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In vitro FRAP reveals the ATP-dependent nuclear mobilization of the exon junction complex protein SRm160

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We present a new in vitro system for characterizing the binding and mobility of enhanced green fluorescent protein (EGFP)-labeled nuclear proteins by fluorescence recovery after photobleaching in digitonin-permeabilized cells. This assay reveals that SRm160, a splicing coactivator and component of the exon junction complex (EJC) involved in RNA export, has an adenosine triphosphate (ATP)-dependent mobility. Endogenous SRm160, lacking the EGFP moiety, could also be released from sites at splicing speckled domains by an ATP-dependent mechanism. A second EJC protein, RNPS1, also has an

ATP-dependent mobility, but SRm300, a protein that binds to SRm160 and participates with it in RNA splicing, remains immobile after ATP supplementation. This finding suggests that SRm160-containing RNA export, but not splicing, complexes have an ATP-dependent mobility. We propose that RNA export complexes have an ATP-regulated mechanism for release from binding sites at splicing speckled domains. In vitro fluorescence recovery after photobleaching is a powerful tool for identifying cofactors required for nuclear binding and mobility.

Introduction

RNA splicing is coupled to RNA export from the nucleus; the mRNAs produced from intron-containing precursors are exported much more efficiently from the nucleus than are identical mRNAs produced without splicing (Luo and Reed, 1999). SRm160 is a coactivator of splicing that remains persistently bound to mRNAs after intron removal (Blencowe et al., 1998) at sites 20–24 nucleotides upstream from exon–exon junctions in a complex also containing DEK, RNPS1, Y14, the mRNA export factor Aly/REF, and Magoh (Kataoka et al., 2000, 2001; Le Hir et al., 2000a,b, 2001). Formation of this exon junction complex (EJC) is splicing dependent. In vitro, at least two proteins of the complex, Y14 and Magoh, bind to the TAP/p15 heterodimer (Kataoka et al., 2001) whose homologues are essential for the nuclear export of most mRNA (Herold et al., 2001). SRm160 stimulates 3' end cleavage of transcripts, and its overexpression causes the appearance of intron-containing RNAs in the cytoplasm (McCracken et al., 2002). The teth-

ering of SRm160 to transcripts lacking an active intron stimulates their 3' end cleavage and enhances their expression (McCracken et al., 2003; Wiegand et al., 2003).

The microscopic observation of fluorescent fusion proteins in living cells is a powerful tool for measuring intracellular dynamics. Time-lapse studies can track the movement of larger cellular structures, whereas FRAP is ideal for measuring the intracellular mobility or binding of fluorescently tagged proteins (Lippincott-Schwartz et al., 2001). The apparent mobility of several nuclear proteins has been reported, including histone H1.1 (Lever et al., 2000; Misteli et al., 2000), the glucocorticoid receptor (McNally et al., 2000), the estrogen receptor (Stenoien et al., 2001), ataxin (Stenoien et al., 2002), the nucleolar protein fibrillarin (Phair and Misteli, 2000), the nucleosomal binding protein HMG-17 (Phair and Misteli, 2000), the transcription factor Runx (Harrington et al., 2002), and the RNA splicing factor ASF/SF2 (Kruhlik et al., 2000; Phair and Misteli, 2000). All of these proteins recover from photobleaching at rates much slower than would be predicted for diffusing proteins of similar size. These decreased apparent mobilities may be imposed either by equilibrium interactions with structures such as chromatin and the nuclear matrix or by transient

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Key words: nuclear matrix; RNPS1; RNA export; RNA splicing; nuclear protein mobility

Abbreviation used in this paper: EJC, exon junction complex.

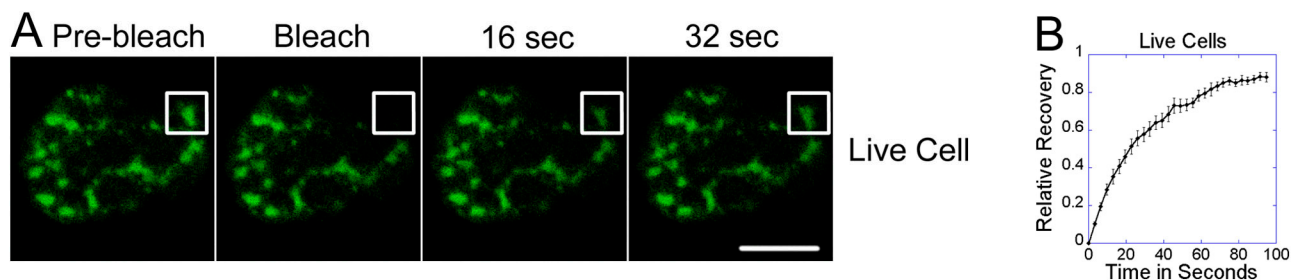


Figure 1. **Recovery of EGFP-SRm160 fluorescence after photobleaching in live cells.** (A) Images of this HeLa cell stably expressing EGFP-SRm160 were taken before (Pre-Bleach), immediately after (Bleach), and at intervals after bleaching (16 and 32 s). The bleached area is outlined by a white square. Bar, 10 μ m. (B) The recovery curve for EGFP-SRm160 in stably expressing HeLa cells presents the SEM for eight cells. The half time of recovery was 20 s with an immobile fraction of \sim 10%.

binding in large complexes (Nickerson, 2001). The regulation of these interactions has been explored by measuring changes in FRAP recovery rates after metabolic or drug manipulations; e.g., in transcriptionally inhibited cells (Kruhlak et al., 2000; Phair and Misteli, 2000), with steroid receptor agonists and antagonists (Stenoien et al., 2001), in the presence of proteasomal inhibitors (Stenoien et al., 2001, 2002), and in energy-depleted cells (Phair and Misteli, 2000; Stenoien et al., 2001; Calapez et al., 2002). It has recently been reported that the FRAP recovery rates of poly(A) binding protein II and TAP are significantly reduced after a treatment designed to reduce cell ATP levels (Calapez et al., 2002).

Here, we present a system for performing FRAP in the nuclei of digitonin-permeabilized cells, allowing the easy identification of cofactors required for nuclear protein binding and mobility. Visualized in this way, the mobilization of EGFP fusion proteins with SRm160 and RNPS1, two constituents of the EJC, required ATP. The spliceosomal SRm160 binding protein SRm300 (Blencowe et al., 2000) was not present in ATP-mobilized complexes, suggesting that ATP animates RNA export rather than RNA splicing complexes.

Results

The apparent mobility of EGFP-SRm160 was measured by FRAP in HeLa cells stably expressing the fusion protein. EGFP-SRm160 was most concentrated in splicing speckled domains, which is consistent with previous results (Wagner et al., 2003). Additionally, EGFP-SRm160 was also visible at a lower concentration within the surrounding nucleoplasm. Photobleaching experiments were exclusively performed by bleaching splicing speckled domains because it was difficult to obtain reliable recovery quantitation for the nucleoplasmic pool without overexpression of the fluorescent protein. The half time of recovery for EGFP-SRm160 at 37°C was 20 s with a 90% recovery and an immobile fraction of \sim 10% (Fig. 1). In comparison, the half time of recovery of the prototypic splicing factor SC35 was shorter, 10 s with an immobile fraction of \sim 10% (unpublished data). It has been reported that the EGFP fusion protein of ASF/SF2, an SR protein and splicing factor, has a photobleaching recovery half time in the range of 3–25 s depending on the cell line (Kruhlak et al., 2000; Phair and Misteli, 2001).

In these FRAP experiments, photobleached protein bound to sites in speckled domains is exchanging with unbleached

protein from other sites. The binding of SRm160 to splicing speckled domains has been reported previously (Blencowe et al., 1994; Wan et al., 1994; Wagner et al., 2003) and is confirmed by experiments reported in Fig. 1 A. In all FRAP experiments where fluorescence recovery was observed in the photobleached speckled domain, a corresponding decrease in fluorescence was measured in unbleached speckled domains. Because diffusion is rapid relative to our measured fluorescence recovery rates, we interpret these experiments as measuring the tightness of binding of fluorescent fusion proteins to sites at bleached and unbleached splicing speckled domains (Phair and Misteli, 2001; Lele, T., P. Oh, and D. Ingber, personal communication). The immobile fraction represents protein bound irreversibly over the time course of the experiment.

To assess the energy dependence of SRm160 mobilization, we first performed FRAP at room temperature. Recovery was slowed only slightly (unpublished data). Next, we reduced ATP levels by treating cells with 10 mM sodium azide plus 6 mM 2-deoxyglucose in the absence of glucose for 15–30 min. This reduction resulted in a small but significant decrease in the FRAP-measured mobility of EGFP-SRm160 (unpublished data). However, measurement of ATP levels by luciferase assay showed that after 30 and 45 min in azide/2-deoxyglucose, ATP concentrations had declined only 71% and 83%, respectively (Table I). Because ATP levels in cultured cells and tissues are 2–5 mM, and because K_m s for ATP are typically on the order of 10–200 μ M, there might be more than sufficient ATP persisting to maintain an ATP-dependent EGFP-SRm160 mobilization.

To resolve this point, we extended the FRAP technique into a novel in vitro system for biochemically characteriz-

Table I. **ATP reduction in HeLa cells**

Time	ATP
	μ M
Control	2,170 \pm 50
30 min	620 \pm 14
45 min	366 \pm 11

Cells were grown in 2 mM 2-deoxyglucose and 5 mM sodium azide in the absence of glucose for the indicated times. Cell ATP levels were measured by luciferase assay as described in Materials and methods. Data points were reported as the mean \pm SD of three replicates.

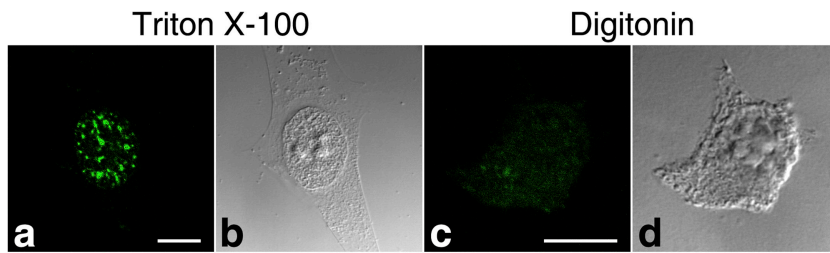


Figure 2. Digitonin permeabilized cells have an intact nuclear envelope. HeLa cells were permeabilized with Triton X-100 (a and b) or digitonin (c and d) before treatment with the B4A11 mAb against SRm300 and an Alexa 488-conjugated second antibody. Micrographs were collected using matched exposures so that cell fluorescence could be quantitatively compared. The antibodies were able to penetrate into the nuclear interior in Triton X-100-permeabilized cells. In contrast, nuclei of digitonin-permeabilized cells were devoid of fluorescence showing that the nuclear envelope remained intact. Bars, 10 μm .

ing the mobility and binding of nuclear proteins. Cells stably expressing EGFP-SRm160 were extracted in 50 $\mu\text{g}/\text{ml}$ digitonin to permeabilize the cell membrane without removing the nuclear envelope, a preparation previously used to study nucleocytoplasmic transport (Adam et al., 1990). We verified that the nuclear envelope remained impermeable to macromolecules by incubation with primary antibodies against the nuclear proteins SRm300 (Fig. 2), SRm160, and NuMA (not depicted) and fluorescently tagged second antibodies. These macromolecules did not penetrate the nuclear envelope. In this preparation, larger molecules, including EGFP-SRm160, are constrained to the nucleus, whereas ATP and other molecules smaller than the diffusion limit for nuclear pores (40–60 kD) are depleted from nuclei. ATP levels decline to 1 nM or less in these nuclei (Ribbeck et al., 1999). After photobleaching EGFP-SRm160 in the regions of single speckled domains, there was no detectable recovery of fluorescence in these digitonin-permeabilized cells (Fig. 3). However, supplementation with 5 mM ATP restored the apparent mobility of EGFP-SRm160 (Fig. 3). Recovery was slower and the fi-

nal recovery diminished to $\sim 60\%$ compared with 90% in living cells (compare Fig. 4 with Fig. 1 B).

After one round of ATP supplementation, extensive rinsing to remove ATP abolished recovery after photobleaching, but mobility could be restored by adding 5 mM ATP for a second time (Fig. 3 B), establishing that the continuing presence of ATP was required for maintaining mobility. However, the second addition of ATP resulted in a significantly increased immobile fraction of SRm160. In some experiments, EGFP-SRm160 in the nucleus of the same cell was photobleached while the cell was alive (mobile), after digitonin permeabilization (immobile), and eventually after ATP supplementation (mobile again).

To characterize the mechanism for the ATP-dependent mobilization of SRm160, digitonin-permeabilized cells were supplemented with either ADP, the nonhydrolyzable ATP analogue AMP-PNP, or GTP. ADP did not significantly restore fluorescence recovery (Fig. 4). The nonhydrolyzable ATP analogue AMP-PNP restored EGFP-SRm160 mobilization in a manner indistinguishable from ATP (Fig. 4), suggesting that ATP-induced conformational changes rather

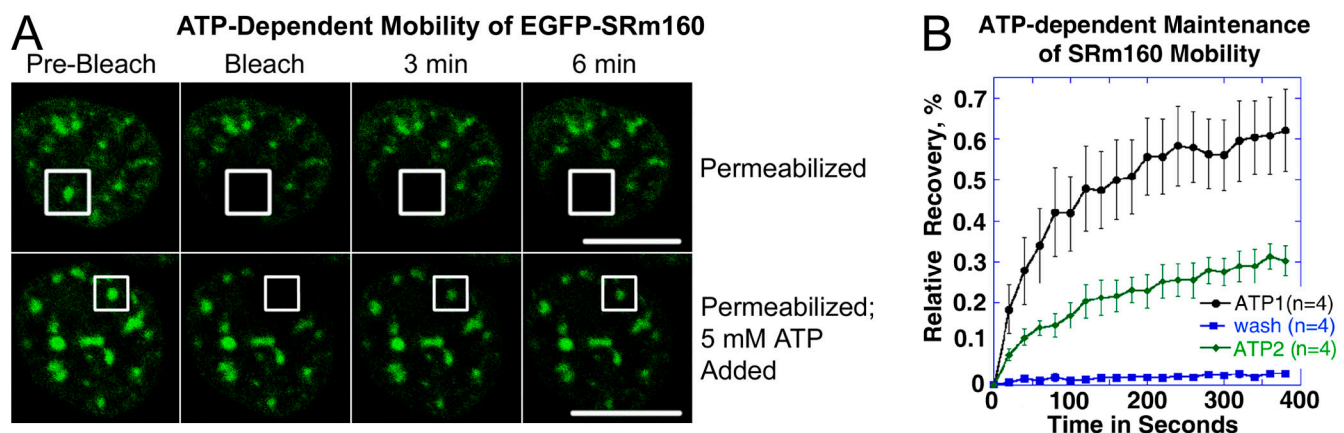


Figure 3. ATP supplementation restores EGFP-SRm160s mobility. (A) HeLa cells stably expressing EGFP-SRm160 were permeabilized with digitonin before photobleaching. Bleached areas are indicated by the white squares. Images were collected before (Pre-Bleach), immediately after (Bleach), and at intervals after photobleaching (3 and 6 min). EGFP-SRm160 was immobile in permeabilized cells, and bleached areas did not recover their fluorescence (top). In contrast, EGFP fluorescence was restored in the bleached areas of ATP (5 mM)-supplemented cells (bottom), indicating that EGFP-SRm160 has an ATP-dependent mobility. Bars, 10 μm . (B) HeLa cells were permeabilized with digitonin, replenished with 5 mM ATP, and photobleached (ATP1). The cells were washed extensively with import buffer to withdraw the ATP and photobleached again (wash). Finally, 5 mM ATP was added for a second time before a second round of photobleaching (ATP2). The curves represent the SEM for the indicated number of cells. ATP withdrawal stops the apparent mobility of SRm160, but readdition of ATP restores mobility again.

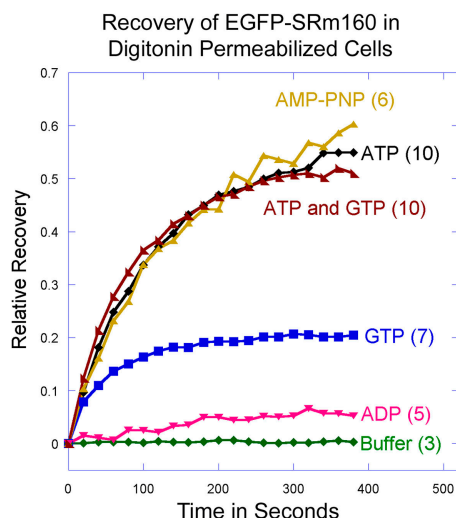


Figure 4. The nucleotide specificity of EGFP-SRm160 mobility in digitonin-permeabilized cells. HeLa cells stably expressing EGFP-SRm160 were permeabilized with digitonin. Before photobleaching, either buffer alone, 5 mM ADP, ATP, GTP, or AMP-PNP, or a combination of ATP and GTP (5 mM each) were added. Each point represents the mean for the number of photobleached cells indicated in parentheses to the right of the curve.

than protein phosphorylation may mediate the relocalization of SRm160 or its complexes. Addition of GTP resulted in only a 20% recovery after bleaching (Fig. 4). ATP and GTP did not work synergistically because they allowed no further or faster recovery than ATP alone (Fig. 4).

Next, we used the *in vitro* FRAP assay to characterize the mobility of other nuclear proteins. Transiently expressed EGFP-hnRNP A2 was a useful control. It was distributed throughout the nucleoplasm, slightly enriched in nucleoplasmic foci, and excluded from nucleoli (Fig. 5 A). The half time of recovery was ~ 15 s with an immobile fraction of $\sim 30\%$ (Fig. 5, A and D). In contrast to EGFP-SRm160, digitonin permeabilization did not slow the fluorescence recovery of EGFP-hnRNP A2 (Fig. 5 A), suggesting that hnRNP A2 mobility does not require ATP or other cofactors small enough to diffuse through nuclear pores.

SRm160 participates in RNA splicing as a coactivator and a constituent of spliceosomes (Blencowe et al., 1998, 2000) and in RNA export as a constituent of the EJC (Le Hir et al., 2000a,b, 2001). The SRm160 binding protein SRm300 is also present in spliceosomes (Blencowe et al., 1994, 2000) but is not detected in the EJC. EGFP-SRm300 in transiently transfected HeLa cells colocalized with SRm160 in splicing speckled domains (unpublished data). After the photobleaching of speckled domains, EGFP-SRm300 fluorescence recovered with a half time of 10 s (Fig. 5, B and D). Approximately 10% of EGFP-SRm300 remained immobile (Fig. 5 D).

In digitonin-permeabilized cells, EGFP-SRm300 fluorescence recovery was greatly reduced with only 8% recovery observed after 6 min (Fig. 5, B and E). Mobilization was not restored by ATP supplementation (Fig. 5, B and E), and the slight recovery observed in permeabilized cells was suppressed by ATP addition. This finding suggests that SRm300 requires factors other than ATP for movement within the nucleus. When SRm160 is relocalizing af-

ter ATP supplementation, it cannot be moving in a complex containing SRm300.

However, the protein RNPS1 is detected in the EJC (Kataoka et al., 2001; Le Hir et al., 2000a,b, 2001). EGFP-RNPS1 was most concentrated in splicing speckled domains with a smaller nucleoplasmic pool when transiently expressed in HeLa cells (Fig. 5 C). The half time of recovery after photobleaching in living cells was <10 s with an immobile fraction of $\sim 40\%$ (Fig. 5 D). Mobility completely stopped after digitonin permeabilization (Fig. 5 C) but could be substantially restored by ATP addition (Fig. 5, C and E). This recovery was slower than in living cells and only reached 30% compared with 60% for living cells (Fig. 5 E).

A second and complimentary approach was developed to assess the ATP dependence of SRm160 release from binding sites at speckled domains. This approach was done to confirm the results obtained from the *in vitro* FRAP assay and to rule out the possibility that the EGFP portion of the fusion protein was contributing to ATP-dependent relocalization. HeLa or SAOS cells were permeabilized and soluble proteins were extracted from both nucleus and cytoplasm with 0.5% Triton X-100 in cytoskeletal buffer with or without ATP. In contrast to digitonin treatment, this extraction removes the nuclear envelope, allowing diffusing nuclear proteins to leave. In some experiments, a soluble nuclear extract was added. As previously reported (Wan et al., 1994; Blencowe et al., 1998), treatment in the absence of ATP did not release SRm160 from either splicing speckles or from the nucleoplasm (Fig. 6 A). However, in the presence of ATP, most SRm160 was released. In contrast, the fluorescence intensity of SRm300 was only modestly reduced (Fig. 6 A). Thus, the endogenous SRm160 is released from splicing speckled domains in an ATP-dependent manner just as the fluorescent fusion protein.

We have shown that the localization of SRm160 to splicing speckled domains and its attachment to the nuclear matrix is conferred by two domains of the protein: amino acids 300–350 and 351–688 (Wagner et al., 2003). Both domains were also important for the ATP dependence of FRAP recovery: EGFP-SRm160 221–350, containing the first speckle targeting sequence and EGFP-SRm160 350–688, harboring the second recovered in an ATP-dependent manner (Fig. 7) but with a larger immobile fraction than the full-length protein (Fig. 4). Thus, the ATP-dependent recovery of SRm160 may depend on both splicing speckle domain targeting sequences.

Discussion

Using a new *in vitro* FRAP assay, we have shown that the apparent mobility of SRm160 depends on ATP. Although we continue to use traditional FRAP terminology, our interpretation of these FRAP experiments is that they are measuring the binding of SRm160 to sites at splicing speckled domains. There is no recovery after photobleaching under conditions where EGFP-SRm160 remains tightly bound. Addition of ATP allows for the unbinding and exchange of bleached and unbleached proteins. A general theoretical model for this interpretation of FRAP experiments has been developed (Lele, T., P. Oh, and D. Ingber, personal communication) and builds on previous

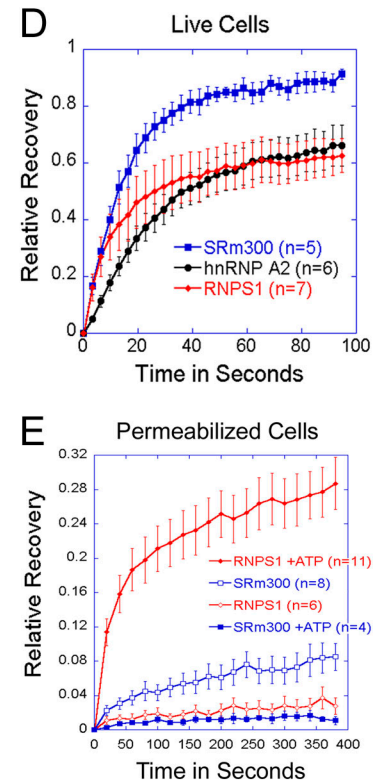
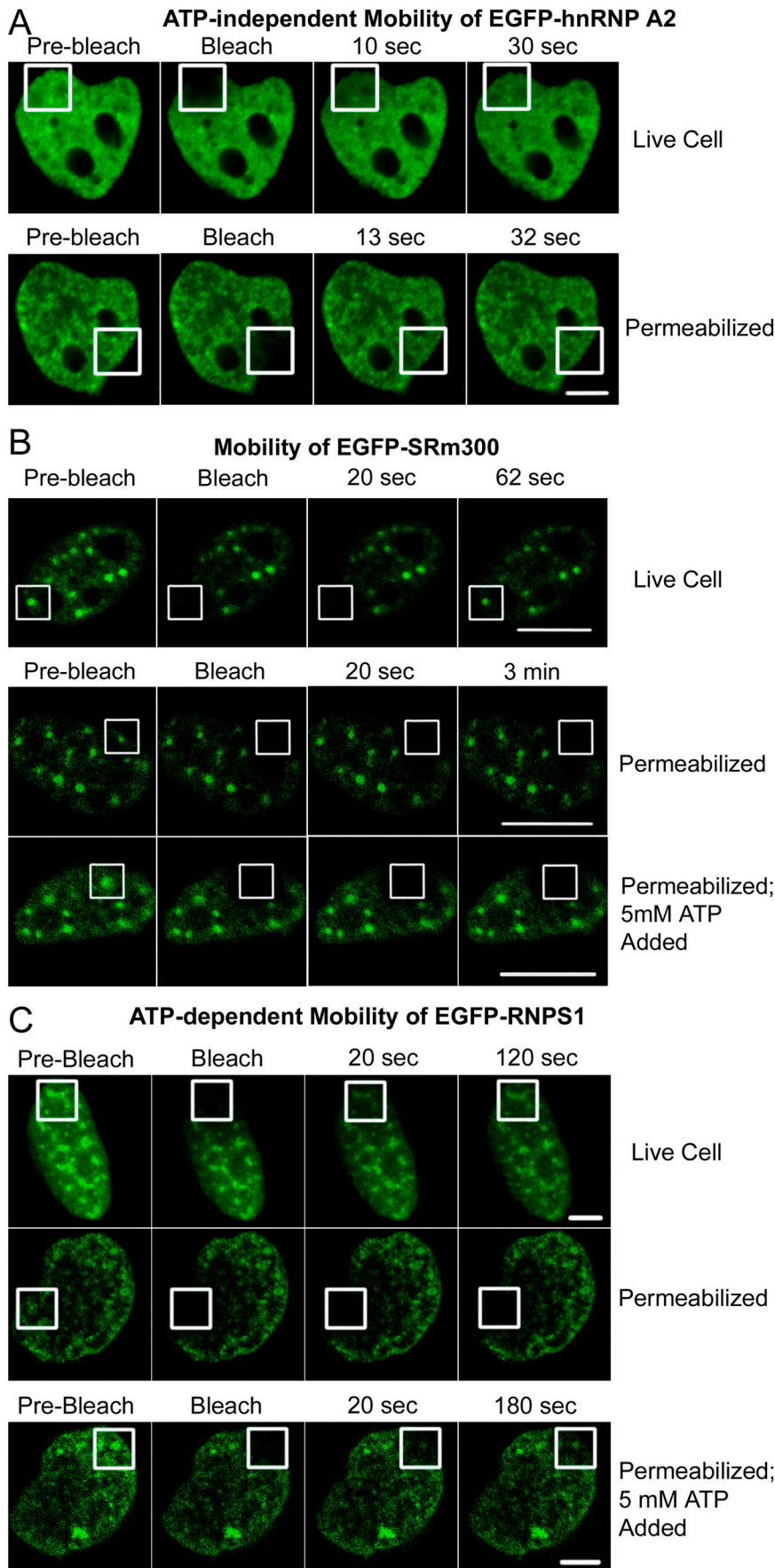
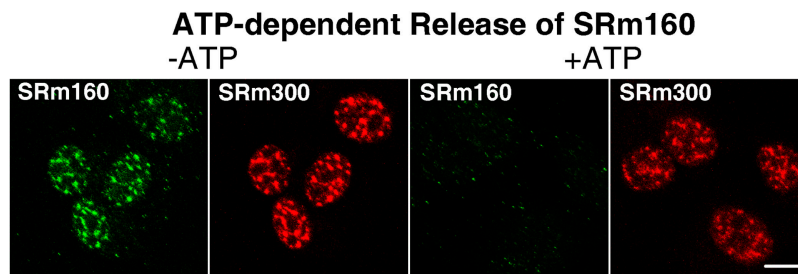


Figure 5. Photobleaching recovery of EGFP-hnRNP A2, EGFP-SRm300, and EGFP-RNPS1 in live and digitonin-permeabilized cells. (A) HeLa cells transiently expressing EGFP-hnRNP A2 were photobleached. Recovery is shown for the same cell before (top) and after (bottom) digitonin permeabilization. Images were taken before (Pre-bleach), immediately after (Bleach), and at the indicated intervals after bleaching. The bleached area is outlined by a white square. Bar, 10 μ m. (B) HeLa cells transiently expressing EGFP-SRm300 were photobleached. Recovery is shown for a live cell (top), after digitonin permeabilization (middle), and after addition of 5 mM ATP to a permeabilized cell (bottom). Images were taken before (Pre-bleach), immediately after (Bleach), and at intervals after bleaching. The bleached area is outlined by a white square. EGFP-SRm300 does not recover in live cells but not in digitonin-permeabilized cells with (bottom) or without (middle) ATP supplementation. Bars, 10 μ m. (C) HeLa cells transiently expressing EGFP-RNPS1 were photobleached. Recovery is shown for a live cell (top), after digitonin permeabilization (middle), and after addition of 5 mM ATP to a permeabilized cell (bottom). Images were taken before (Pre-Bleach), immediately after (Bleach), and at the indicated intervals after the bleach. The bleached area is outlined by a white square. EGFP-RNPS1 becomes immobile in digitonin-permeabilized cells (middle), but substantial recovery was restored upon ATP addition (bottom). Bars, 10 μ m. (D) Photobleach recovery curves for EGFP-hnRNP A2, EGFP-SRm300, and EGFP-RNPS1 in live cells are plotted as the SEM for the indicated number of cells. The half times for recovery were 15 s for EGFP-hnRNP A2, 10 s for EGFP-SRm300, and <10 s for EGFP-RNPS1. (E) Photobleach recovery curves for EGFP-SRm300 and EGFP-RNPS1 in digitonin permeabilized cells before and after addition of ATP are plotted as the SEM for the indicated number of cells. ATP supplementation restored mobility to ~30% of EGFP-RNPS1 but did not increase EGFP-SRm300 recovery.

Figure 6. The release of endogenous SRm160 from Triton X-100-permeabilized cells is ATP dependent. HeLa cells were permeabilized in 0.5% Triton X-100 in the presence of 20 mM ATP (+ATP) or without ATP (-ATP). SRm160 and SRm300 were detected by immunofluorescent staining using the mAbs B1C8 and B4A11, respectively. In the presence of ATP, the large majority of SRm160 is released during the permeabilization, whereas most SRm300 is retained. Projections of confocal data stacks are shown. Images were collected using identical machine settings so that the images could be compared quantitatively. Bar, 10 μ m.



interpretations of FRAP data as binding measurements (Phair and Misteli, 2001).

When SRm160 is exchanging and diffusing in the presence of ATP, it could be alone, in an RNA splicing complex (Blencowe et al., 1994, 2000), or in an RNA export complex (Kataoka et al., 2001; Le Hir et al., 2000a,b, 2001). SRm300 is a binding partner of SRm160 and, with SRm160, a constituent of spliceosomal complexes but has not been detected in the RNA export-related EJC. SRm300 apparent mobility was not restored by ATP, so SRm300 cannot be in a complex with SRm160 during ATP-induced mobilization. In contrast, RNPS1 is together with SRm160 in RNA export complexes (Kataoka et al., 2001; Le Hir et al., 2000a,b, 2001) and had a similar ATP-dependent unbinding of SRm160-containing RNA export but not of splicing complexes.

ATP hydrolysis was not required for SRm160 mobilization because the nonhydrolyzable analogue AMP-PNP restored fluorescence recovery as well as ATP (Fig. 4). Thus, ATP-induced conformational changes rather than phosphorylation events may mediate the mobilization of SRm160 or its complexes. GTP only partially restored the mobilization of SRm160 and there was no synergistic effect of ATP and

ATP-dependent Release of SRm160

-ATP

+ATP

GTP. There may be two NTP-dependent mechanisms for SRm160 fluorescence recovery, each regulating the mobilization of distinct forms or complexes of SRm160. One mechanism would have a strict requirement for ATP, whereas the second would use either ATP or GTP. There would be approximately twice as many complexes with ATP-dependent compared with NTP-dependent mobility (Fig. 4).

SRm160 and the other splicing and RNA export factors studied here are most concentrated in splicing speckled domains, and our experiments directly measure its binding at those sites. Regulated binding at these sites, where a majority of pre-mRNAs are spliced (Smith et al., 1999), might represent a mechanism for retaining transcripts until their processing is completed. Further, if similar ATP-dependent binding mechanisms exist at nucleoplasmic sites between splicing speckled domains and the nuclear pores through which mRNA leaves the nucleus, then a simple mechanism for channeling RNA export could be proposed. Export complexes containing SRm160 and RNPS1 might diffuse in the nucleoplasm but be constrained to defined paths by occasional, transient docking to binding sites along a path. A similar controlled diffusion mechanism has been proposed for the export of NOP140-containing complexes (Meier and Blobel, 1992). Consistent with this model, we have observed SRm160 strung along long nuclear tracks by immunoelectron microscopy (unpublished data). In this model of nuclear export regulation, complexes with bound ATP would be diffusing but complexes with ADP would be docked on structures. Regulation of ATP exchange or hydrolysis would select complexes for release and export. Consistent with this model for regulated RNA export is the recent demonstration by *in vivo* FRAP that the intranuclear mobilities of poly(A) binding protein II and TAP are reduced in cells after treatment with azide and 2-deoxyglucose or after temperature reduction (Calapez et al., 2002). Both proteins may be in mRNP complexes during export.

More generally, the binding and exchange of small regulatory molecules like ATP might control the relocation of other complexes in the nucleus. This relocation might be achieved by regulating the release of these complexes from one binding site on a relatively immobile nuclear structure, like chromatin or the nuclear matrix, and permitting their subsequent binding at a new site. The advantage of regulated release might be that complexes are retained until they are completely assembled or processed. Further, the intranuclear geometry of specific binding sites might channel diffusion of molecules or complexes along pre-

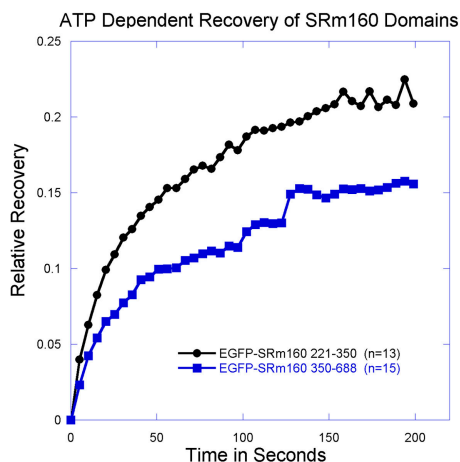


Figure 7. SRm160 domains conferring ATP-dependent mobilization. HeLa cells transiently expressing the indicated EGFP-SRm160 deletion mutants were permeabilized with digitonin and FRAP performed in the presence of 5mM ATP. In the absence of ATP, there was no detectable recovery. Each point represents the mean for the number of photobleached cells indicated in parentheses to the right of the curve.

ferred paths. In vitro FRAP provides an assay for uncovering such mechanisms and identifying the small molecules that may regulate them.

The energy dependence of nuclear protein mobility has previously been assessed by measuring photobleaching recovery rates after metabolic inhibitor treatments to reduce ATP levels or after reducing cell temperature. The intranuclear mobilities of ASF/SF2, fibrillarin, and HMG-1 are unchanged after cooling cells from 37° to 23°C and after treatment of cells with sodium azide (Phair and Misteli, 2000). In contrast, the estrogen receptor immobilizes in cells treated with sodium azide and 2-deoxyglucose, suggesting an energy-dependent mobility. ATP involvement in the nuclear mobility of Cajal bodies is supported by their faster movement in sodium azide/2-deoxyglucose-treated cells studied by time-lapse microscopy (Platani et al., 2002). A negative result in metabolic inhibitor studies is difficult to interpret without a direct measurement of the resulting ATP levels. Treatment with sodium azide and 2-deoxyglucose for 45 min did not reduce measured ATP levels (Table I) sufficiently to severely compromise SRm160 mobility. Therefore, inhibitor experiments in living cells may underestimate, or miss entirely, the ATP dependence of mobility for some proteins and their complexes.

A preferable approach to measure the ATP dependence of nuclear protein binding and mobility is the in vitro FRAP assay we introduce here. This same system can be used to deplete, and then supplement molecules, other than ATP so long as they are small enough to diffuse through nuclear pores. Even smaller nuclear proteins (<40–60 kD) can be depleted. This system offers an unprecedented ability to biochemically manipulate and characterize mobility processes in the nucleus. The rate of both EGFP-SRm160 and RNPS1 mobilization was reduced in ATP-supplemented permeabilized cells compared with living cells and there was a higher percentage of each protein that remained in the immobile fraction. This finding might be attributable to depletion of other mobility cofactors, which are either smaller than the diffusion limit of pores or which are exported from the nucleus by ATP or its analogs (Englmeier et al., 1999). FRAP of digitonin-permeabilized cells offers an assay for the future identification of such additional cofactors. More generally, mobility studies using digitonin-permeabilized cells are a valuable tool for identifying cofactors affecting the nuclear dynamics of proteins, complexes, and structures.

Materials and methods

Plasmids

The expression vector and method used to establish EGFP-SRm160-expressing cells has been described previously (Wagner et al., 2003). EGFP-SRm300 was established by assembling a full-length cDNA for SRm300 that was subsequently cloned into the pEGFP-C3 expression vector (CLONTECH Laboratories, Inc.). The SRm300 sequence was verified by sequencing. EGFP-hnRNP A2 was obtained by inserting the hnRNP A2 cDNA (provided by G. Dreyfus, University of Toronto, Toronto, Canada) from pET28a-hnRNP A2 (Kamma et al., 1999) into pEGFP-C1 (CLONTECH Laboratories, Inc.). To generate EGFP-RNPS 1, the cDNA for RNPS1 (provided by B. Blencowe, University of Pennsylvania, Philadelphia, PA) was amplified by PCR and inserted into pEGFP-C1 (CLONTECH Laboratories, Inc.).

Cell culture

HeLa cells were cultured in DME supplemented with 10% FBS. Transfections were performed using Superfect (QIAGEN). HeLa cells stably ex-

pressing EGFP-SRm160 were obtained by selection with G418 followed by FACS® analysis for EGFP-positive cells and analysis of cell lines derived from single cell clones. For FRAP experiments, cells were grown on 40-mm glass coverslips and transferred to the FCS2 live cell chamber (Bioptechs) mounted on an SP laser scanning confocal microscope (Leica) with objective heater. The temperature was maintained at 37°C. To permeabilize cells, the medium was replaced with 50 µg/ml digitonin in permeabilization buffer (20 mM Hepes, pH 7.3, 110 mM potassium acetate, and 2 mM magnesium acetate) for 6 min. Afterward, the cells were washed with permeabilization buffer for 10 min. In some experiments, cells were incubated in permeabilization buffer containing either 5 mM ATP (Sigma-Aldrich), GTP (Sigma-Aldrich), AMP-PNP (Roche), or ADP (Calbiochem) for at least 5 min before FRAP measurements.

Antibody penetration assay

Antibody penetration assay was performed as described by Adam et al. (1990). HeLa cells grown on coverslips were extracted with either 0.5% Triton X-100 or 50 µg/ml digitonin in permeabilization buffer at RT for 6 min. Cells were treated with the mAb B4A11 against SRm300 for 10 min and washed with permeabilization buffer. Next, the cells were incubated with an Alexa 488-conjugated secondary antibody (Molecular Probes) for 10 min, washed with permeabilization buffer, and fixed with 4% formaldehyde in cytoskeletal buffer (Blencowe et al., 1998).

ATP depletion and measurement

To reduce ATP levels, HeLa cells were incubated in glucose-free Opti-MEM (GIBCO BRL) with 6 mM 2-deoxyglucose (Sigma-Aldrich) and 10 mM sodium azide for up to 45 min. Cell extracts were prepared by incubation in 100 mM Tris, pH 7.75, and 4 mM EDTA at 100°C for 2 min. ATP levels were measured by luciferase assay using an ATP assay kit (Calbiochem).

Fluorescence recovery after photobleaching

Images of the nucleus were scanned before and after bleaching at low laser power. Routinely, two images were taken before and 20–30 images after the bleach at intervals of 1.6 or 20 s depending on the time of recovery. To destroy EGFP fluorescence, maximal laser power was applied to a region of interest for 3 s. Confocal Software (version 2; Leica) was used to measure the intensity of fluorescence in the bleached area and in the whole nucleus for the whole stack of images. For analysis, these data were transferred into a Microsoft Excel spreadsheet. Any remaining fluorescence in the bleached area after the bleach was normalized to zero. The relative fluorescence intensity (I_{rel}) in the previously bleached area was calculated as described by Phair and Misteli (2000): $I_{rel} = T_0 I_t / T_t I_0$, with T_0 being the total cellular intensity before bleach, T_t the total cellular intensity at time t , I_0 the intensity in the bleached area before bleach, and I_t the intensity in the previously bleached area at time t . Recovery curves were drawn using Kaleidagraph 3.5 (Synergy Development). The percentage of immobile protein was determined as the difference between the plateau of any given recovery curve and a relative recovery of 1, a value that would reflect complete recovery without an immobile fraction. For example, a plateau at 0.8 reflects an immobile fraction of 20%.

Nuclear protein release assay

HeLa cells (CCL2) grown on coverslips were washed in PBS at 37°C for 2 min. They were permeabilized at 37°C for 10 min in 0.5% Triton X-100 in cytoskeletal buffer in the presence of 20 mM ATP or without ATP before fixation at 4°C in 4% formaldehyde. SRm160 was detected by immunofluorescent staining using the SRm160-specific mAb B1C8 (mouse IgM), and SRm300 was detected using mAb B4C11 (mouse IgG), followed by an Alexa 488-conjugated goat anti-mouse IgM and Alexa 568-conjugated goat anti-mouse IgG secondary antibody (Molecular Probes) for 1 h at RT. Coverslips were mounted with Prolong (Molecular Probes).

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