cells depleted of REF1 (Fig. 5 C, lane 2), but are consistently slightly reduced in cells depleted of RNPS1 alone or in combination with REF1 or with REF1, Y14, and SRm160 (Fig. 5 C, lanes 3, 4, 7, and 8; unpublished data). The levels of hsp70 and hsp83 mRNAs before and after shifting the cells for 1 h to 37°C were also analyzed. Both in control and in REF1 depleted cells, these mRNAs were induced to comparable levels after heat shock (Fig. 5 C, lanes 5 and 6 vs. 1 and 2). The induction levels are slightly reduced in cells codepleted of REF1 and RNPS1 or of all EJC proteins (Fig. 5 C, lanes 7 and 8 vs. 5 and 6).

The hsp83 mRNA differs from all other heat shock mRNAs in that it is normally expressed at relatively high levels even without exposure to heat stress, and in being the only heat shock gene containing an intron (Echalier, 1997). When cells are shifted to 37°C, hsp83 mRNA synthesis is induced, but because splicing is inhibited at 37°C (Yost and Lindquist, 1986), the unspliced hsp83 pre-mRNA accumulates (Fig. 5 C, lanes 5–8, asterisk). The accumulation of unspliced hsp83 pre-mRNA occurs in depleted cells as well as in control cells at 37°C, but is not observed in depleted cells at temperatures below 37°C even after prolonged exposures (Fig. 5 C, lanes 1–4; unpublished data). Together, these results indicate that simultaneous depletion of REF1, RNPS1, SRm160, and Y14 proteins does not lead to a general block of transcription or splicing of bulk pre-mRNAs. Nonetheless, a decrease of steady-state mRNA levels is observed, which may result from a reduced transcriptional activity or an increased turnover rate of mRNAs in depleted cells. This decrease on mRNA levels certainly contributes to the overall inhibition of [^{35}S]Met incorporation into newly synthesized proteins.

Codepletion of EJC proteins leads to a partial nuclear accumulation of poly(A)$^+$ RNA

The distribution of poly(A)$^+$ RNA in cells depleted of REF1 and RNPS1 or of all EJC proteins (except DEK) was analyzed 8 d after addition of dsRNAs. Consistent with the partial inhibition of protein synthesis, in both populations an increased number of cells exhibiting a significant nuclear accumulation of polyadenylated RNAs was observed (Fig. 6, E and G, arrowheads). The percentage of cells in which the poly(A)$^+$ signal was predominantly nuclear or evenly distributed between the nucleoplasm and cytoplasm (Fig. 6, M and O) was determined in two independent experiments. After REF1 depletion a significant nuclear accumulation of poly(A)$^+$ RNA was observed in ~10% of the cell population. Codepletion of REF1 and RNPS1 resulted in ~20% of cells showing a clear nuclear staining. This percentage increased to 40% when Y14 and SRm160 were also depleted. In contrast, depletion of NXF1 results in a strong nuclear accumulation of poly(A)$^+$ RNA in about 94% of the cell population (Fig. 6 C; Herold et al., 2001; Braun et al., 2002). Moreover, although depletion of NXF1 results in a homogeneous phenotype (Fig. 6, C and K), depletion of all EJC proteins leads to a heterogeneous pattern of oligo(dT) staining (Fig. 6, E and G). Because significant cytoplasmic staining was evident in most cells 8 d after depletion (Fig. 6, E, G, M, and O), we conclude that bulk mRNA export can proceed in the absence of REF1, RNPS1, SRm160, and Y14, although with a reduced efficiency.

Discussion

Before this study, REF1 and the additional components of the EJC were largely believed to provide a molecular link between splicing and mRNA export (for review see Reed and Hurt, 2002). However, we show here that neither REF1 nor the individual EJC proteins are essential for export of bulk mRNA. Furthermore, simultaneous depletion of REF1, RNPS1, SRm160, and Y14 only results in a partial accumulation of polyadenylated RNAs within the nucleus, indicating that besides REF1 or the EJC proteins, additional adaptor protein(s) bridge the interaction between NXF1:p15 and cellular mRNAs in higher eukaryotes.

Role of REF1 in mRNA export

Although REF1 is not essential for mRNA export in vivo, several lines of evidence indicate that REF1 can nevertheless contribute to the recruitment of NXF1 to cellular mRNAs. First, antibodies specific to REFs that prevent their interaction with RNA in vitro reduced the export rate of mRNA after microinjection into *Xenopus* oocytes (Rodrigues et al., 2001). Second, microinjection of recombinant REF1 into *Xenopus* oocytes stimulates the export of mRNAs that are otherwise exported inefficiently (Zhou et al., 2000; Rodrigues et al., 2001). Finally, it has recently been shown that the Herpes virus ICP27 protein promotes export of viral RNAs by recruiting REF1 directly and NXF1 indirectly (Koffa et al., 2001). Thus, REF1 is able to promote NXF1 binding to RNA cargoes. Together with these observations, our results suggest that in higher eukaryotes REF1 is not essential for the export of bulk mRNA (i.e., mRNA export can still take place in its absence), but may contribute to the overall efficiency of this process. We cannot rule out that REF1 plays in addition a crucial role in the export of specific mRNAs. Moreover, REF1 may play a role in other posttranscriptional processes as discussed below.

Role of EJC proteins in posttranscriptional mRNA metabolism

Depletion of the individual EJC proteins leads to different effects on cell proliferation, mRNA export, and protein synthesis. In particular, individual depletion of RNPS1 or Y14 inhibits cell proliferation without significantly affecting bulk mRNA export (as judged by oligo[dT] in situ hybridizations). This suggests that these proteins may play a crucial role in the posttranscriptional metabolism of specific transcript(s), which in turn are required for cell proliferation or survival.

Simultaneous depletion of REF1 and RNPS1 or of these two proteins together with SRm160 and Y14 significantly inhibits both cell proliferation and the incorporation of [^{35}S]Met into newly synthesized proteins (Fig. 5). The inhibition of protein synthesis may be in part due to a partial inhibition of mRNA export, but a reduction in steady-state mRNA levels also contributes to the observed phenotype. Alternatively, the overall reduction of protein synthesis could be due to a role of these proteins in the translation activity of exported mRNAs rather than in the export process itself (B. Cullen and M.J. Moore, personal communication). Consistently, a role for Y14 in modulating the translation activity of cytoplasmic mRNAs has been proposed (Dostie and Dreyfuss, 2002). Finally, the accumulation of poly(A)$^+$ RNA within the nucleus may not only reflect an export block but the nuclear retention of pre-mRNAs whose processing is inhibited. Although splicing is not generally inhibited in the absence of REF1, RNPS1, SRm160, and Y14, removal of specific introns and/or 3′-end processing of specific transcripts may require the activity of some of these proteins. In particular, both RNPS1 and SRm160 were originally identified as activators of pre-mRNA splicing and SRm160 has been reported to influence polyadenylation in mammalian cells (Blencowe et al., 1998; Eldridge et al., 1999; Mayeda et al., 1999; Longman et al., 2001; McCracken et al., 2002).

Although the *Drosophila* genome encodes homologues of all known vertebrate EJC proteins, it has not been established whether the EJC is assembled in this organism, so our conclusions are applicable to the individual proteins and not to the EJC. The possibility that in vertebrates these proteins would play an essential role in bulk mRNA export through their assembly into the EJC is nonetheless unlikely. Indeed, the effect of splicing (and thereby of EJC assembly) on multiple steps of gene expression including transcription, polyadenylation, mRNA export, translation efficiency, and mRNA decay has recently been systematically analyzed in mammalian cells (B. Cullen and M.J. Moore, personal communication). These studies show that mRNAs generated through the splicing pathway are more efficiently incorporated into polyribosomes. In contrast, splicing and EJC formation has no significant effect on nuclear mRNA export. In agreement with these observations in mammalian cells, in

this study we show that REF1/Aly, RNPS1, SRm160, and Y14 are not essential for the formation of a export-competent mRNP in *Drosophila* cells.

UAP56: a specific recruitment factor or a general mRNP remodeling protein?

Although in *S. cerevisiae* the role of Sub2p in mRNA export has been shown to be related to its ability to recruit Yra1p (Strässer and Hurt, 2001), further work is needed to clarify the role of this protein in higher eukaryotes. Analysis of the association of UAP56 and REF1 with the Balbiani ring RNPs of *Chironomus tetans* has shown that UAP56 is recruited cotranscriptionally along the mRNA independently of the presence of introns (Kiesler et al., 2002). In contrast, the recruitment of REF1 is restricted to the regions of the transcript in which introns have been removed. This indicates that binding of UAP56 to nascent transcripts does not necessarily lead to the recruitment of REF1.

In this and previous studies (Gatfield et al., 2001) we found that although UAP56 is essential for the export of bulk mRNA, REF1 is dispensable. There are several possible explanations for this observation. In higher eukaryotes, UAP56 may recruit an unidentified essential protein (besides REF1), which in turn promotes binding of NXF1:p15 heterodimers. Alternatively, UAP56 may not act as a recruiting factor but its role in mRNA export could be more general. Indeed, as expected for a helicase, UAP56 could trigger ATP-dependent rearrangements of the mRNP at specific stages of the export process. This may then facilitate binding of proteins that act as adaptors for NXF1. Although this is an attractive hypothesis, it has not yet been shown whether UAP56 has helicase activity or whether its putative ATPase activity is required for its essential export function.

The missing link in the mRNA export pathway of higher eukaryotes

The essential role of NXF1 or Mex67p in the export of poly(A)$^+$ RNA is well established in *S. cerevisiae*, *C. elegans*, and *D. melanogaster*. In these organisms, inactivation or depletion of these proteins results in a strong accumulation of bulk mRNA within the nucleoplasm (Segref et al., 1997; Tan et al., 2000; Herold et al., 2001; Wilkie et al., 2001). In yeast and *Drosophila* cells, this nuclear accumulation of poly(A)$^+$ RNA correlates with a strong inhibition of protein synthesis, indicating that export of most mRNAs is affected (Hurt et al., 2000; Vainberg et al., 2000; Herold et al., 2001; Wilkie et al., 2001). Similarly, as mentioned above, both Dm UAP56 and its yeast homologue Sub2p are essential for export of bulk mRNA (Gatfield et al., 2001; Jensen et al., 2001; Strässer and Hurt, 2001).

The link between Sub2p and Mex67p is provided by Yra1p, which is also essential for the export of poly(A)$^+$ RNA in *S. cerevisiae* (Strässer and Hurt, 2000, 2001; Stutz et al., 2000). In this paper we show that, despite strong conservation, REF1, the metazoan homologue of Yra1p, is dispensable for bulk mRNA export. This raises the possibility that another essential adaptor protein or (alternatively) multiple and partially redundant adaptor proteins link UAP56 and NXF1 in higher eukaryotes. The identification of this additional adaptor protein(s) will provide important insights into the molecular mechanisms by which mRNAs are exported in higher eukaryotic cells.

Materials and methods

Cloning of *Drosophila* cDNAs

Genes encoding *Drosophila* homologues of components of the EJC were retrieved in EST databases and from the *Drosophila* genome using the Blast suite of programs (Altschul et al., 1997). Multiple sequence alignments were constructed using CLUSTAL X (Thompson et al., 1994). *Drosophila* genes described in this study correspond to the following genes in the FlyBase (http://flybase.bio.indiana.edu): REF1 (*Aly*; CG1101); REF2 (CG17031); Y14 (*Tsu*; CG8781); DEK (CG5935); RNPS1 (CG16788); SRm160 (CG11274); UAP56 (*Hel25E*); and NXF1 (*sbr*). Full-length *Drosophila* REF1 (Aly), REF2, Y14, RNPS1, and DEK cDNAs were amplified by PCR using *D. melanogaster* quick-clone cDNA (CLONTECH Laboratories, Inc.) as a template and primers introducing unique restriction sites. All PCR reactions were performed with the Expand high-fidelity PCR system (Roche). The complete cDNAs were cloned into pBS-SK (Stratagene) and sequenced. The cDNA present in these plasmids was used for all further subcloning steps.

Although the sequences of REF1, Y14, and SRm160 are identical to those present in the FlyBase, we have obtained DEK, REF2, and RNPS1 cDNA clones that differ from those present in the FlyBase. However, our cDNA sequences are identical to sequences present in several ESTs. The corrected DEK, REF2, and RNPS1 cDNA sequences have been submitted and are available at EMBL/GenBank/DDBJ under accession numbers AJ459408 (DEK), AJ459410 (REF2), and AJ459409 (RNPS1).

dsRNAi in cultured *Drosophila* cells

SL2 cells were propagated at 25°C in Schneider's *Drosophila* medium supplemented with 10% fetal bovine serum, 50 U/ml penicillin and 50 μg/ml streptomycin. dsRNAi was performed essentially as described by Herold et al. (2001). dsRNAs used in this study correspond to full-length REF2 and Y14 cDNAs, or to fragments comprising amino acids 33–231 of REF1; 75–369 of SRm160; 6–251 of DEK; 1–184 of UAP56; 1–229 of RNPS1; and 137–354 of NXF1. 15 μg of dsRNA were used per 2×10^6 cells growing in suspension at a density of 10^6 cells/ml.

RNA isolation and Northern blots

Total RNA was isolated using TRIzol Reagent (Life Technologies). Total RNA was separated in denaturing formaldehyde agarose gels and blotted onto a positively charged nylon membrane (GeneScreen Plus; NEN Life Science). [^{32}P]-labelled probes were generated by random priming using standard methods. REF1, REF2, RNPS1, and Y14 probes correspond to the full-length cDNA, whereas SRm160 and DEK probes correspond to a 3′-end cDNA fragment of 1,000 and 1,100 nucleotides. Hybridizations were carried out in ULTRAhyb solution (Ambion), following the recommended protocol for hybridization and washing.

Preparation of total cell extracts and Western blotting

Drosophila total cell extracts were prepared as described by Herold et al. (2001). Rabbit antisera (KJ58 and KJ70) raised against murine REF1-II has been described before (Rodrigues et al., 2001). KJ58 recognizes Dm REF1 but not Dm REF2. Rabbit antibodies raised against recombinant Dm NXF1 have been described before (Herold et al., 2001). Antibodies to Dm DEK, Dm UAP56, and Dm RNPS1 were raised in rats immunized with GST fusions of the full-length proteins expressed in *E. coli*. Antibodies to Dm Y14 were obtained by immunizing rats with full-length Dm Y14 expressed in *E. coli* as a hexa-histidine-tag fusion. Antibodies to SRm160 were raised in rats immunized with a GST fusion of a fragment of the protein encompassing amino acids 1–192. For Western blots, the polyclonal antibodies were diluted 1:1,000, with the exception of anti-Dm RNPS1 diluted 1:400 and KJ58 diluted 1:2000. Bound primary antibody was detected with alkaline phosphatase-coupled anti–rabbit or anti–rat antibodies (1:50,000 dilution; Western-Star kit; Tropix).

FISH and indirect immunofluorescence

FISH in *Drosophila* cells were performed as described by Herold et al. (2001). Indirect immunofluorescence with specific antibodies was performed as described by Almeida et al. (1998). Affinity-purified anti-REF antibodies (KJ58; Rodrigues et al., 2001) were diluted 1:20,000. Cells were mounted using Fluoromount-G (Southern Biotechnology Associates, Inc.).

Images were taken with a confocal laser-scanning microscope (Carl Zeiss LSM 510).

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